In Situ Bioremediation Techniques to Reduce Total Organic Matter Oversaturation of Fluvial Sediments: An Experimental Study

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Abstract: An in situ experiment was performed in sediments of River Magro (east Spain) in order to evaluate the usefulness of microbial bioremediation, both bioaugmentation and biostimulation, as a tool for reducing the excessive organic matter (OM) content in dammed river stretches due to historical wastewater spilling. The study had a prospective approach focused on the application of a biologically active commercial product (BAP), consisting of a mix of bacterial strains, ectoenzymes, and nutrients, where a range of concentrations and temporal dosages of the product were experimentally assayed in situ. They were further combined with the addition of potential organic enhancers, such as acetate, as well as of inhibitors of specific microbial guilds. On the other hand, inorganic electron acceptors for the anaerobic respiration of the organic matter were additionally amended. In additional assays, the BAP additions were combined with inorganic nutrients amendments, or even the latter were tested alone. These combinative treatments aimed at exploring the possible enhancement of synergistic or antagonistic interactions among the amended compounds, as well as the eventual effect of growth limiting factors. The single BAP additions of 50 g/m³ led to OM reductions of up to 17%, and significant removals of nitrogen or phosphorus were additionally observed by increasing or by fractioning the BAP dosage, respectively. However, a better response using the same amount of the BAP was obtained by supplementing it with sodium acetate. In this case, reductions of the OM content reached up to 35% of the accumulated OM, thus indicating that a complementary stimulus is still necessary to run out barriers towards the final steps of the anaerobic OM digestion. This treatment was also linked to the strongest significant drop in the TP content of the sediments. Neither the addition of inorganic electron acceptors nor inorganic nutrients improved the results, or they were even antagonistic of the degradative potential of the BAP product. Apparently, the occurrence of acetoclastic microorganisms, which was demonstrated by high throughput DNA-sequencing, was critical for the optimal OM reductions in the sediments. This exploratory study demonstrates that the applicability of BAPs can be extended to cover the remediation of fluvial ecosystems, and support the complementarity of different bioremediation strategies.

Keywords: River Magro; organic matter pollution; bioaugmentation; biostimulation; biologically active commercial product (BAP); microbial syntrophy; methanogenesis; sulfate reduction

1. Introduction

The organic matter (OM) pollution of river sediments is a major concern for environmental safety. This has a negative effect on the ecological functioning of the riverine ecosystem, as creating a high oxygen demand that diminishes oxygen availability for aquatic biota [1]. This OM also contributes to a sustained release of nutrients into the overlying water, thereby worsening its trophic status [2].
The organic pollutants can also be diffusively transported to the groundwater [3], further spreading the contamination.

A case of a polluted ecosystem is the River Magro, a Mediterranean river located in eastern Spain that suffered historical inputs of domestic sewage and industrial wastewater [4–6]. Apparently, the degradation of organic matter in the sediments of this river progressed at lower rates than its accumulation, especially in dammed sections, and this caused the exacerbated increase of OM contents in the sediments [5]. These concerns are extensive to other freshwater ecosystems [1,5], and therefore, suitable solutions could be applied more widely. This encouraged the river basin authority to explore the use of bioremediation techniques to decrease the undesirable organic loads in the riverine sediments, provided that they should be adapted to the specific characteristics of the polluted site [7].

Minimally invasive in situ solutions for bioremediation of polluted riverine sediments may include bioaugmentation and biostimulation techniques [8]. The former implies the addition of specialized degradative microbes, whereas the latter is intended to enhance the pollutant degradative capacity of the native microbial populations by adjusting the environmental conditions. Both are comparatively low-cost technologies that can be assayed even in sites with reduced accessibility, such as the upper-middle course of many rivers. A widely used commercial product for reducing pollution is the bacterial-enzymatic lyophile complex commercially known as Acti-zyme™ (also licensed as Hycura™). This biologically active product (BAP) is manufactured by Acti-Zyme Products, Ltd. This BAP has been commonly used to improve the functioning of wastewater treatment plants for the removal of chemical pollutants and pathogens [9,10], as well as to promote the generation of biogas [11,12]. However, utilization of this BAP for the remediation of polluted natural riverine ecosystems has been rare so far, despite some promising results having been reported [5].

The anaerobic degradation of the OM via methanogenesis has been advised as practical for bioremediation purposes in highly reduced aquatic sediments with high OM content [13]. In these environments, CO₂ is almost the only inorganic electron acceptor available for energy conservation. This restricts the options to methanogenesis and fermentation, the former completing the final step in the decay of OM. However, there are several environmental constraints that may prevent the growth of methanogens and other pollution-degrading anaerobic microorganisms. One of them is the availability of their metabolic precursors [14]. Among these, acetate (CH₃COO⁻) is a key intermediate compound for the degradation of OM that drives a predominant pathway of methane production in freshwater ecosystems [14,15]. Sulfate-reducing bacteria, some of which can use acetate, outcompete methanogens when sulfate is available, these interactions being a major concern for microbial environmental engineering [16]. Nevertheless, both microbial guilds can co-exist if concentrations of shared substrates are high enough to avoid a competitive exclusion [17,18], which appears to be feasible if there is a high content of OM and enough acetate availability [19]. An analogous competitive scenario might occur between denitrifying bacteria and methanogens [20], though this would depend on the availability of nitrate, which, however, can be limited under the usual low-redox conditions of OM-rich sediments. Experimental additions of acetate have been occasionally carried out to address these concerns and enhance the OM degradative activity [21]. All these issues deserve consideration for the design of bioremediation strategies, in order to adjust them to the conditions of the site.

Accordingly, our study focused on the investigation of the performance of a BAP as a remediation solution to reduce the exceeding OM loads of heavily contaminated streambeds, also suitable for the sediments of other types of aquatic ecosystem. This was examined under different experimental conditions. A first set of treatments included a prospection for an optimized use of the BAP (i.e., amount, temporal dosage, spatial dosage). On the other hand, other treatments consisted in combination of the BAP with other compounds that can act as nutrient amendments or as electron acceptors, or even their use without BAP. This second set of treatments aimed for the assessment of its suitability for complementing the application of the BAP under eventual natural availability limitations of nutrients and/or electron acceptors, and consequently, they were amended at a non-limiting concentration. Finally, inhibitors of sulfate-reduction and methanogenesis were used together with the BAP to show
the relative relevance of these anaerobic respiration processes when the BAP was used. The suitability of each of the treatments is reported and discussed here.

2. Materials and Methods

2.1. Study Site

A mesocosms experiment was conducted in situ in the middle course of the River Magro (1544 km² catchment area, 130 km long), one of the main tributaries of the River Jucar, located in east Spain (Figure 1). The experimental stretch of the river (Figure 1a) was located downstream the towns of Requena and Utiel, where historical pollution has originated mainly from domestic sewage and wineries [4], although farming may have contributed as well [6]. The high OM pollution accumulated in the river sediments is a legacy of periods when wastewater was directly poured into the river [5]. Climate characteristics of the site are typical of the Mediterranean region, showing a marked seasonality, with mean annual precipitation around 500 mm that mainly concentrates in autumn and spring. As typical for the summer months in the site, the weather was mainly sunny and little windy during the assay conducted in the summer, with the water temperature ranging 20–24 °C.

Figure 1. Location of the study site (39.465° N, 1.068° W): (a) Google Earth image of the middle course of River Magro (east Spain) where the experiments were conducted, (b) Image of the river channel upstream the dam where the mesocosms were settled, (c) Tubes used as mesocosms when stacked in the sediments. Note the round holes in its upper part allowing water flow.

This section of the river was chosen because it had a higher content of biodegradable OM compared to other sites, and was not affected by heavy metals as observed in locations nearby, as shown by an extensive survey performed to assess the organic matter and heavy metals content of sediments of this and other rivers nearby. The study area was located just upstream a small dam that caused sediment
retention and the slowing down of the water flow, which were both desirable conditions for the setting up of the experimental mesocosms (Figure 1). Water depth was consistently kept by the dam at around 70 cm over the sediments, and water velocity was very low under these water retaining conditions.

2.2. Characteristics of the Proprietary Biocatalyst

A proprietary BAP provided by Acti-Zyme Products Ltd. (Grand Forks, Canada) was used as the bioaugmentation source in this study. Depending of the country, the owner uses different trademark names as Acti-zyme or Hycura, although the product composition is the same. Description of Hycura in the United States Patent and Trademark Office (Registration Number 5505843) indicates that it contains biologically active enzymes for industrial purposes, such as catalases, amylases, and proteases [11]. The bacterial strains composing the BAP include the spore-forming genus *Bacillus*, as well as other aerobic, facultative anaerobic, and anaerobic bacteria. The product is presented in solid state and it is composed of a lyophilized granular powder. Once the product is re-hydrated, different microbial morphologies can be observed under the microscope inspection of DAPI-stained samples (Figure S1). The safety data sheet of Acti-zyme/Hycura [22] already indicates that it is not expected to produce significant ecotoxicity upon exposure to aquatic organisms and aquatic systems. This was specifically assayed in ecotoxicological tests conducted by the University of Valencia with different bio-indicator organisms (i.e., *Nannochloris oculata*, *Daphnia magna*, *Brachionus calyciflorus*, *Thamnocephalus platyurus*, *Salmo trutta*, and *Anguilla anguilla*), which proved the lack of acute toxicity at concentration levels lower than 1 g/L (much higher than the highest dosage used in our experiments), so it was considered as not representing a risk for the micro and macrofauna, neither to vegetation [22].

2.3. Experimental Design and Setup

All the experiments were developed simultaneously in situ during 45 days after an acclimation period of 2 days. Incubations were conducted during July and August, when temperatures in the river are the highest, and microbial metabolic rates of mesophilic microorganisms are assumed to increase accordingly. Previous assays (data not shown) showed that these higher temperatures optimized the effectiveness of treatments within the assayed timeframe. Mesocosms were made using cylindrical PVC mesocosms (50 cm diameter and 100 cm high) opened both at its top and its bottom. The mesocosms had round holes in its upper part to allow water circulation without affecting sediments (Figure 1c), in order to avoid complete water isolation. They were submerged and partially staked into the sediments, in such a way that its mouth was located around 20 cm centimeters below the water surface. The average depth and volume of the sediment enclosed below the overlaying water were 55 ± 14 cm and 108 ± 27 L, respectively. The immersion in water and the tubes round holes allowed that the flow and natural settling process continued during the experiment. Each of the mesocosms were assigned to an individual treatment, which are summarized in Table 1, to test (1) the effect of the BAP addition, (2) the effect of varying the concentration and spatial distribution of the BAP, (3) the effect of temporally dosing the BAP, (4) the effect of adding electron acceptors for anaerobic respirations, (5) the role of the acetoclastic microbial metabolisms, sulfate-reduction, and methanogenesis, and their potential limitations, and (6) the occurrence of potential inorganic nutrient limitations. Two additional mesocosms contained untreated sediments and operated as controls. All 6 experiments were conducted in parallel. The assay solutions were prepared by dissolving the BAP and/or other amended compounds in river water. Each solution was mixed thoroughly for 2 h, then additions were done by injecting each assayed aqueous solution in three points of the sediments of the corresponding mesocosms to a depth of 50 cm. This proved to be efficient in spreading the solutions inside the sediments confined in the mesocosms. The detailed setting up of each experiment was as follows:
Table 1. Summary of the different mesocosms used to conduct the experiments made in the middle course of the River Magro. The table also indicates the treatments tested by ANOVA (post hoc Bonferroni’s pairwise comparisons) in each experiment. The $H_0$ in the table footnote are the null hypotheses for each experiment. The codes shown here are used as labels for plotting cases in the chart of the principal coordinates analysis (PCO). Two control mesocosms were shared for all experiments. "$\checkmark$" was used for indicating if each treatment is part of an experiment.

| Treatment                                      | Code          | Experiment |
|------------------------------------------------|---------------|------------|
| Non-amended controls (2)                      | Control       | ✓          |
| One dosage of 50 g/m$^3$ of BAP                | BAP-50        | ✓          |
| One dosage of 25 g/m$^3$ of BAP                | BAP-25        | ✓          |
| One dosage of 75 g/m$^3$ of BAP                | BAP-75        | ✓          |
| One dosage of 50 g/m$^3$ of BAP, more spatially distributed | BAP-SP        | ✓          |
| Two dosages of 25 g/m$^3$ of BAP               | BAP-2inj      | ✓          |
| Three dosages of 20, 15, and 15 g/m$^3$ of BAP | BAP-3inj      | ✓          |
| 50 g/m$^3$ KNO$_3$                             |               |            |
| BAP-50 + 50 g/m$^3$ KNO$_3$                    | BAP-NO$_3$    | ✓          |
| 10 g/m$^3$ FeCl$_3$                            | Fe            | ✓          |
| BAP-50 + 10 g/m$^3$ FeCl$_3$                   | BAP-Fe        | ✓          |
| BAP-50 + Na$_2$MoO$_4$ 5 mM                    | BAP-Mo        | ✓          |
| BAP-50 + BES 3.5 mM                            | BAP-BES       | ✓          |
| BAP-50 + 5 g/m$^3$ CH$_3$COONa                 | BAP-Ace       | ✓          |
| BAP-50 + NH$_4^+$                              | BAP-NH$_4$    | ✓          |
| BAP-50 + PO$_4^{3-}$                           | BAP-P         | ✓          |
| BAP-50 + Trace elements                        | BAP-Olig      | ✓          |
| NH$_4^+$ + PO$_4^{3-}$ + Trace elements         | AllNut        | ✓          |
| BAP-50 + AllNut                               | BAP-AllNut    | ✓          |

$H_0$ experiment 1: There is no effect of the BAP. $H_0$ experiment 2: The effect of BAP is not concentration-dependent at the conditions assayed, nor depending on the spatial distribution. $H_0$ experiment 3: There is not an effect of temporally dosing the BAP. $H_0$ experiment 4: There is not an effect of adding NO$_3^-$ and Fe$^{3+}$ as alternative electron acceptors. $H_0$ experiment 5: The acetoclastic metabolisms are not involved in the process. $H_0$ experiment 6: Inorganic nutrients are not limiting factors of the process.

Experiment 1 (effect of the product): This experience aimed to test the efficiency of the BAP in accelerating the digestion of OM accumulated in sediments. This consisted in the addition of a standard injection of 50 g/m$^3$ of the BAP, which uses the concentration previously assayed in the same river basin that resulted in significant effects [5]. This was the concentration of BAP used for the standard additions in the rest of experiments unless otherwise indicated.

Experiment 2 (dosage and spatial distribution of the product): This experiment had optimization purposes for the BAP additions, and was made to assess the dependence of the treatment efficiency on the dose of the product used and its spreading on the sediment. To do this, two concentrations of 25 and 75 g/m$^3$ of the BAP were assayed additionally to the standard dose already assayed in experiment 1. Furthermore, a treatment was also assayed where the standard dose of 50 g/m$^3$ was divided into 10 equivalent dosages (5 g/m$^3$ each) that were injected in 10 different places of the sediment, in order to assess the effect of a higher spatial spread of the inoculum.

Experiment 3 (temporal dosage of the product): These treatments also had a BAP optimization target, in this case, to check the requirement of temporal maintenance of the BAP additions in a natural environment with long renewal times. Thus, the intermediate dose of 50 g/m$^3$ was divided to be added according to two temporal patterns: (1) in two injections of 25 g/m$^3$ each, conducted at the beginning of the experiment and after 15 days; or (2) in three injections, one of 20 g/m$^3$ done at the beginning of experiment, and two of 15 g/m$^3$ performed after 15 and 30 days, respectively.

Experiment 4 (a supplementary addition of electron acceptors for anaerobic respirations): This experimental setting was designed to evaluate the potential of alternative anaerobic metabolisms...
other than methanogenesis and sulfate-reduction to degrade OM, such as the dissimilatory denitrification and the reduction of ferric iron, respectively, which are more thermodynamically favorable than the former. Thus, four different treatments were conducted, whether containing or lacking 50 g/m³ of BAP, and combined with (1) 50 g/m³ of potassium nitrate (KNO₃); or (2) 1 g/m³ of ferric chloride (FeCl₃).

Experiment 5 (role of the acetoclastic metabolisms): A set of incubations were carried out to test the relative role and eventual inhibition of the main acetoclastic microorganisms potentially involved in the process. Treatments consisted in the injection of 50 g/m³ of the BAP accompanied by (1) an addition of sodium molybdate (Na₂MoO₄) at a final concentration of 5 mM, which is a sulfate analogue extensively used to inhibit the growth of sulfate-reducing bacteria [18,23,24]; (2) an addition of 2-bromoethane-sulfate (BES) at a final concentration of 3.5 mM, which is a specific inhibitor of methanogenesis [18,25]; and (3) an addition of 5 g/m³ of sodium acetate (CH₃COONa) as enhancer for acetoclastic microorganisms.

Experiment 6 (a supplementary addition of inorganic nutrients): This experiment was designed to assess a potential inorganic nutrient limitation for the performance of the BAP, which would require a redesign of the remediation protocol. Accordingly, five different treatments were established consisting in the injection of the BAP at the standard dose of 50 g/m³ plus (1) 50 g/m³ of ammonium chloride (NH₄Cl); (2) 12.5 g/m³ of monosodium phosphate (NaH₂PO₄) and 12.5 g/m³ of disodium phosphate (Na₂HPO₄); (3) a solution of trace elements added at a dose of 1 liter of solution per m³ of sediment, this was prepared with the following compounds (in mg/L): H₃BO₃ (50), FeCl₂-4H₂O (2000), ZnCl₂ (50), MnCl₂-4H₂O (50), (NH₄)₂Mo₇O₂₄-4H₂O (50), AlCl₃-6H₂O (90) CoCl₂-6H₂O (2000), NiCl₂-6H₂O (50), CuCl₂-2H₂O (30), Na₂SeO₃-5H₂O (100), EDTA (1000), and 36% HCl (1 mL/L); (4) a combination of all the former nutrient (1+2+3) supplies without BAP and; (5) the same combination as in treatment 4 but including the standard BAP dose.

As described in Table 1, the effect of treatments in the experiments 2 to 6 was compared to both the controls without any amendment, as well as with the treatment containing only the injection of the BPA at the standard dose of 50 g/m³, as performed in experiment 1.

2.4. Sediment and Water Sampling, and In Situ Analyses

Sediment samples from each mesocosm were obtained in 9 replicates, both just before treatments injections, as well as 45 days after, at the end of the experiments, to determine the variations of the studied variables. The sediment samples were obtained with a Russian peat borer sampler [26] provided of extension rods to integrate the sediment sample from surface sediments to the desired depth. This was stacked in the sediments to cut through them by turning clockwise. Accordingly, a semi-cylindrical shaped sediment core was obtained that maintained intact its structure. Depth-integrated sediment samples were obtained from the upper 20 cm and kept frozen until analysis. The variables measured in these sediment samples were the percentage (w/w) organic matter content (%OM), the chemical (COD) and the biochemical (BOD₅) oxygen demand, total nitrogen (TN), total phosphorus (TP), ammonium (NH₄⁺), nitrite (NO₂⁻), and loosely-binding phosphorus (loosely-P). The pH of sediments was measured on a 1:2.5 sediment to water ratio aliquot after the stabilization of the pH measurement in the mix. Measurements were performed with a Thermo Scientific Orion 8102BN Ross combination pH electrode (Thermo Fisher, Waltham, MA, USA). Dissolved oxygen (DO) and temperature in the overlying water inside mesocosms were measured with a WTW Oxi-3310 (WTW, Weilheim, Germany) device provided with an optical oxygen sensor and an electrothermic sensor.

The main sampling effort was dedicated to the characterization of sediments as they were the target of the study. However, an additional survey was conducted to monitor the suspended solids in water and its content in particulate OM to assess possible impacts of OM deposition on the mesocosms. Thus, a volume of unfiltered water sample was obtained from each microcosm at the end of the experiment to measure the total suspended solids (TSS) concentration and the amount of settling matter. Additionally, both at the beginning and the end of the experiments, a subsample of unfiltered
water was kept in high density polyethylene (HDPE) bottles of 125 mL for the total organic carbon (TOC) analysis, whereas another bottle was filled with water filtered through Whatman GF/F glass filters (VWR, Barcelona, Spain), corresponding to a retention of approximately 0.7 µm nominal particle size, for the analysis of the dissolved organic carbon (DOC). All water samples were transported refrigerated to the lab for further analyses. A sediment trap was also installed inside each control mesocosm to assess the extent of the time-integrated settling and possible re-suspension of sediments during the experiment.

2.5. Chemical Analyses of Water and Sediments

Water analyses were performed either on filtered or non-filtered water depending on the variable to be determined, as explained above. All chemical reagents were purchased from VWR (Barcelona, Spain). TSS were determined by filtering non-filtered water samples through a previously weighted Whatman AH-934 glass fiber filter (VWR, Barcelona, Spain), with 1.5 µm nominal particle retention. Concentration of TSS was obtained by considering the final weight of the filter after drying it for 2 h at 105 °C, and the volume of filtered sample. Thereafter, the particulate organic matter content was determined by the difference in weight after burning the filters at 460 °C for 6 h (LOI, loss on ignition). The amount of settleable solids (SS) was measured on non-filtered water samples by the Imhoff cone method [27]. The volume index (VI, mL/g), which is a measure of the settling ability of suspended solids, was calculated by multiplying the SS (mL/L) by 1000, then divided by the TSS (mg/L). TOC and DOC were measured from the respective water samples in a Shimadzu total organic carbon analyzer TOC-V CSN (Shimadzu, Tokyo, Japan) using potassium hydrogen phthalate as a standard.

Sediment analyses were performed from homogenized sediment samples (9 replicates per mesocosms at each sampling). The %OM in sediments was quantified gravimetrically after ignition at 460 °C during 6 h. The COD and BOD5 in sediments were determined as a measure of total and biodegradable OM, respectively, following standard methods [27] adapted to sediments. The relationship between BOD5 and COD was used as proxy of biodegradability of the OM accumulated in the sediment. The TN in sediments was measured as nitrate by the second derivative UV-spectrometry method [28] after an alkaline persulfatic digestion of the sample. The TP was measured as orthophosphate by the ascorbic acid reduction method [27] after an acid persulfatic digestion of the sample. The loosely-binding P (loosely-P) was extracted from sediments with 0.5 M NaCl for 1 h following Corrales-González et al. [29], then measured as for the TP. Ammonium and nitrite were extracted from the pore water with a 2 M KCl solution [30], then ammonium was determined by the phenate method using salicylate [31], and nitrite was measured after reaction with sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride [27]. All spectroscopic analyses were conducted using a DU-7 UV-Visible spectrophotometer (Beckman Instruments, Brea, CA, USA).

2.6. Statistical Analyses

An analysis of variance (ANOVA) was conducted for each experiment to compare the effect of the different treatments in reducing the pollution loads of the analyzed variables (Table 1). The Bonferroni test was performed as a post hoc test for multiple comparisons of treatments within each experiment. The effect of each of the treatments in the experiments 2 to 6 was compared to both the controls without any amendment, as well as with the treatment containing only the BPA at the standard dose of 50 g/m³ used in experiment 1 (Table 1). Replicates of both control mesocosms were joined and processed as one experimental condition, with 18 replicates. Extreme outliers were removed from the descriptive and statistical analysis after an outlier detection analysis. They were considered as such when exceeded 1.5 times the interquartile range below the 1st quartile or above the 3rd quartile respectively. A total of 9 outliers were detected following this procedure, which represented around 0.6% of our dataset. All the statistical analyses were performed using the University of Valencia licensed statistical package SPSS v.22 (SPSS Inc., Chicago, IL, USA).
Furthermore, an analysis of principal coordinates (PCO) was carried out using a squared Euclidean distances matrix. The Euclidean distance was used as the dissimilarity measure between samples. Accordingly, the PCO clustering pattern was used to discern homogeneous experimental results among treatments from those showing a differential effect. This analysis was performed using the vegan package of R software.

2.7. DNA Extraction, Illumina 16S RNA Gene Sequencing, and Taxonomic Classification

A next generation-targeted amplicon sequencing (NG-TAS) analysis of archaeal and bacterial communities was performed in sediments (300–500 mg of sample) of the studied site prior to starting the experiment. The extraction of the environmental DNA was performed using the EZNA Soil DNA isolation kit (Omega Bio-Tek Inc., Norcross, GA, USA). After a NanoDrop quantification of samples, the sequencing of the region V4 of the 16S rRNA gene was done using the Illumina MiSeq system (2 × 250 bp) as previously described in Picazo et al. [32]. Briefly, Illumina compatible, dual indexed amplicon libraries of the 16S-V4 rRNA hypervariable region were created with primers 515f/806r. The PCR reactions were made following Kozich et al. [33]. Completed libraries were batch normalized using Invitrogen SequallPrep DNA Normalization Plates. Then, the Qubit quantified pool was loaded on a standard Illumina MiSeq v2 flow cell and sequencing was performed in a 2 × 250 bp paired end format using a MiSeq v2 500 cycle reagent cartridge. The sequences obtained were then processed using the UPARSE pipeline implemented in USEARCH v11.0.667 [34]. After merging of read pairs, the dataset was filtered by a maximum number of expected errors of 0.5. Chimeric sequences were removed with USEARCH v11.0.667 utilizing the UCHIME [34], against the SILVA 132 database. Filtered sequences were clustered in zero-radius operational taxonomic units (ZOTUs), which are sequences with 100% of identity. Later on, the alignments and taxonomic assignments were done with SINA v1.2.1152 using SILVA 132 database [35]. SINA uses the lowest common ancestor method (LCA). We configured a “Min identity” of 0.8 and a maximum number of search results of 1 per sequence results in “best match” type. Sequences with low alignment quality (<75%) and sequences identified as mitochondria or chloroplasts were removed from the analysis. All the sequence data from this study have been deposited in the Sequence Read Archive (SRA) of the National Centre for Biotechnology Information (NCBI), with BioProject accession number PRJNA635639.

3. Results

3.1. Settling Characteristics of the Suspended Solids and Other Water Features

Anoxic conditions prevailed within the sediment, as the DO concentrations measured in the sediments’ overlying water were always lower than 0.3 mg/L. The TSS concentration in the flowing water at the end of the experiment was 118 ± 25 mg/L on average, from which 2.8% was organic matter. Regarding settleable solids (SS), overall records that ranged between 3.0 and 4.6 cm thickness were obtained for the whole experimental period in the sediment traps set inside the control mesocosms (Figure 2a). SS estimated with the Imhoff cone method (Figure 2c) were 0.6 mL/L on average. Accordingly, a volume index (VI) of 5.08 mL/g on average was calculated. TOC concentrations in water at the beginning and the end of the experiment were 3.93 ± 0.17 and 4.06 ± 0.52 mg/L on average, respectively, where DOC accounted for around 85% of TOC.
3.2. Organic Matter Content, Nutrients, and Other Features of the Studied Sediments at the Beginning of the Experiments

The studied sediments had a thickness of around 1 m and a pH that averaged 8.00 ± 0.27. The average %OM content was 9.26 ± 2.29, whereas average values of COD and BOD5 were 103.09 ± 18.40 mg O2/g dw and 16.96 ± 5.76 mg O2/L·g dw, respectively. The average content of TN and TP were 3.06 ± 0.69 mg/g dw and 1.10 ± 0.28 mg/g dw, respectively. Inorganic fractions of nitrogen varied more among mesocosms and averaged 0.24 ± 0.16 mg/g dw and 0.6 ± 0.49 µg/g dw for NH4+ and NO2−, respectively. The average value of the loosely-binding P (loosely-P) extracted from interstitial water was 0.31 ± 0.09 mg/g dw, thus representing slightly less than 30% of TP.

3.3. Prokaryotic Community Composition of the Studied Fluvial Sediment

The NG-TAS analysis provided a complete and accurate picture of the prokaryotic community structure (Bacteria and Archaea) of the studied sediments. An interactive visualization is provided as a Krona html chart in the supplementary material (Figure S2). This chart has embedded the taxonomic hierarchy of the sample, in such way that taxonomic categories are subdivided into sectors of a pie-type diagram. Accordingly, taxonomy nodes are arranged from the top level of the hierarchy at the center of the pie and progressing outwards. The analysis showed a bacterial assemblage dominated by obligate or facultative anaerobes, most of them belonging to microbial guilds related to the anaerobic degradation of organic matter.

A total of 16,437 sequence reads were recorded, where those assigned to Bacteria represented nearly 99% of the mapped sequences. Among them, Proteobacteria was the dominant phylum, accounting for ca. 58% of the reads. It was by far followed by other phyla such as Epsilonbacteraeota (10%) and Bacteroidetes (6%). The Gammaproteobacteria class dominated among Proteobacteria and mainly consisted on unclassified members of families Steroidobacteraceae, Burkholderiaceae, and Rhodanobacteraceae, whereas sulfur oxidizers (Thiobacillus), sulfate reducers (Desulfurhabdus), and denitrifiers (Dechloromonas) were also relatively abundant. The Alphaproteobacteria, the other
significant though less abundant class of Proteobacteria, also displayed the occurrence of denitrifiers such as *Rubrimonas*.

Among the Epsilonbacteraeota, abundant groups related to the sulfur cycle also occurred, as the Campylobacterales genera *Sulfurovum* and *Sulfuricurvum*, and to a lesser extent, also *Arcobacter*. The Bacteroidetes cluster was composed by diverse groups, dominated by *Asinibacterium* and unclassified members of the family Flavobacteriaceae. The former belongs to the facultatively anaerobic members of the family Chitinophagaceae. Other members of this family were also found, though less abundant, such as *Sediminibacterium*, *Dinghuibacter*, and *Flavihumibacter*. Another moderately abundant phylum, Firmicutes, was predominantly represented by strict anaerobes as well, where diverse families of the order Clostridiales were clearly dominant. Among them, fermentative and proteolytic bacteria such as *Youngiibacter* and *Proteiniclasticum* composed mainly the contribution of this cluster.

The archaeal assemblage represented nearly 1% of the prokaryotic community in the sediments and was dominated by methanogens of phylum Euryarchaeota. All metabolic pathways for methane production were potentially present, which means that either acetate, formate, or H\textsubscript{2} could be used as a methanogenic substrate. The genus *Methanobacterium* and several members of the family Methanoregulaceae were the dominant members of the archaeal community. The later, that particularly uses H\textsubscript{2} and formate as substrates to produce methane but requires acetate for growth, included the genera *Methanoregula*, *Methanolinea*, and *Methanosaeta*. Other Archaea present in the sample that are able to use acetate are those of the family Methanofastidiosaceae. On the other hand, the less abundant group of methanogens recorded in the sample, Methanomassiliicoccales, was phylogenetically distant from all other orders of methanogens.

### 3.4. Experiment 1 (Effect of a Standard Injection of the BAP)

The injection of the BAP as a single dose of 50 g/m\textsuperscript{3} was able to reduce significantly (\(p\)-values \(\leq 0.007\)) both the %OM and COD content of the sediments by 17% and 16%, respectively, compared to the non-amended controls (Figure 3). Reductions of around 28% of the BOD\textsubscript{5} were also observed, but this did not statistically differ from those of the controls because of the high dispersion in the data of the control replicates for this variable, which did not occur for the %OM and COD. TN and the inorganic fractions of nitrogen also decreased (Figure 4), but statistical significance (\(p\)-value = 0.036) was only found for nitrite compared to the control, decreasing by around 14%. Contrastingly, this did not entail a significant net reduction of the TP nor loosely-binding P content of sediments, that even increased slightly though not significantly.
Figure 3. Box and whisker plots of the variation of the percentage of organic matter content (%OM), the chemical oxygen demand (COD), and the biological oxygen demand (BOD$_5$) after 45 days of incubation for the different experiments conducted in the River Magro. The box and whisker plot show the minimum value, first quartile, median (solid line), third quartile, and maximum value of the data set. The gray dotted line shows the zero-line for the variations, built-up from the average values for the variable at the beginning of the experiment. Outliers are indicated as black crosses. The codes of samples are described in Table 1. One and two asterisks denote significance at confidence levels of 95% and 99%, respectively. The color of asterisks refers to pairwise comparison between any treatment with either the control (red) or with the standard dose of 50 g/m$^3$ of BAP treatment shown in experiment 1 (blue).
Figure 4. Box and whisker plots of the variation of the total nitrogen (TN), ammonium (NH$_4$), nitrite (NO$_2^-$), total phosphorus (TP), and loosely-binding phosphorus (Loosely-P), after 45 days of incubation for the different experiments conducted in the River Magro. The box and whisker plot show the minimum value, first quartile, median (solid line), third quartile, and maximum value of the data set. The gray dotted line shows the zero-line for the variations, built-up from the average values for the variable at the beginning of the experiment. Outliers are indicated as black crosses. The codes of samples are described in Table 1. One and two asterisks denote significance at confidence levels of 95% and 99%, respectively. The color of asterisks refers to pairwise comparison between any treatment with either the control (red) or with the standard dose of 50 g/m$^3$ of BAP treatment shown in experiment 1 (blue).

3.5. Experiment 2 (Concentration and Spatial Distribution of BAP)

As shown in Figure 3, an increase in the amount of BAP used for injections to a dose of 75 g/m$^3$ did not improve with statistical significance the effects already reached by the standard dosing of 50 g/m$^3$, though the achieved reductions in %OM and COD slightly increased up to 20% and 24% respectively. On the other hand, reducing the dose of the BAP to 25 g/m$^3$ did not achieve organic matter variations significantly different from the controls, meaning that the effect of the BAP was blurred when the dose was lowered from 50 to 25 g/m$^3$. Similarly, a higher spread of the standard
50 g/m$^3$ BAP dose did not show observable reductions of the OM-related variables compared to the control without BAP injections. Instead, both the %OM and BOD$_5$ in this treatment showed slightly significant increases ($p$-values $\leq 0.032$) compared to the standard 50 g/m$^3$ dose. All these results show that the effects of lowering the dose, or to spread it excessively, result in negligible effects of the BAP additions for reducing the organic matter content in the riverine sediments.

Concerning TN and TP (Figure 4), only the amendments with the highest dose of BAP (75 g/m$^3$) achieved significant reductions of TN, up to 20%, compared to the control, whether neither this treatment nor any of the others amended with the BAP achieved a statistically significant difference in its final TP content compared to the control. Regarding the N and P-forms, the only treatment showing statistically significant differences with either the control or the standard BAP dose (50 g/m$^3$) was that where the standard dose was spread, where ammonium decreased by 68% ($p$-value = 0.001) and the loosely-binding P increased by 13% ($p$-value = 0.02) compared to the control, whereas the nitrite increased ($p$-value = 0.034) compared to the non-spread standard dose treatment.

3.6. Experiment 3 (Temporal Dosing of the BAP)

The temporal dosing of the BAP did not significantly improve ($p$-values $> 0.05$) the removal of organic matter compared to the single standard dose of BAP (50 g/m$^3$), though both alternative dosages also significantly reduced ($p$-values $\leq 0.003$) the %OM (as well as COD for the splitting in three additions) content when compared to the controls, as did the standard dose (Figure 3). Statistically significant reductions of TP were detected, both distributing the standard dose in two or three injections, compared to both the controls ($p$-values $\leq 0.018$) and the standard BAP dose ($p$-values $\leq 0.003$). These reductions were higher, around 55%, with two injections of the BAP (Figure 4). By contrast, none of the alternative dosage treatments significantly varied the TN content. Concerning the N and P forms, both alternative dosages decreased significantly the ammonium content compared to the control ($p$-values $\leq 0.004$), though a significantly higher reduction ($p$-values $< 0.001$) of ~70% compared to the standard 50 g/m$^3$ dose was also observed in the treatment temporarily fractioned in three injections. In this treatment, this occurred in parallel to a significant ($p$-value $< 0.001$) increase of nitrite compared to both the controls and the standard BAP dose.

3.7. Experiment 4 (A Supplementary Addition of Inorganic Electron Acceptors)

The addition of inorganic electron acceptors (nitrate and oxidized iron) in order to enhance anaerobic respiratory processes other than sulfate reduction and methanogenesis, not only did not improve the performance of the standard BAP dose (50 g/m$^3$) in reducing the organic matter content, but instead blurred its effect. Thus, even when BAP was added together with the respective electron acceptor, no differences were found with the values of the organic matter variables of the controls ($p$-values $> 0.05$), whereas all treatments gave significantly higher OM values than those of the standard dose without amendments ($p$-values $< 0.05$ or $p$-values $< 0.001$ depending on the variable). Such way, these additions clearly difficult the positive effect of the BAP for organic matter degradation. The main significant changes promoted by the treatments made in this experiment were related to the increase of nitrite in the treatment with KNO$_3$ addition (Figure 4), increasing by one order of magnitude (until 1.2 µg/g dw), compared to both the non-amended controls ($p$-value = 0.003) and the standard dose of BAP ($p$-value $< 0.001$).

3.8. Experiment 5 (Assessing the Role of Acetoclastic Metabolisms, Sulfate Reduction, and Methanogenesis)

The addition of acetate as substrate for the acetoclastic metabolism improved the performance of the standard dose of BAP in reducing the organic content of the assayed sediments (Figure 3). When acetate was added, the %OM and COD decreased by 35.0% and 22.7%, respectively, both yielding significantly higher decreases ($p$-values $\leq 0.01$) compared to the control and even, a significantly higher %OM decrease ($p$-value $< 0.001$) compared to the standard BAP (50 g/m$^3$) dose. These reductions were the highest observed in the whole study. Contrastingly, all OM-related parameters increased compared
to the control, though not achieving statistical significance, when the BAP was supplemented either with the inhibitors BES or with molybdate. Moreover, these amendments even showed significantly higher values of the organic matter variables compared to the standard dose treatment (p-values ≤ 0.01). These responses indicate that inhibition of either methanogens or sulfate-reducing bacteria affected notably the performance of the BAP treatment, but also that they difficult the already existing anaerobic respiratory processes through these pathways.

Although none of the treatments showed any statistically significant difference in TN compared to the controls and the standard BAP dosage, a noticeable significant reduction (p-values ≤ 0.003) of around 70% of the ammonium content in the sediments compared to the control and standard was observed when the acetate amended to the standard BAP treatment was compared to all the other treatments, or even higher when BES was added (Figure 4). Moreover, for the BES amended treatment, a concomitant increase in nitrite was significant compared to both the control and the standard BAP dosage. Contrarily, in the molybdate amended treatment, the ammonium increased compared to both the control and the standard BAP dose, whereas nitrite was significantly lower than in the control for this treatment. Concerning phosphorus, TP concentration showed the highest (~ 50%) and highly significant (p-values ≤ 0.001) decreases when the BAP was supplemented with acetate compared to both the control and the standard BAP dose treatments (Figure 4). The BES amended treatment showed a similar behavior. The increases in loosely-binding P, though not statistically significant for all comparisons in this experiment, may show that the decreases in TP can be explained by a higher P-mobilization capacity induced by the BAP, as also shown by the rest of experiments.

3.9. Experiment 6 (A Supplementary Addition of Inorganic Nutrients)

The addition of inorganic nutrients did not yield significant variations in the organic matter variables when compared to the controls nor with the standard BAP dose, except a slight facilitation of the aerobic digestion of OM when compared to the standard BAP dose (Figure 3), as indicated by the significant BOD₅ increases in most of these treatments (p-values ≤ 0.048). However, this was not translated into net reductions of the %OM content nor COD. Concerning N and P variables, most treatments did not show significant differences neither with the controls nor with the standard BAP dose.

3.10. Multivariate Analysis

Figure 5 shows a two-dimensional principal coordinate analysis (PCO) performed on the Euclidean distances based on the response of the different treatments, namely, the variation of the measured variables after 45 days of incubation.

The PCO analyses confirmed the main differences shown by the ANOVAs. The grouping and loadings of variables in the PCO must be interpreted as the variation responses of the tested variables at the end of the experiment. The analysis explained a percentage of the variance in the first and second axes of 43.1% and 25.9%, respectively, and showed clear separate clusters. The treatments with the standard BAP dose (50 g/m³), either on a single temporal dosage or splitting it in two equivalent injections of 25 g/m³, as well as the increased dose (75 g/m³) and, especially, the treatment where the standard dose of the BAP was amended with acetate, were fairly distinct to the others. These, by far, showed the highest effectiveness in the reduction of organic matter variables and major nutrients, as it is clearly shown by the variables linked to these samples. On the other hand, ineffective treatments regarding organic matter reduction, such as the addition of BES with its inhibitory effect, were partially useful for the reduction of some of the nutrient forms, such as TP and ammonium. Finally, the most unsatisfactory results in reducing the organic and nutrients loads were found for the rest of the treatments, which are grouped on the right part of the PCO biplot.
impacted the river’s aquatic biodiversity and ecological functioning [5]. Nowadays, the implementation of wastewater treatment plants (WWTP) has improved the quality of water poured into the river, and few native microbial communities are not totally able to handle this excess of organic pollution following its accumulation rhythm and, consequently, OM accumulates in the sediments of dammed river stretches [5].

Figure 5. Principal coordinate ordination (PCO) based on the Euclidean distances for the response to the studied variables in the different experiments. The color lines numbered from 1 to 3 represent the hierarchical structure (i.e., subdivision points in the tree) for the Euclidean distances among cases. Abbreviations of treatments (in blue bold) are as follow: Control (non-amended control), BAP-50 (one dosage of 50 g/m$^3$ of BAP), BAP-25 (one dosage of 25 g/m$^3$ of BAP), BAP-75 (one dosage of 75 g/m$^3$ of BAP), BAP-SP (one dosage of 50 g/m$^3$ of BAP, more spatially distributed), BAP-2inj (two dosages of 25 g/m$^3$ of BAP), BAP-3inj (three dosages of 20, 15, and 15 g/m$^3$ of BAP), NO$_3$ (50 g/m$^3$ KNO$_3$), BAP-NO$_3$ (BAP-50 + 50 g/m$^3$ KNO$_3$), Fe (10 g/m$^3$ FeCl$_3$), BAP-Fe (BAP-50 + 10 g/m$^3$ FeCl$_3$), BAP-Mo (BAP-50 + Na$_2$MoO$_4$ 5 mM), BAP-BES (BAP-50 + BES 3.5 mM), BAP-Ace (BAP-50 + CH$_3$COONa 5 g/m$^3$), BAP-NH$_4$ (BAP-50 + NH$_4^+$), BAP-P (BAP-50 + PO$_4^{3−}$), BAP-Olig (BAP-50 + Trace elements), AllNut (NH$_4^+$ + PO$_4^{3−}$ + Trace elements), BAP-AllNut (BAP-50 + AllNut). Abbreviations of variables (in black bold) are as follow: %OM (percentage of organic matter content), COD (chemical oxygen demand), BOD$_5$ (biological oxygen demand), TN (total nitrogen), TP (total phosphorus), Loosely-P (loosely-binding P).

4. Discussion

In the past decades, previous to the construction of wastewater treatments plants, the River Magro suffered regular sewage inputs, either domestic or industrial, that resulted in a great disturbance of its natural ecological functioning [4–6,36]. In most of its upper-middle course, the high oxygen demand of the streambed makes the site an ideal candidate for the use of bioremediation techniques that stimulate the anaerobic degradative metabolisms of organic matter. The NG-TAS analysis demonstrated the natural occurrence of a relatively diverse prokaryotic assemblage where different microbial guilds involved in the anaerobic degradation of organic matter dominated the native prokaryotic community of the River Magro sediments. However, these native microbial communities are not totally able to handle this excess of organic pollution following its accumulation rhythm and, consequently, OM accumulates in the sediments of dammed river stretches [5]. Nowadays, the implementation of wastewater treatment plants (WWTP) has improved the quality of water poured into the river, and few
inputs of organic matter occur. Accordingly, the analysis of the overlying water during the experiment indicates a relatively low concentration of particles experiencing a rapid settling. Still, some minimal deposition of OM might occur inside mesocosms during incubations, which otherwise did not affect the results of the experiment. The improvement of the quality of waters poured into the river now puts the focus on the main pollution problems in the river sediments, which keep a high organic and nutrient load, thus challenging the recovery of the river ecological quality.

The BAP selected for our experiments (Acti-zyme™) has been used to treat wastewater under both aerobic and anaerobic conditions [5,9–11]. Biochemical tests conducted with this product show the occurrence of a general hydrolytic activity of catalases, amylases, and proteases. However, other enzymatic activities related to the nitrogen cycle (i.e., tryptophanase, urease) are apparently lacking [11]. Attending to that composition, this BAP is expected to accelerate the initial hydrolytic phases of OM degradation, which are followed by a sequential process of acidogenesis, acetogenesis, and finally, methanogenesis [37]. The treatment operating optimally in our study consisted in the addition of BAP supplemented with sodium acetate. This efficacy was primarily assessed in terms of %OM and COD decreases and, in general, both parameters behaved similarly. Overall, the BOD₅ values showed the same pattern except for the treatment containing acetate, where it also decreased but to a lesser extent compared to COD. This yielded a higher BOD₅/COD relationship and suggests that, in this treatment, there was still enough biodegradable material to further increase the reduction of OM. However, it should be noted that, in anaerobic environments, BOD₅ could not be a totally appropriate parameter to show the real biodegradability as in sediments OM degradation should be more related to metabolisms that do not use oxygen as an electron acceptor. On the other hand, the highest decrease in TP content was also shown by the treatment including BAP amended with acetate, which together with the increase in loosely-P, may show the release of inorganic-P from the organic phosphorus as organic matter is degraded.

In previous studies using Acti-zyme™ in wastewater treatment plants [10,12], the concentrations employed were usually lower (only occasionally around 50 g/m³) than the standard dose used in our experiments. Concentrations assayed here were selected based on earlier studies carried out in rivers also located in the Jucar River basin [5]. Our findings indicate possible shortcomings of using lower amounts (i.e., 25 g/m³), or of diluting excessively the product by a high spatial spreading, in such a way that the threshold concentration for the effective functioning of the BAP in natural environments should not be much lower than that used here as standard (i.e., 50 g/m³). This could be the case, at least, for river sediments, which have renewal times much longer than those of the sewage systems. For treating sewage, however, using lower amounts of BAP than those assayed here, but with sustained maintenance amendments, would be enough.

Although our study was not clearly able to solve if the performance of the BAP could be optimized by modifying some basic aspects of the application protocol, a certain improvement in the degradation of organic matter was found by increasing the dose of BAP. However, differences did not commonly reach the statistical significance with the standard BAP treatment. Only for the removal of N and P, some statistical significance was obtained that could motivate further exploration of this possibility. Differently to the assessments conducted for the application of BAP (i.e., changes in the amount, temporal dosage, spatial dosage), the gradual effects of the other treatments were not explored in this study. This is because they had an exploratory purpose to test its suitability for accompanying the standard BAP application. Accordingly, these compounds were provided at non-limiting concentration, in such way that its optimal utilization could be appraised in future experiments once they have proven its effectiveness, or neglected when ineffective.

An important outcome of our study is that acetate clearly benefits the performance of the BAP in reducing the organic and nutrient content. This could have different causes, which might all lead to the production of methane. Acetoclastic methanogens, sulfate-reducing and denitrifying bacteria, all share acetate as electron donor [16,38], and all them were present to some extent in the studied sediments as demonstrated by the NG-TAS analysis. On the other hand, acetate is also used by the syntrophic
acetate oxidizing bacteria [37,39], which in turn produce H\(_2\) or formate that can later be used by both hydrogenotrophic methanogens and sulfate-reducers. An experiment conducted with different inocula from biogas reactors showed that additions of acetate caused a shift from a predominant pathway of biogas generation by syntrophic acetate oxidizing bacteria and hydrogenotrophic methanogens, to another consisting in the direct utilization of acetate by acetoclastic methanogens [40]. Thorough examinations of the functioning of the assayed BAP have shown that this product did not stimulate the production of hydrogen sulfide because of the absence of thiosulfate reductase activity, but instead promoted methane production [11,41]. These assumptions on the role of the different microbial guilds would still require further demonstration supported by additional sequencing studies. Even so, our experiments may shed some light on this subject. The separate addition of molybdate and BES was done to differentially suppress the activity of sulfate-reducing bacteria [18] and methanogens [18,24], respectively. Both inhibitors significantly affected the performance of the BAP, at least regarding the net removal of organic matter, thereby demonstrating that both microbial guilds were involved in the process triggered by the BAP. It is worth mentioning again the likely co-existence rather than competition of both acetoclastic groups [17,18], which is possible even in sulfate-rich environments when shared substrates are abundant [42].

The regulation of methanogenesis might also originate from other causes. A poor performance of methane production has been commonly observed in anaerobic digesters with elevated ammonium concentrations [43,44]. This agrees with the results found in our study, since the higher increases of ammonium were found in those treatments being less effective in reducing OM. Electron acceptors as nitrate, and its denitrification products such as NO\(_2\), can also have adverse effects on methanogenesis [45]. This is because nitrogen oxides increase the redox potential above that suitable for methane production, which otherwise could be additionally related with a harmful effect of these compounds [46]. This is consistent with the results of our experiments, as the experimental additions of nitrate and, in some cases, the increases of nitrite, occurred in those treatments that less efficiently reduced the OM.

Hence, treatments supplemented with either nitrate or iron to be used as electron acceptors were conducted, aiming to facilitate anaerobic metabolic pathways other than sulfate-reduction and methanogenesis. This was part of the prospective study and intended to explore the possibility of taking advantage of the biostimulation of native denitrifying or iron-reducing bacteria, respectively. However, our results were not promising in that sense, as no significant reductions of organic matter were achieved in any of these cases. Only a partial increase in the mineralization of nutrients was deduced in view of the increasing concentrations observed for some of them. Similarly, the additions, in other treatments, of nitrogen, phosphorus, or oligoelements, did not improve perceptibly the reduction of OM, which indicates that neither the action of the BAP nor that of the native microbial guilds dominating the studied sediments were strongly limited by these nutrients. Only the slight improvement of the BOD\(_5\) relative to the COD observed in these treatments, which occurred in other treatments as well, as commented above, led to consider that these amendments could be covering some nutritional requirements for the aerobic heterotrophic microorganisms. On the other hand, variations of inorganic N in the experiments were strongly related to the pH of sediments, which is also an important factor in the degradation of OM [37]. The pH correlated positively to ammonium and negatively with nitrite concentrations, respectively, as displayed by the correspondent vectors in the PCO (Figure 5). This is because an alkalinization of the environment firstly occurs by the release of ammonium via the microbially catalyzed urea hydrolysis [47]. However, pH decreases again as ammonium is later removed during nitrification. Moreover, anaerobic ammonium oxidations can also occur in anoxic environments [48]. Concerning phosphorus, the increases of loosely-binding P usually found when the BAP was treated are likely related to the capacity of the BAP to enhance organic matter degradation, where part of the organic-P is released as this easily bioavailable P-form.
5. Conclusions

Our mesocosms experiments set a suitable system to assess the performance of the tested BAP (Acti-zyme™) as a remediation solution to treat OM-polluted river sediments. This represents an uncommon use of this biocatalyst product. As deduced from the study, an integrated treatment appears to be more convenient than the single use of the BAP, in such way that an optimization is possible with special focus on the syntrophic/methanogenic steps from acetate. Again, this is a novel capacity for the product. The consequences of treatments could already be found in a medium-short time frame, notwithstanding the fact that removal efficiencies could increase even more in a longer time frame. Outcomes indicate that the inoculum should be above threshold concentrations to trigger the degradation process of OM, preventing the occurrence of rate limiting steps. Furthermore, these concentrations appear to be higher than those usually used for conventional applications of the BAP for wastewater treatments, indicating that a stronger initial dose is likely required in natural ecosystems where the degradation of highly preserved OM is blocked. Our outcomes provide then new information on the applicability of Acti-zyme™ as a bioremediation product in natural environments, where research on this subject is still scarce. But beyond this application, our findings can also be useful for its more conventional uses. We claim for further studies incorporating molecular-based analyses to fully understand the role of the microbial populations involved.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/12/4308/s1. Figure S1: Microphotographs of the BAP cells stained with 4′-6-Diamidino-2-phenylindol (DAPI) after 18 h of rehydration. White bar indicates 5 µm. Figure S2: Krona chart showing the relative abundance and diversity of Bacteria and Archaea in a sample of the studied fluvial sediment with a total number of 16,437 assigned reads. This html file consists in a multilevel pie chart that allows an interactive summary and visualization of the complete taxonomic annotation of the reads obtained.

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