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Physiological Profiling and Functional Diversity of Groundwater Microbial Communities in a Municipal Solid Waste Landfill Area

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Abstract: The disposal of municipal solid wastes in landfills represents a major threat for aquifer environments at the global scale. The aim of this study was to explore how groundwater geochemical characteristics can influence the microbial community functioning and the potential degradation patterns of selected organic substrates in response to different levels of landfill-induced alterations. Groundwaters collected from a landfill area were monitored by assessing major physical-chemical parameters and the microbiological contamination levels (total coliforms and fecal indicators—Colilert-18). The aquatic microbial community was further characterized by flow cytometry and Biolog EcoPlates™ assay. Three groundwater conditions (i.e., pristine, mixed, and altered) were identified according to their distinct geochemical profiles. The altered groundwaters showed relatively higher values of organic matter concentration and total cell counts, along with the presence of fecal indicator bacteria, in comparison to samples from pristine and mixed conditions. The kinetic profiles of the Biolog substrate degradation showed that the microbial community thriving in altered conditions was relatively more efficient in metabolizing a larger number of organic substrates, including those with complex molecular structures. We concluded that the assessment of physiological profiling and functional diversity at the microbial community level could represent a supportive tool to understand the potential consequences of the organic contamination of impacted aquifers, thus complementing the current strategies for groundwater management.

Keywords: Biolog EcoPlates™; flow cytometry; microbial community; metabolic fingerprint; groundwater quality; hydrogeochemistry

1. Introduction

Groundwater is the second largest freshwater deposit representing 14% of all inland waters and one of the most important drinking water resource [1,2]. Several factors may impair groundwater environment including drought, excessive withdrawals, and pollutant contamination derived by anthropogenic activities [3–5]. Awareness of the importance of groundwater resources led the European Commission to define legislative tools for ensuring the groundwater ecosystem quality and fostering the related research efforts [6]. The environmental policy started to consider aquifers as a living ecosystem to be preserved [7]. To date, the anthropogenic impact on groundwater biological communities is largely disregarded, although a wide range of organisms are adapted to live under the limiting conditions imposed by darkness and low trophic resources [8,9]. Among those, prokaryotic microorganisms are

the most relevant in terms of biomass [10]. The environmental factors that influence the dynamics of aquatic microbial communities are increasingly attracting the research interest, given the key role played by microorganisms in the ecosystem while processing and cycling organic carbon and nutrients [11,12]. Changes in water physical-chemical characteristics were found to affect the structural and functional properties of the microbial communities [13–15].

Among the global threats for the aquifer environment, the municipal solid waste disposal in landfills is of major concern for either human or environmental health, since pollutants can promptly migrate into groundwater by the leaching of percolate [11,16,17]. To counteract and prevent "significant adverse environmental effects" derived from landfill practices, a detailed characterization of groundwater quality is required and based on representative hydrogeological conceptual models (European Landfill Directive; 1999/31/EC) [18]. However, despite the good chemical status is determined by compliance with water quality standards, the ecological approach to evaluate the anthropogenic impact on groundwaters has been poorly considered [19].

Different approaches are suitable for monitoring and predicting the effects of anthropogenic groundwater alterations, offering an insight into the relationships occurring between geochemical parameters and the microbial functional properties [13,20,21]. The microbial phylogenetic changes, observed so far in many literature studies [22–24], were not likely exhaustive to explain the microbial functional responses to the anthropic impact. A fundamental understanding of the microbially-driven processes and the following ecosystem responses to anthropogenic stress factors is still needed.

Starting from the outcomes of one-year monitoring activities in a municipal solid waste landfill in Central Italy [21], we aimed to explore the functional properties of groundwater microbial communities and their responses to different levels of landfill-induced geochemical alterations. The hypothesis is that changes in groundwater quality will affect the functioning of the resident microbial communities by inducing preferential metabolic pathways. This approach could complement the current geochemical assessments to better elucidate the effects of the anthropic activities on the aquifer environment.

2. Materials and Methods

2.1. Field Sampling and Major Water Characteristics

The study was carried out at a groundwater-monitoring network located within the area of a municipal solid non-hazardous waste landfill facility in Central Italy (Figure 1). The landfill, duly equipped with liners and leachate collection systems, is operating since 2002, following the Landfill Directive 1999/31/EC. Between 2003 and 2018, it received 2.9 million tons of waste in 4 different basins, deriving mainly from the mechanical-biological treatment of municipal waste (65%), and non-hazardous waste of other origin. The yearly amount varied from 170,000 tons to 2,700,000 tons. A water table aquifer (depth 5–15 m) was found only in the northern part of the facility. A confined aquifer (depth 5–27 m) underlies the entire landfill area, with groundwater flowing from North to South through lacustrine sediments formed by clays, silts, and sandy layers [21].

During two sampling campaigns (October 2016 and May 2017), 22 water samples were collected from 12 piezometers (Figure 1), by following specific guidelines for groundwater sampling in industrial areas [25]. The landfill is composed of four different basins, three of which are currently in the "after-care" phase and only the fourth basin was active during sampling. Basin 4 (840,000 m^3) is located between Pz07 and Pz01. The distance of Pz07 from the waste load area is approximately 50 m. Pz01 is at about 50 m from the southern end of the basin 4.

Major physical-chemical water characterization was assessed on-site by measuring electrical conductivity, oxidation-reduction potential (ORP), temperature, pH, dissolved oxygen with a flow cell equipped with AQUAREAD probes. The concentration of NO_3, SO_4, F, Cl (Ionic Chromatography—Dionex DX-120), HCO_3 (HCl titration), Si, Mg, Ca, Na, K (Inductively Coupled Plasma—Optical Emission Spectrometry—Perkin Elmer P400), DOC (Shimadzu TOC-5000A analyser) were successively measured in laboratory [21].

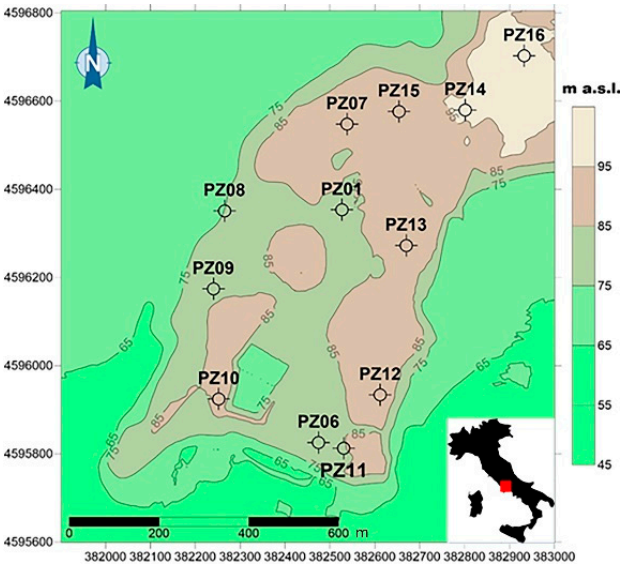

Figure 1. Location of the waste landfill facility area (Central Italy), with the indication of sampling sites.

The occurrence of total coliforms (TC) and fecal indicator bacteria (*E. coli*) was estimated using the Colilert-18 test kit (IDEXX Laboratories Inc., Westbrook, Maine, USA) [26]. Duplicate samples were collected in 100-mL sterile polystyrene bottles and analyzed within 6 h. The positive wells for the activities on ONPG (yellow) and MUG (fluorescent) substrata were counted and referred to the Quanti-Tray/2000 Most Probable Number (MPN) table to quantify the total coliforms and *E. coli*, respectively.

2.2. Microbial Community Characterization by Flow Cytometry

Subsamples for flow cytometric analyses were collected in 2-mL Eppendorf safe-lock tubes, fixed with formaldehyde (2%, final concentration) and analyzed within 24 h. The microbial community characterization was performed by the Flow Cytometer A50-micro (Apogee Flow System, Hertfordshire, UK) equipped with a solid-state laser set at 20 mV and tuned to an excitation wavelength of 488 nm. The total prokaryotic cell counts (TCC) were measured by staining with SYBR Green I (1:10,000 dilution; Molecular Probes, Invitrogen) for 10 min in the dark at room temperature. The light scattering signals (forward and side scatters), the green fluorescence (530/30 nm) and red fluorescence (>610 nm) were acquired with a threshold sets on the green channel. Samples were run at low flow rates to keep the number of events below 1000 event s^{-1}.

The data were analyzed using the Apogee Histogram Software (v2.05). The TCC was determined by their signatures in a plot of the side scatter vs. the green fluorescence. The intensity of green fluorescence emitted by SYBR positive cells allowed for the discrimination among cell groups exhibiting two different nucleic acid content (cells with low NA content—LNA; cells with high NA content—HNA) [27]. This method was successfully applied for prokaryotic cell counting in different groundwater settings [15,21].

2.3. Metabolic Potential, Functional Diversity and Community Level Physiological Profile

The Biolog EcoPlates™ assay (Biolog, Inc., Hayward, California, USA) was used to detect the microbial degradative activity of 31 organic carbon sources, contained in triplicate in 96 micro-well plates (plus three control wells) and labeled with the respiration-sensitive tetrazolium dye. Within 6 h from sampling, 150 µL of sampled water was introduced in each well. The plates were incubated at 20 °C in the dark. The analysis of the color development for each substrate along the incubation time provided information on the degradation rate of each substance. The development of formazan was detected through the measurement of the absorbance at 590 nm (VICTOR™ X3 Multilabel plate reader.

PerkinElmer Inc., Waltham, Maine, USA). Data acquisition was performed at the starting time (time 0) and every 24 h, over a period of 15 days. The optical density (OD 590 nm, expressed as absorbance units) of each substrate was subtracted by the corresponding initial OD value (t = 0) to eliminate the intrinsic absorbance signal [28]. Negative values were set to 0.

The mean degradative activity on all 31 substrates was intended as the microbial metabolic potential, expressed as Average Well Color Development (AWCD), the average of adjusted absorbance of the whole microplate [29]:

$$\mathrm{AWCD} = \frac{1}{31}\sum(\mathrm{OD}_{590}) \quad (1)$$

The AWCD kinetic profiles were calculated over the entire incubation time, based on the density-dependent logistic growth equation:

$$y = \mathrm{AWCD_t} = \frac{\mathrm{K}}{1 + e^{-\mu(\mathrm{t}-\mathrm{s})}} \quad (2)$$

where:

- K = the asymptote/carrying capacity
- μ = exponential rate of AWCD change
- t = time (h)
- s = the time when y = K/2

These parameters were utilized to compare the microbial functional properties in different samples [30]. Data elaboration were performed in the interval between 0 and 168 h of incubation, representing the exponential phase before the stationary phase. In particular, the 24 h endpoint represented the prompt microbial responsiveness in substrate degradation, while the 168-h endpoint represented the maximum potential degradation activity.

Following Weber et al. [31], the functional diversity was related to the rates at which the Biolog substrates were utilized by the aquatic microbial community. Herein, the functional diversity was estimated in terms of equitability (E), taking into account both Shannon index (H) and substrate richness (i.e., number of positive wells in the microplates—S):

$$\mathrm{E} = \frac{\mathrm{H}}{\ln \mathrm{S}} \quad (3)$$

where:

- $\mathrm{H} = -\sum p_i\,(ln\ p_i)$
- p_i = the ratio of the activity of a particular substrate to the sums of activities of all substrates
- S = number of positive wells in the microplates

The standardization of initial density inoculum was carried out by normalization of optical density (OD_{590}) of each substrate by the corresponding Average Well Color Development (AWCD) value [28,32,33]. To simplify the interpretation of the results, the 31 substrates utilized by the Biolog test, were grouped into six categories of chemical compounds as follows: carbohydrates, polymers, carboxylic acids, phenolic compounds, amino acids and amines [34]. The Area Under the Curve (AUC) describing the variation of OD with time was calculated for each well using a trapezoidal approximation [35]. All values, hereafter reported both for classes of compounds and single substrates, were expressed as AUC and utilized for the univariate analysis.

2.4. Statistical Analysis

Geometric means were used to characterize physical, chemical and microbial characteristics of the groundwater groups. In the case of *E. coli* < 1 MPN/100 mL, we assumed 0.5 MPN/100 mL as lowest value.

The multivariate analysis of similarities (ANOSIM) was used to test the difference among the sample groups by considering all major physical, chemical, and microbial parameters. A multi-group similarity percentage test (SIMPER), through the Bray-Curtis similarity measure (multiplied by 100), was run to identify the percentage contribution of selected data to the average dissimilarity.

The non-parametric Kruskal-Wallis univariate test was performed on all parameters in order to identify statistical differences between the groundwater groups.

A non-metric multi-dimensional scaling ordination plot (nMDS), based on the Gower dissimilarity matrix, was used to graphically visualize hydrogeochemical and microbiological variation patterns [36]. The values of the degradation activity performed on the different categories of organic compounds were incorporated in a nMDS analysis with a vector-fitting procedure, in which the length of the arrow is proportional to the correlation between the nMDS-axes and each variable. This method allowed determining which degraded class of compounds were significantly correlated with the nMDS ordination. In particular, we focused on those substrates that contributed for 50% of the overall SIMPER dissimilarities between the groundwater groups.

A non-parametric multivariate analysis of variance (NPMANOVA) was performed on AWCD-normalized Biolog data (i.e., on either substrate classes or single substrates) to highlight differences in the community-level physiological profiles (CLPP) between the identified groundwater groups.

3. Results

3.1. Groundwater Physical, Chemical and Microbial Characteristics

The major groundwater physical, chemical and microbial characterization (Tables S1 and S2) allowed identifying three statistically distinct groundwater groups (ANOSIM, $p < 0.05$), herein defined as "pristine", "mixed", and "altered".

The pristine groundwater group included ten samples from six piezometers (Pz6-2016, Pz6-2017, Pz12-2016, Pz12-2017, Pz13-2017, Pz14-2016, Pz14-2017, Pz15-2016, Pz15-2017, and Pz16-2017) (Figure 1). These samples were anoxic groundwaters characterized by the lowest mean values of ORP and DO, the lowest mean EC along with the lowest mean concentration of SO_4 and Ca (Table 1).

Table 1. Physical and chemical characteristics of different groundwater groups presented as geometric means. The median was used in case of negative ORP values. Data are shown in order of contribution to the variability among groups obtained through the SIMPER test. EC = electrical conductivity; ORP = redox potential; T = temperature; DO = dissolved oxygen; DOC = Dissolved Organic Carbon). a, b, c indicate statistical differences among groups (Kruskal-Wallis, $p < 0.05$).

Parameter	Unit	Pristine	Mixed	Altered
EC	µS/cm	766.4 ^{a}	915.6	1452.7
ORP	mV	−119.3 ^{a}	74.3 ^{b}	−40.8 ^{c}
T	°C	17.8	19.1	17.3
pH		7.2 ^{a}	7.0 ^{b}	6.7 ^{c}
DO	mg/L	0.2 ^{a}	3.1 ^{b}	0.4 ^{a}
NO_3	mg/L	0.9 ^{a}	18.2 ^{b}	0.8 ^{a}
SO_4	mg/L	11.7 ^{a}	45.9 ^{b}	232.5 ^{c}
K	mg/L	10.5 ^{a}	2.1 ^{b}	5.1 ^{ab}
DOC	mg/L	2.2 ^{a}	0.8 ^{b}	2.0 ^{a}
F	mg/L	0.4 ^{a}	0.2 ^{b}	0.2 ^{ab}
Cl	mg/L	7.9 ^{a}	19.5 ^{b}	11.3 ^{a}
Si	mg/L	16.4 ^{a}	7.5 ^{b}	19.7 ^{a}
Mg	mg/L	23.1 ^{b}	22.0 ^{b}	45.7 ^{a}
Ca	mg/L	94.1 ^{b}	135.4 ^{c}	238.3 ^{a}
Na	mg/L	9.9	8.2	8.8
HCO_3	mg/L	419.8 ^{b}	424.5 ^{b}	632.4 ^{a}

The mixed groundwater group included six samples from 3 piezometers, where the water table aquifer is probably mixed with the lower one (Pz7-2016, Pz7-2017, Pz8-2016, Pz8-2017, Pz9-2016, and Pz9-2017). These samples were more oxidized groundwaters and showed the highest mean values of ORP, DO, NO_3 and Cl, along with the lowest mean DOC and Si concentrations.

The altered groundwater group, including six samples from four piezometers (Pz1-2016, Pz10-2016, Pz10-2017, Pz11-2016, Pz11-2017, Pz13-2016), was characterized by anoxic, slightly acidic groundwaters with the highest EC, SO_4, Mg, Ca and HCO_3 concentrations.

Fecal contamination was detected only in the altered groundwater samples in the 2016 campaign. Total prokaryotic cell counts (TCC = $7.93 \times 10^4 - 1.69 \times 10^7$ cells/mL) showed a large variability among the groundwater groups, with relatively high values in altered conditions, along with significant high percentages of LNA cells (Table 2).

Table 2. Microbial community characteristics (geometric means) of the three groundwater groups. In case of negative *E. coli*, half of detection limit was used for mathematical calculation (i.e., 0.5 MPN/100 mL). TCC = Total Cell Counts; HNA cells = cells with high nucleic acid content; LNA cells = cells with low nucleic acid content. a, b, c indicate statistical differences among groups (Kruskal-Wallis, $p < 0.05$).

Parameter	Unit	Pristine	Mixed	Altered
Total Cells Count	10^5 cells/mL	4.4	3.1	6.6
HNA Cells	% of TCC	52.8 ^{b}	52.6 ^{b}	27.1 ^{a}
LNA Cells	% of TCC	41.6 ^{b}	46.4 ^{b}	65.5 ^{a}
Total Coliforms	MPN/100 mL	157.8	103.0	292.6
E. coli	MPN/100 mL	<1	<1	3.3

The different contribution of the macro-chemical elements and microbial characteristics to the identified groundwater groups was graphically summarized in an ordination plot (Figure 2). The multivariate approach allowed for the testing of inter-group dissimilarity, along with highlighting the variation patterns of the groundwater major characteristics.

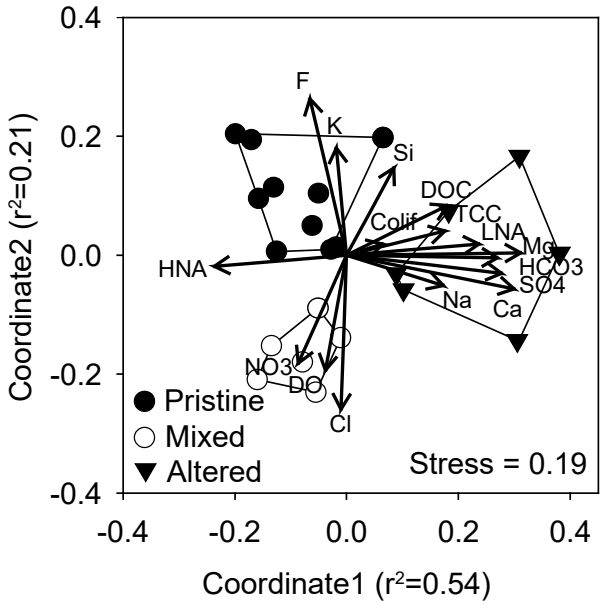

Figure 2. NMDS ordination plot of sampled waters. Vectors represent the major geochemical and microbial community characteristics that concurred to the identification of the three different groundwater groups. Stress value indicates the significant concordance between the distance among samples in the NMDS plot and the actual Gower distance among samples.

3.2. Kinetic Profiles of Metabolic Potential and Functional Diversity

The kinetic parameters, calculated from the sigmoidal curves of the AWCD values over time, highlighted the overall differences in the metabolic responses of microbial communities from the three groundwater groups (Figure 3a and Table 3). The microbial potential activity reached a similar theoretical maximum K value in all groundwater groups. The microbial community from the pristine groundwater differed significantly, showing the highest exponential rate of AWCD change (μ) (Table 3). In altered conditions, the microbial community showed $AWCD_{24h}$ values two-fold higher than that developed under pristine and mixed conditions and the lowest time to reach K/2, hence indicating a higher affinity for the available substrates.

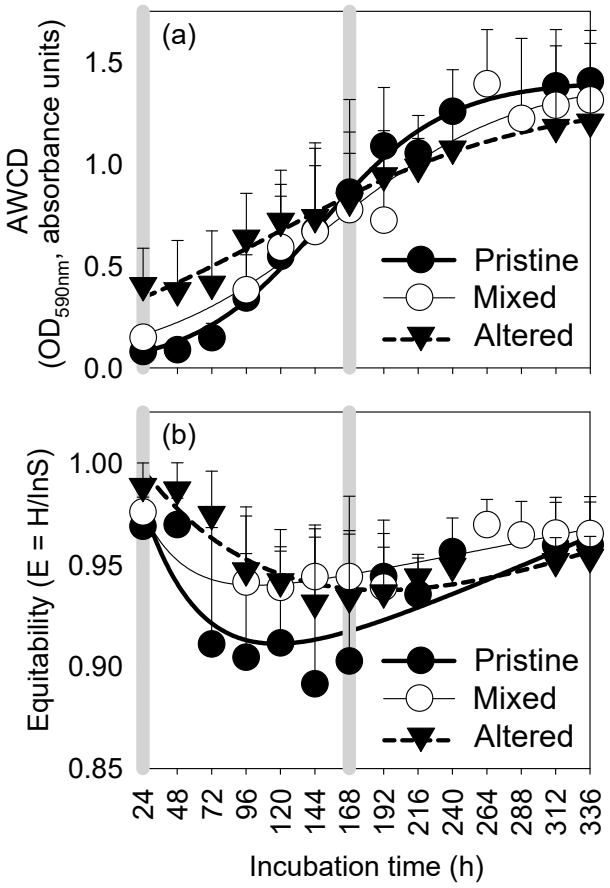

Figure 3. Kinetic profiles of (**a**) metabolic potential (Average Color Well Development—AWCD) and (**b**) functional diversity (substrate utilization equitability—E) (Equation (3)) of the identified groundwater groups. Gray lines indicate 24 h and 168 h incubation time.

Table 3. Kinetic parameters of the fitted logistic growth equations (Equation (2)). K = asymptote, s = time to reach K/2, μ = exponential rate of AWCD change, r^2 = correlation coefficient.

Parameter	Unit	Pristine	Mixed	Altered
K	OD_{590nm}	1.42	1.41	1.33
s	h	157.1	147	117.1
μ	h^{-1}	0.029	0.023	0.021
r^2		0.98	0.99	0.99

After 24 h of incubation, the functional microbial diversity, indicated by the equitability values, of microbial communities from pristine and mixed sample groups showed similar values (E = 0.93 and 0.92, respectively), which were significantly lower than those found in altered conditions (E = 0.96).

The equitability under pristine conditions reached the lowest values with respect to altered and mixed conditions, indicating a heterogeneous distribution in substrate degradation (Figure 3b and Table 4). The equitability showed lower values at 168 h (E = 0.903, 0.944, and 0.934, respectively for pristine, mixed groundwaters) than at 24 h.

Table 4. Utilization patterns of organic substrates in different groundwater groups after 24 h and 168 h of incubation. Values are given as percentage of the total OD_{590}.

24 h																							
Classes of Substrates	**Substrates**	**Pristine**										**Mixed**								**Altered**			
Carbohydrates	β-methyl-D-glucoside	4.7	6.4	4.8	4.9	4.6	5.1	5.3	3.8	5.6	4.4	4.8	5.6	3.3	3.4	3.6	4.8	3.3	3.1	4.9	3.5	3.5	3.5
	D-xylose	4.1	1.5	3.2	2.9	4.0	1.6	0.0	5.7	1.5	3.0	3.6	3.6	1.9	3.7	4.5	3.4	3.9	3.1	3.6	3.7	3.3	3.6
	i-erythritol	7.0	4.2	5.2	5.7	6.3	6.6	8.1	5.6	7.0	4.6	5.8	5.3	6.7	4.9	6.0	5.9	4.1	3.7	4.5	4.0	3.7	6.5
	D-mannitol	5.5	7.2	5.1	5.5	5.0	5.2	7.1	4.4	8.6	4.9	5.6	6.8	5.7	3.6	5.3	5.3	3.9	2.9	4.1	3.4	3.6	5.2
	N-acetyl-D-glucosamine	4.9	6.9	5.5	5.4	5.0	4.4	7.3	4.0	6.1	6.2	5.3	4.6	3.8	4.5	4.3	4.1	3.8	2.9	3.3	3.6	3.8	5.0
	D-cellobiose	5.0	2.9	5.1	5.6	6.6	3.7	6.2	4.8	5.9	5.3	5.5	3.2	1.7	4.6	5.0	5.7	3.5	4.7	4.2	3.6	3.7	4.7
	α-D-lactose	6.0	3.3	5.0	6.1	5.2	5.5	1.7	4.7	6.7	5.5	5.2	7.7	1.8	4.1	5.2	6.2	3.2	5.0	4.0	2.9	4.1	4.4
	glucose-1-phosphate	2.7	1.9	1.5	2.7	1.9	0.0	7.3	2.3	0.0	2.0	2.5	1.1	2.9	2.5	1.4	1.8	2.5	2.5	2.0	2.2	3.0	1.6
	D,L-α-glycerol phosphate	2.0	2.8	0.0	0.6	3.1	0.0	0.0	0.9	2.8	1.1	1.5	2.0	2.7	1.8	1.9	1.7	2.5	1.6	2.1	2.5	2.9	0.5
Polymers	tween40	5.7	5.1	5.7	4.1	6.7	3.0	3.3	4.5	4.6	7.6	4.6	4.0	5.3	4.5	7.8	4.2	4.4	5.7	3.6	3.3	4.0	6.4
	tween80	0.0	2.4	3.4	3.6	0.0	2.4	0.1	2.6	0.0	1.6	0.0	0.0	1.7	3.1	2.9	3.4	3.7	3.3	3.0	3.3	3.8	3.3
	α-cyclodextrin	2.3	1.7	2.3	2.3	1.7	1.1	3.1	2.4	2.7	2.4	2.0	2.5	3.7	2.2	1.3	3.0	2.3	2.8	3.1	3.6	2.9	1.7
	glycogen	5.0	6.3	3.8	2.6	2.8	3.8	4.4	3.6	4.2	4.4	3.9	5.5	2.7	2.8	3.3	3.9	3.0	3.4	3.0	3.3	3.7	3.9
Carboxylic Acids	D-galactonic acid γ-lactone	4.5	2.5	4.6	3.0	3.7	3.9	4.3	2.9	2.4	3.4	4.3	2.8	4.4	2.6	4.2	3.5	3.7	3.8	3.4	3.5	2.5	4.3
	D-galacturonic acid	4.6	2.8	0.7	2.4	2.8	2.8	2.2	3.1	2.8	0.0	2.6	3.8	3.5	3.1	3.0	3.5	1.9	2.9	3.2	3.0	3.1	1.9
	γ-hydroxybutyric acid	4.1	1.8	4.3	5.3	3.9	11	1.0	2.7	9.3	7.6	5.0	2.6	2.7	3.8	2.7	3.8	4.1	3.9	2.6	3.7	3.9	5.1
	D-glucosaminic acid	4.3	2.7	3.1	4.2	3.8	3.2	7.0	3.2	5.8	4.4	4.1	4.1	4.5	3.3	3.7	3.6	2.5	2.4	3.1	3.1	3.2	3.0
	itaconic acid	2.6	2.0	2.9	2.6	2.7	2.1	2.7	2.4	3.2	2.2	2.6	3.4	1.9	2.2	2.0	2.2	3.5	3.1	2.6	2.8	2.4	2.4
	α-ketobutyric acid	0.0	2.7	3.7	3.0	0.0	5.9	0.8	3.2	0.0	2.6	4.6	0.0	2.3	2.9	1.5	1.2	2.0	1.5	2.1	2.8	3.1	2.7
	D-malic acid	0.0	2.1	2.8	1.6	3.0	0.0	0.5	2.3	1.7	1.5	0.0	3.4	3.7	3.5	3.3	1.8	2.6	3.8	3.0	3.4	3.6	1.8
	pyruvic acid methyl ester	0.0	1.7	2.9	0.0	0.0	0.0	0.0	0.7	0.0	0.8	0.0	0.0	2.8	1.0	0.5	0.0	2.6	2.2	2.0	3.4	3.6	0.4
Phenolic Compounds	2-hydroxy benzoic acid	0.0	1.9	2.1	0.0	2.2	0.0	0.0	2.2	0.0	0.0	0.0	2.2	3.3	3.5	3.5	5.0	3.2	2.5	4.4	3.3	3.4	1.7
	4-hydroxy benzoic acid	0.0	1.5	3.2	1.0	0.0	1.2	0.2	3.6	0.0	1.4	0.0	0.0	1.2	1.6	0.8	0.0	3.2	1.9	2.2	2.9	2.7	2.8
Aminoacids	L-arginine	4.1	3.6	3.0	4.1	3.8	4.4	6.4	3.7	2.9	5.9	3.6	4.6	4.1	3.2	3.9	3.7	2.8	3.7	3.5	3.5	3.3	3.9
	L-asparagine	3.8	3.8	2.8	3.0	3.4	3.6	3.1	2.7	1.2	2.8	3.5	3.7	4.6	4.1	3.1	2.5	3.8	4.2	3.7	3.1	3.0	3.5
	L-phenylalanine	3.0	2.3	1.6	2.3	2.0	2.4	1.4	2.8	1.9	1.8	2.4	2.0	2.9	2.5	1.5	1.4	3.3	2.1	2.0	2.9	1.3	2.0
	L-serine	3.4	3.8	2.3	3.1	3.5	3.9	5.0	2.7	2.6	2.5	3.6	4.2	3.5	4.2	3.1	3.3	3.4	3.6	3.2	3.3	3.3	2.9
	L-threonine	3.8	2.8	4.7	4.3	4.2	4.0	4.3	3.9	3.7	4.6	4.1	1.5	3.9	4.2	3.7	4.3	4.1	4.1	3.5	3.7	3.6	4.2
	glycyl-L-glutamic acid	3.6	2.6	1.8	2.8	2.2	0.0	1.5	2.3	2.6	2.4	3.0	3.3	3.1	3.4	2.1	2.9	2.3	3.2	3.0	2.7	2.3	2.0
Amines	phenylethyl-amine	0.0	2.7	0.0	2.5	2.3	5.7	1.2	3.9	2.3	0.9	3.3	3.0	2.1	2.9	2.2	1.2	3.8	3.0	4.2	3.1	2.7	2.5
	putrescine	3.2	4.0	2.7	2.8	3.4	3.8	3.6	2.6	2.1	2.3	2.8	3.5	1.5	2.2	2.7	2.7	3.1	3.4	2.9	3.0	3.1	2.6
168 h																							
Classes of substrates	**Substrates**	**Pristine**										**Mixed**								**Altered**			
Carbohydrates	β-methyl-D-glucoside	4.8	4.4	8.8	7.6	1.3	2.6	5.8	2.1	11	5.8	3.4	5.7	3.8	2.8	1.5	5.6	4.8	5.5	4.5	5.3	2.6	8.2
	D-xylose	3.7	3.8	0.3	0.5	3.4	0.3	0.0	1.9	0.1	0.2	1.2	0.6	0.9	1.3	0.9	1.5	1.2	1.2	2.7	1.8	2.3	0.7
	i-erythritol	1.7	1.5	1.2	1.7	1.4	1.1	2.9	2.5	0.9	2.4	2.7	2.4	1.7	2.9	2.1	4.2	2.7	1.6	3.4	2.5	2.8	2.9
	D-mannitol	5.0	5.1	8.2	9.2	2.1	3.1	7.6	3.7	5.3	10	6.7	3.4	6.0	5.6	6.1	5.3	5.6	5.3	3.7	4.7	5.9	5.7
	N-acetyl-D-glucosamine	4.0	4.9	6.1	8.1	3.0	6.8	7.4	7.4	1.4	6.0	4.3	7.0	5.1	4.9	4.2	4.0	5.0	5.4	3.0	3.0	7.2	5.3
	D-cellobiose	5.7	4.7	5.5	3.1	4.6	2.0	3.6	3.2	5.0	7.4	4.2	2.2	3.0	4.0	2.3	4.9	4.9	7.5	4.6	6.2	2.6	6.2
	α-D-lactose	4.3	3.8	2.1	2.9	3.0	6.9	2.2	5.0	1.9	4.2	3.8	2.5	1.8	2.4	1.8	4.9	2.3	2.5	3.5	3.0	2.9	2.1
	glucose-1-phosphate	2.2	2.9	2.1	4.6	0.4	3.7	4.0	2.3	0.3	2.0	2.1	0.5	2.8	2.4	0.8	1.6	3.3	2.8	1.6	3.8	4.5	3.8
	D,L-α-glycerol phosphate	1.0	2.0	0.3	1.6	1.7	1.3	1.2	1.7	1.4	0.7	1.0	1.1	1.4	1.2	1.6	1.6	2.4	1.3	2.1	2.2	2.0	1.4
Polymers	tween40	3.2	3.7	3.9	5.8	2.9	6.0	4.0	4.1	7.3	4.0	4.9	8.3	3.0	4.9	7.6	6.1	3.7	5.1	2.7	4.0	4.7	3.3
	tween80	4.4	3.8	4.1	2.4	0.0	8.7	6.7	1.6	7.4	2.5	4.4	5.9	4.1	4.5	8.6	2.8	5.1	3.1	3.4	4.8	5.1	4.6
	α-cyclodextrin	4.5	3.6	2.3	1.5	1.0	3.7	3.2	4.1	5.4	2.6	3.2	3.8	3.9	3.8	3.2	1.8	1.0	2.1	3.2	2.7	3.2	2.5
	glycogen	4.7	4.3	11	3.2	0.4	1.9	2.4	2.8	1.4	7.1	3.0	4.5	4.7	3.0	3.8	3.6	4.8	6.8	2.6	4.2	2.6	10
Carboxylic Acids	D-galactonic acid γ-lactone	1.9	2.9	1.0	1.3	2.3	1.0	2.6	0.8	0.5	6.1	2.8	3.2	2.9	3.7	3.1	5.3	3.5	1.5	3.3	2.0	1.9	2.0
	D-galacturonic acid	3.4	3.0	6.4	2.6	8.2	1.5	3.0	3.4	5.0	0.3	1.6	2.9	3.6	3.2	2.6	2.5	2.8	1.4	2.6	2.0	2.2	3.9
	γ-hydroxybutyric acid	0.8	1.1	0.5	1.5	3.1	1.2	0.4	0.6	1.4	1.1	3.1	0.5	0.6	1.7	1.0	2.9	2.3	1.2	2.2	1.6	2.4	1.9
	D-glucosaminic acid	2.3	2.6	0.7	2.1	3.3	2.9	1.7	1.0	1.4	3.1	3.3	4.0	4.7	4.3	6.3	4.4	1.9	1.7	2.3	2.2	2.2	2.9
	itaconic acid	4.1	3.9	6.5	4.2	5.3	6.3	2.0	6.3	1.9	3.3	3.9	3.2	4.4	3.9	3.8	4.9	4.5	5.1	9.1	3.5	2.0	3.8
	α-ketobutyric acid	1.9	3.0	0.8	1.6	0.1	3.4	0.3	1.4	0.1	0.6	5.8	1.3	1.5	1.7	0.8	0.6	0.9	0.6	1.3	1.7	2.0	0.4
	D-malic acid	1.8	1.5	0.6	0.9	1.9	0.7	0.6	1.0	0.6	2.4	1.0	1.6	5.3	4.5	3.4	1.2	2.3	1.9	2.8	2.7	2.7	0.7
	pyruvic acid methyl ester	4.3	3.0	3.9	2.9	9.7	9.9	3.9	5.5	4.1	3.0	5.1	5.6	3.5	2.9	4.0	2.8	4.5	4.9	5.5	4.3	4.3	4.7
Phenolic Compounds	2-hydroxy benzoic acid	1.0	1.0	0.2	0.0	0.5	0.0	4.0	0.4	0.0	0.1	0.6	0.8	2.0	2.1	0.8	3.6	1.6	0.9	2.9	2.1	2.2	0.5
	4-hydroxy benzoic acid	3.3	4.4	2.1	3.0	6.8	4.4	5.4	6.4	0.9	4.7	3.8	3.9	5.3	3.4	4.4	1.9	4.1	6.1	2.0	3.8	1.7	4.9
Aminoacids	L-arginine	5.1	2.9	1.4	3.4	1.6	1.8	2.1	3.9	0.3	1.4	3.8	4.2	2.6	3.7	3.8	5.5	3.6	3.9	3.4	4.6	2.9	1.9
	L-asparagine	4.2	4.2	5.3	2.4	3.5	1.6	5.4	6.8	3.9	4.4	4.4	6.8	4.6	4.5	3.6	2.3	5.6	3.5	3.7	4.7	3.5	4.2
	L-phenylalanine	3.6	3.2	1.8	4.8	1.6	2.6	1.6	2.3	2.1	1.3	2.1	1.5	2.6	1.6	1.3	1.6	1.9	1.6	1.2	2.7	1.3	1.5
	L-serine	3.6	4.1	5.7	2.0	3.2	1.6	3.0	2.3	0.6	8.0	2.7	2.9	3.6	4.2	1.9	2.9	3.9	4.3	3.7	3.6	6.9	1.1
	L-threonine	1.5	2.0	1.3	2.3	3.7	0.9	2.0	2.9	0.6	0.9	2.3	2.1	2.1	2.6	1.4	2.9	2.6	2.2	3.2	2.4	2.6	2.0
	glycyl-L-glutamic acid	1.6	1.9	1.8	3.9	1.6	1.1	0.7	1.4	1.5	0.5	2.0	1.6	2.1	2.2	2.2	1.6	1.6	3.0	2.4	2.2	1.8	1.1
Amines	phenylethyl-amine	3.8	2.8	0.1	2.2	1.3	7.4	6.1	5.2	0.3	0.2	2.5	2.3	3.8	2.7	4.6	0.4	3.3	1.1	3.6	1.8	1.8	0.7
	putrescine	2.9	4.1	4.1	6.8	0.9	3.6	4.2	5.9	8.2	3.7	4.4	4.2	2.8	3.5	6.4	4.9	2.3	5.2	4.0	3.9	6.9	4.7

<1	1–2	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	>10

3.3. Community Level Physiological Profiles

The CLPP data were expressed as percentage contribution of the degradative activity on each substrate to the total. The data integration by nMDS representations summarizes how changes in the hydro-geochemical conditions corresponded to a shift in the utilization of organic substrates provided (Figure 4).

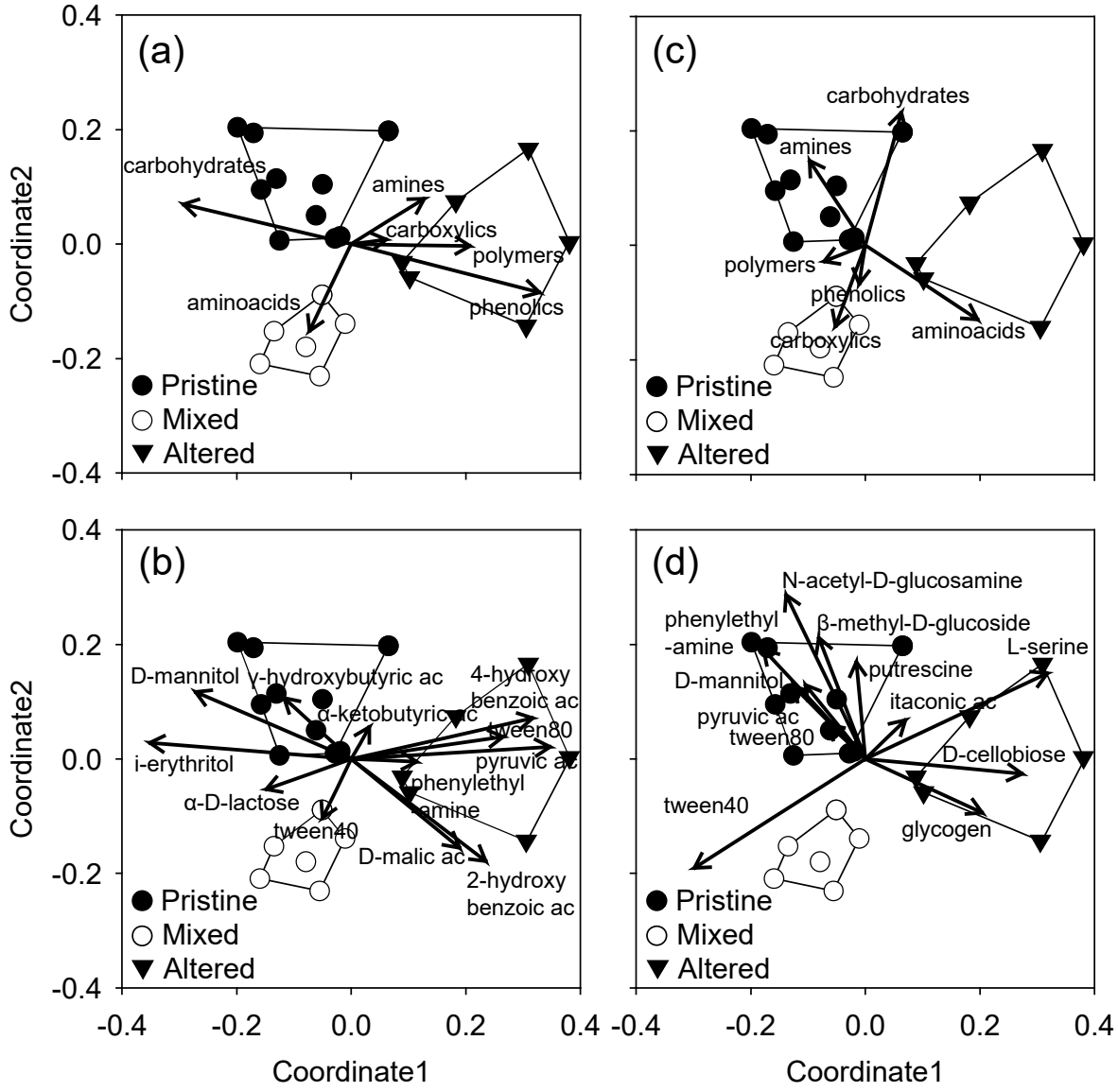

Figure 4. Variation patterns of the six class of compounds and of a selection of 12 substrates (i.e., those explaining 50% of dissimilarity among groups) at 24 h (**a**), (**c**) and 168 h (**b**), (**d**). Data were expressed as AUC values normalized for the AWCD. NMDS ordination plot of sampled waters is based on geochemical and microbial community characteristics (see Figure 2).

The utilization patterns of organic substrates in the early stage of incubation ($CLPP_{24h}$) showed that microbial communities from pristine conditions fed preferentially on carbohydrates (Table 4; Figure 4a), which contributed on average for 38.8% of the total absorbance. Inside this class of compounds, i-erythritol was the most degraded compound (6.0%), followed by D-mannitol and D-lactose, (5.9% and 5.5% respectively) (Figure 4b). Carbohydrates utilization decreased significantly to 33% at 168h incubation (NPMANOVA; $p < 0.01$) and shifted toward the utilization of other compounds such as β-methyl-D-glucoside and N-acetyl-D-glucosamine (Figure 4d). Carboxylic acids contributed

to the $CLPP_{24h}$ for 22.0%, although only four out of eight compounds were utilized (Table 4). Most of the contribution was assigned to the γ-hydroxibutyrric acid (5.0%) (Figure 4b). The utilization of pyruvic acid methyl ester increased consistently at 168 h, passing from 0.6% to 5.0%. Aminoacids and polymers contributed for 18.9% and 12.9% of the total absorbance, respectively. The $CLPP_{168h}$ changed significantly due to the significant high utilization (NPMANOVA; $p < 0.05$) of polymers, phenolic compounds and amines (Figure 4c). In particular tween 40 (4.5%), phenylethyl-amine (2.9%) mostly drove this change (Figure 4d).

The $CLPP_{24h}$ in mixed conditions relied preferentially on carbohydrates (36.5%), mainly driven by i-erythritol (5.8%) and D-mannitol (5.4%) (Table 4), that declined after 168 h incubation (NPMANOVA; $p < 0.01$) (27.3%) (Table 4). The carboxylic acids contribution varied significantly in the $CLPP_{24h}$ and $CLPP_{168h}$ (22.1% and 25.1%, respectively) and relied mostly on D-glucosaminic acid (3.9% and 4.5%, respectively) (Table 4). Polymer utilization (13.0%) at 24 h was driven mainly by tween40 degradation (5.1%) (Figure 4a,b). As for pristine conditions, amines and phenolic compounds, contributed at lesser extent to the $CLPP_{24h}$ (5.0% and 3.5% respectively). Polymers and phenolic compounds increased significantly (NPMNOVA; $p < 0.05$) from $CLPP_{24h}$ to $CLPP_{168h}$ (17.9% and 5.4%, respectively) and mainly contributed, together with carboxylic acid degradation, to define the CLPP at 168h (Figure 4c).

In altered conditions, the $CLPP_{24h}$ was characterized by a wide utilization of the provided substrates (Table 4) and no statistical changes were recorded in the degradation patterns over the incubation time (Table 4). Carbohydrates (31.5%) were utilized preferentially, although at lesser extent with respect to the pristine and mixed conditions (NPMANOVA; $p < 0.05$). A relatively higher contribution of carboxylic acids (23.5%), polymers (14.1%), amines (6.2%) and phenolic compounds (5.7%) was also observed (Figure 4a). Compounds that mostly contributed to the $CLPP_{24h}$ were D-malic acid and pyruvic acid methyl ester (3.0% and 2.4%, respectively), tween-80 (3.4%), phenylethyl-amine (3.2%) and 2-hydroxy benzoic acid and 4-hydroxy benzoic acid (3.1% and 2.6% respectively) (Figure 4b). The relative $CLPP_{168h}$ was characterized for enhanced degradative skills toward specific substrata such as: D-cellobiose (5.4%), glycogen (5.2%), itaconic acid (4.7%), and L-serine (3.9%) (Figure 4d).

4. Discussion

In this study, we found that the landfill-induced geochemical alteration determined changes in the microbial functional diversity and metabolic physiological profile with an increased ability in the substrate utilization passing from pristine to altered conditions (i.e., altered > mixed > pristine). Although partly affected by the limitations of all experimental approaches performed in laboratory conditions, the Biolog assay and the kinetic profiles of substrate degradation allowed assessing the potential metabolic capacity of groundwater microbial communities, as a suitable information to better understand the effects of potential inputs of allochthons organic substrates on the aquifer. This methodological approach was consistently applied in several aquatic settings [37–40].

The Biolog assay relies on the different capacity of aquatic microorganisms to adapt to the spatial and temporal variations in quantity and quality of dissolved OM pool available in the aquatic environment [41,42]. Overall, the bioavailable OM fraction decrease by the transit from surface to groundwater, passing through several soil and sediment layers [43–45]. Therefore, groundwater ecosystems can show oligotrophic conditions and scarce productivity [10,46,47].

Microbial communities from the pristine and mixed groundwater showed the typical traits found in subsurface aquatic environments, with a relatively lower affinity for most of the Biolog substrates [10,48,49]. The heterogeneity in substrate utilization and the decreasing values of functional diversity over time suggested a relatively lower metabolic versatility to adapt to newly available substrates with respect to those in altered conditions. The physical-chemical characteristics in pristine conditions were compatible with the preferential utilization of carbohydrates by microbial communities, being their utilization associated to unbalanced nutrient conditions [50] and decreasing redox potential [36,39]. Carbohydrates represent an important energy-rich carbon source, and constitute storage molecules for the aquatic bacterial metabolism [51], being promptly and preferentially utilized

through the catabolic heterotrophic pathway that involve oxidation of simple or complex carbohydrates and oxygen as a terminal electron acceptor [52,53]. According to the degradation patterns of C-rich substrates, the microbial communities in pristine and mixed condition were likely to be affected by C-limitation. Only after 168 h, microbial communities in pristine conditions developed the ability to degrade polymers (i.e., tween 80) and amines (phenylethyl-amine and putrescine). Polymers are complex carbon substrates, including compounds of synthetic origin. In particular, tween 40 and tween 80 are nonionic surfactants used as oil-in-water emulsifiers in pharmaceuticals, cosmetics, and cleaning compounds. The delay in utilizing tween 80 may reflect the metabolic plasticity [54] that modulates the microbial community functions, suggesting a higher sensitivity of pristine communities to contingent events of contamination by anthropogenic organic pollutants. The shift toward the utilization of amines in the pristine condition might indicate the presence of opportunistic classes of bacteria and a condition of nitrogen deprivation [37,55]. Unlike C-enriched substrates (i.e., carbohydrates and polymers), amines are enriched in nitrogen and utilized by bacteria to synthetize organic molecules such as amino acids and proteins [56].

In altered conditions, microbial communities were relatively more efficient in metabolizing most of all provided substrates, since the early stage of incubation. Although the degrading activity on Biolog carbon sources did not reflect the presence of these specific compounds in the groundwater, a higher homogeneity in substrates utilization and a relatively higher affinity with organic substrates, suggested a higher number of metabolic pathways, which may allow microbial communities to exploit several carbon sources [20,57,58]. The input of OMs of different origins and quality can induce the development of a well-structured community and rare taxa that contribute to enhance the OM biodegradation [42,59]. In addition, the occurrence of fecal indicator bacteria (i.e., *E. coli*) in altered conditions indicated the input of microorganisms originating from the surface environment, suggesting the co-presence of microorganisms with different functional traits other than those originally available in groundwater. In altered groundwater a higher prokaryotic cell abundance and a structural shift toward LNA cells was observed as similarly found for impacted groundwater [24,57,60]. Notably, LNA cells were found to be specifically composed by selected microbial taxa, which were also considered differently responsive to environmental changes than HNA cells [24,61].

In altered groundwater, the metabolic profile relied mainly on carboxylic acids, phenolic compounds, polymers, and amines. Carboxylic acids can be considered part of the labile pool of the organic matter in the aquatic environment, which is an important carbon source for the aquatic microorganisms [62,63]. These substrates are naturally occurring as products of the bacterial degradation and photochemical degradation of the organic matter in surface waters [64,65]. The prompt ability (24 h) in the utilization of complex polymers (tween 80) might indicate the presence of organic pollutants in altered groundwater, being this functional trait linked to polluted environment [55,63]. In this study, organic pollutants were detected at low concentration in some of the altered groundwater sites [25].

Interestingly, glycogen was one of the polymers utilized by microbial communities in altered conditions in the last stage of incubation. Glycogen, representing a source of readily available glucose for many organisms, is a highly branched polysaccharide that consists of glucose units linked in a linear chain. Glycogen is accumulated in bacterial cells during the stationary phase or during inorganic nutrient limitation, conditions that very likely occurred in the last stage of incubation [66]. Moreover, the presence in these groups with specific functional abilities might be deduced, being the degradation of glycogen performed by a set of extracellular enzymes (i.e., glycogen phosphorylases and glycosidases such as α-amylases) owned only by those bacteria that complete the degradative processes to carbohydrates [67]. The consumption of N-rich substrates (phenylethyl-amine and L-serine) across the incubation time of samples from altered conditions, was compatible with a strong nitrogen depletion of the microbial degradative processes given the relatively high activity occurring with respect to pristine and mixed conditions.

5. Conclusions

Changes in groundwater quality can directly affect the functional properties of the aquatic microbial communities with implications on the pattern of the biogeochemical cycling. The Biolog assay provided valuable information for tracking different levels of groundwater alterations. It is topical to understand the structural and functional diversity of the microbial community implied in the C-cycling and ecosystem services (i.e., nitrogen removal, pollutants degradation, and DOC assimilation). The microbial metabolic potential and the environmental factors shaping microbial community functioning are important to understand the potential drivers of biodegradation, thus helping to develop advanced strategies for groundwater management.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/11/12/2624/s1, Table S1: Values of physical and chemical parameters measured in the sampling sites in two sampling campaigns, Table S2: Microbial parameters measured in the sampling sites in two sampling campaigns.

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