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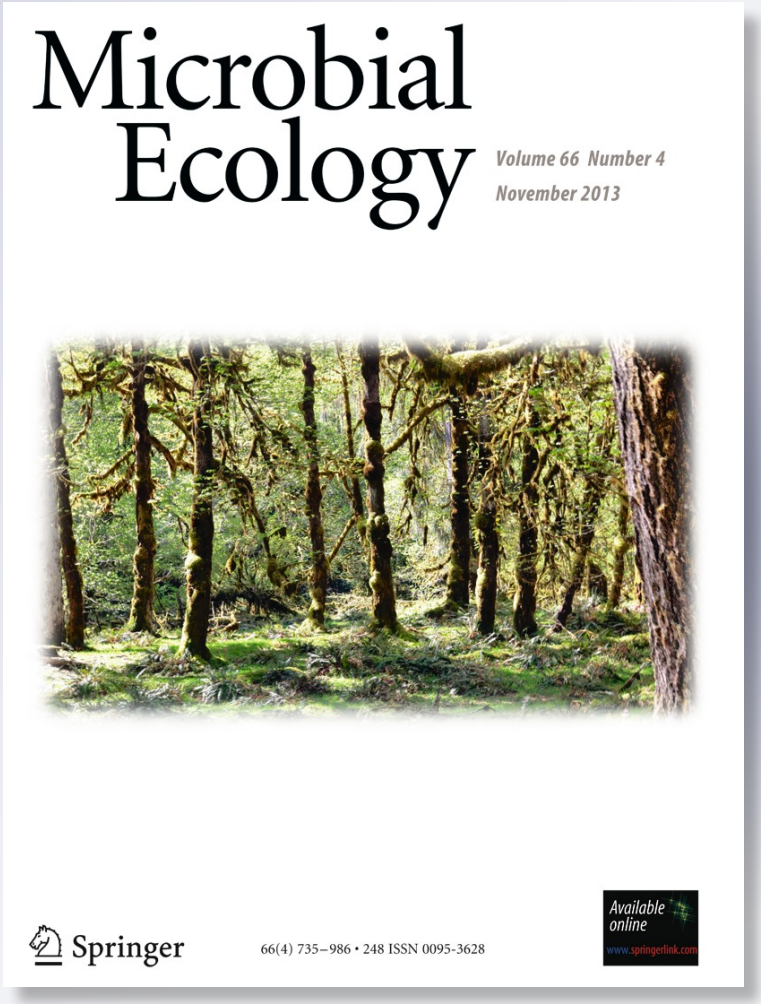

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The Combination of Different Carbon Sources Enhances Bacterial Growth Efficiency in Aquatic Ecosystems

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Abstract The dissolved organic carbon (DOC) pool is composed of several organic carbon compounds from different carbon sources. Each of these sources may support different bacterial growth rates, but few studies have specifically analyzed the effects of the combination of different carbon sources on bacterial metabolism. In this study, we evaluated the response of several metabolic parameters, including bacterial biomass production (BP), bacterial respiration (BR), bacterial growth efficiency (BGE), and bacterial community structure, on the presence of three DOC sources alone and in combination. We hypothesized that the mixture of different DOC sources would increase the efficiency of carbon use by bacteria (BGE). We established a full-factorial substitutive design (seven treatments) in which the effects of the number and identity of DOC sources on bacterial metabolism were evaluated. We calculated the expected metabolic rates of the combined DOC treatments based on the single-DOC treatments and observed a positive interaction on BP, a negative interaction on BR, and, consequently, a positive interaction on BGE for the combinations. The bacterial community composition appeared to have a minor impact on differences in bacterial metabolism among the treatments. Our data indicate that mixtures of DOC sources result in a more efficient biological use of carbon. This study provides strong evidence that the mixture of different DOC sources is a key factor affecting the role of bacteria in the carbon flux of aquatic ecosystems.

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Introduction

Dissolved organic carbon (DOC) is an important component of the total carbon pool in aquatic ecosystems, including lakes, rivers, seas, groundwater, and marshes [1]. Bacterioplankton is the major biological component involved in the degradation and mineralization of the DOC pool and can significantly affect the fate of DOC in aquatic systems [2]. Incorporated DOC has two metabolic pathways in bacterial cells: mineralization into CO_2 via bacterial respiration (BR, catabolism) or incorporation into bacterial biomass through bacterial production (BP, anabolism). The proportion of DOC incorporated by bacterial cells that is assimilated into bacterial biomass comprises an index known as bacterial growth efficiency (BGE) (BGE = BP/[BP + BR]; for a review, see del Giorgio and Cole [3]).

Changes in BP, BR, and BGE directly affect the relative contribution of bacterioplankton to the flux of energy and matter through aquatic trophic chains or to DOC mineralization and release of CO_2 from aquatic ecosystems. BP, BR, and

BGE are usually regulated by temperature, the availability of inorganic nutrients, such as nitrogen and mainly phosphorous, and characteristics of the DOC pool, such as its origin, quality, and composition [4–13]. For instance, autochthonous DOC from phytoplankton is considered to be the most important source of organic matter for BP [7, 14–16], whereas allochthonous DOC is more biologically refractory and usually fuels BR rather than BP [17]. The bulk DOC can be composed of different sources that present different degrees of biological availability. However, the majority of studies focused on the influence of DOC quality on bacterioplankton metabolism did not take into account the potential interactive effects between DOC molecules of different origin.

Studies on the soil microbial community suggest that the different organic substrates present in the soil synergistically interact, affecting the composition and diversity of the soil microbial community [18] and increasing the ecosystem functions driven by these organisms (e.g., carbon decomposition) [19, 20]. Similarly, this process has a high potential to occur in aquatic systems, particularly in lotic and shallow lentic ecosystems in which several different autochthonous and allochthonous carbon sources (e.g., phytoplankton, periphyton, aquatic macrophytes, sediment bed, and drainage area) may contribute to the bulk DOC. Changes in the proportion and composition of autochthonous and allochthonous DOC in the water column occur constantly in nature, e.g., through temporal and spatial changes in the contribution of autochthonous compartments or community succession over the year, local and regional rainfall influences on the input of allochthonous carbon from the drainage basis, seasonal or stochastic fluctuations of terrestrial primary production, variation in autochthonous DOC quality due to fluctuations in phytoplankton community composition, and the confluence of rivers with distinct characteristics [21].

To our knowledge, there is only one study that consistently evaluated the effects of combined DOC sources on BGE in aquatic systems [22]. These authors observed higher BGE in cultures that combined different DOC sources than that expected from single-DOC source cultures [22], though only combined leachates of aquatic macrophytes with natural water samples were evaluated. Thus, investigations on the effects of DOC sources that vary in their chemical composition are still missing. Furthermore, different DOC sources usually support different bacterial communities [23, 24], but no study has focused on the effects of a mixture of DOC sources on bacterial communities. In the present study, we hypothesized that the mixture of different DOC sources would increase the efficiency of carbon use by bacteria (BGE). The main goal of this study was to evaluate the response of bacterial metabolism, bacterial community composition, and bacterial carbon consumption on the presence of different DOC sources that strongly vary in their chemical compositions.

Methods

In the current study, we simulated a mixture of contrasting DOC sources, with algae-originated DOM as the autochthonous end-member and humic substances as the allochthonous end-member; lake water was used as an intermediate DOC source in a biologically available gradient. The treatments were arranged with the addition of each DOC source separately and combined in a full factorial substitutive design.

Experimental Arrangement

Three stock solutions (each of approximately 333 μM C final concentration) were prepared from each DOC source by dilution in Milli-Q sterile water. The experimental set-up consisted of a full-factorial substitutive design composed of seven different treatments: algal extract (Al); humic substances (Hs); Cabiúnas Lagoon water (Ca); Cabiúnas Lagoon water + humic substances (Ca + Hs); Cabiúnas Lagoon water + algal extract (Ca + Al); humic substances + algal extract (Hs + Al); and algal extract + humic substances + Cabiúnas Lagoon water (Al + Hs + Ca). Because a substitutive design was established, the contribution of each DOC source to the cultures varied between treatments, from 0 % or 100 % in single DOC treatments (Al, Hs and Ca), 0 % or 50 % in double DOC treatments (Ca + Hs, Ca + Al and Hs + Al) and 33.3 % in the triple DOC treatment (Al + Hs + Ca). Four replicates were prepared for each treatment and all the treatments were established with the same final DOC concentration (333 μM C).

The bacterial cultures were composed of 95 % of 0.22-μm filtered DOC source (cellulose ester filters; Millipore) and 5 % bacterial inoculum. The bacterial inoculum was obtained by filtering approximately 500 ml of Cabiúnas Lagoon water through 0.7-μm fiberglass filters (Whatman GF/F, New Jersey, USA) and then through 0.22-μm cellulose ester filters (Millipore) on which most of the natural bacterial community was retained. The bacteria on the 0.22-μm filter were resuspended in 100 ml sterile saline solution (0.9 % NaCl), and the inoculum was preserved in the dark at 4 °C until the establishment of the cultures (less than 12 h). This procedure was previously tested and showed a reduction of only 35 % of initial bacterial density (data not shown). The cultures were poured into BOD bottles (150 ml), previously washed with HCl 10 % and Milli-Q water. No internal atmosphere was left in the bottles.

All the cultures were amended with 50 μM N-NH_4NO_3 and 5 μMP-KH_2PO_4 to avoid bacterial growth limitation by inorganic nutrients. The initial bacterial density in all the cultures was 1.91×10^8 cells l^{-1} (SD, 5.6×10^6), which is approximately 10 % of the natural abundance in Cabiúnas Lagoon [10]. The cultures were incubated in the dark at room

temperature (25 °C) for 120 h. We measured the bacterial density, biomass, and oxygen concentration (to estimate BR) in all cultures every 24 h. At the end of the experiment, the replicates of each treatment were combined into one sample and subsequently filtered through 0.22-μm sterile nitrocellulose membranes (Millipore, Massachusetts, USA) to analyze the final bacterial community composition in the treatments. These filters were packed into sterile Eppendorf® tubes and kept frozen at −20 °C for approximately 2 months until the molecular analysis of the bacterial community.

Preparation of DOC Sources

Three different carbon sources (algal extract, dissolved humic substances, and natural DOC from a coastal lagoon) were used in this study and were chosen according to their chemical composition and suitability for bacterial growth [17]. Briefly, the algal extract was obtained from a monoculture of *Ankistrodesmus* sp. grown in Z8 medium until the stationary phase according to the methodology described by Farjalla et al. [17]. The cultures were placed in 15-ml centrifugation tubes and centrifuged at 10,000 rpm for 20 min. The supernatant was discarded, and the pellets were suspended in 10 ml sterile saline solution (0.9 % NaCl). This procedure was repeated four times to prevent possible contamination with nutrients from the culture medium. The pellets were subsequently suspended in Milli-Q water, and the cells were thermally fractured (freezing at ~4 °C and rapid heating to ~36 °C); the procedure was repeated three times. The tubes were centrifuged at 8,000 rpm for 30 min, and the supernatant was considered to be the algal extract DOC.

Humic substances were extracted from a natural groundwater upwelling named Atoleiro, located in Restinga de Jurubatiba National Park, in the northern part of Rio de Janeiro State, Brazil. The groundwater presents a dark color, and the DOC concentrations reach 14 mM C [25]. A water sample was collected and filtered through 0.7-μm fiberglass filters (Whatman GF/F). The humic compounds were extracted from the filtered water by liquid chromatography using XAD-8 resin, and the humic substances were operationally defined as those compounds that adhered to the resin at pH 2 (in protonated form) [26]. The natural lagoon DOC was obtained from Cabiúnas Lagoon, a coastal lagoon located in Restinga de Jurubatiba National Park (RJ, Brazil), a few miles away from Atoleiro. The concentration of DOC in Cabiúnas Lagoon is approximately 833 μM C, and its littoral zone is densely colonized by aquatic macrophytes [10]. A water sample was collected and filtered through 0.7-μm fiberglass filters (Whatman GF/F) and 0.22-μm cellulose ester filters (Millipore) to remove the bacteria. The Cabiúnas filtered water sample and the algal and humic substance extracts were stored in the dark at 4 °C until the beginning of the experiment (48 h).

Analytical Methods and Bacterial Parameter Calculations

The DOC concentration was measured by catalytic combustion in a Total Carbon Analyzer (TOC-5000, Shimadzu). The oxygen concentrations were measured with a picoamperimeter (Unisense, Aarhus, Denmark) coupled to a high-precision micro-oxygen probe [27]. BR was estimated by the O_2 consumption in the cultures ($[O_2]_{initial}-[O_2]_{final}$) every 24 h and then transformed to carbon using a respiration quotient (O_2 to CO_2) of 1 [3, 7]. The aliquots collected to estimate the bacterial density and biomass were preserved with buffered formalin (final concentration of 3.7 %). The samples were stained with acridine orange (final concentration of 0.01 %) on slides, and the bacteria were counted and measured using epifluorescence microscopy (Axiovert Zeiss Universal) at 1,600× magnification [28]. The bacteria were divided into four classes (rods, cocci, vibrios, and spirilla), and at least 300 bacteria or 30 fields per slide were counted. The biovolume estimation followed the methods proposed by Fry [29] using a biomass conversion factor of 5.6×10^{-13} g C μm^{-3} [30]. Bacterial production (BP) was estimated during the exponential growth phase of the cultures from the difference of the final and initial bacterial biomass at each time point.

We estimated the bacterial growth efficiency (BGE), the rate of bacterial DOC removal (DOC_{rem}), and the DOC bioavailability (DOC_L) from the values of BP and BR. BGE is the bacterial biomass produced per unit of organic carbon substrate assimilated, BGE=BP/(BP+BR). DOC_{rem} was estimated as the amount of DOC removed by the bacterial community for both respiration and production in 1 day (DOC_{rem}= BR+BP). DOC_L is the fraction that is readily available for bacterial growth and is given by the ratio between DOC_{rem} and the initial DOC concentration ($DOC_L=DOC_{rem}/DOC_{total}$). All the rates were calculated during the exponential growth phase of the cultures.

The bacterial community composition profile was determined using denaturating gradient gel electrophoresis (DGGE [31]). PCR-produced bands in the DGGE gel are not a precise measurement of bacterial abundances and richness in the samples, due to possible bias during PCR denaturation, annealing and extension steps, but comprise a useful tool to rapidly analyze the similarity of different bacterial communities [32, 33]. In aquatic microbial ecology, DGGE technique has been used to access the spatial and temporal changes in the profile of microbial community composition [34–36].

Bacterial DNA was extracted from the filters using the FastDNA SPIN Kit for soil (BIO 101, California, USA, following [37]), and PCR amplifications were performed using a thermal cycler (Mastercycler®; Eppendorf). The 16S rDNA primers used were 968 F and 1401R, and a CG clamp was added to the forward primer. The PCR mixtures were prepared with 1 μl template DNA, 1× Taq buffer, 2.5 mM $MgCl_2$ (Invitrogen), 200 μmol each deoxynucleoside triphosphate,

20 pmol each primer, 5 μg bovine serum albumin (Sigma), 1 % formamide, 2.5 U Taq polymerase (Invitrogen), and sterile filtered Milli-Q water to a final volume of 50 μl. The PCR procedure was as follows: a denaturing step of 94 °C for 3 min, followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 1 min at 72 °C, followed by a final extension at 72 °C for 10 min. The DNA amplification and denaturing gradient gel electrophoresis (DGGE) was performed using a Dcode Universal Mutation Detection System (Bio-Rad) at 70 V and 60 °C for 16 h in 0.5× TAE buffer. The 6 % polyacrylamide gels were prepared with a denaturing gradient ranging from 40 % to 70 % for the analysis of the PCR products. After electrophoresis, the gels were stained for 40 min with SYBR Green I nucleic acid gel stain (Molecular probes) and visualized using a STORM (Amersham) image capture system. To investigate the degree of dissimilarity between the bacterial communities established in the different treatments, the DGGE band patterns were analyzed using BIOnumériс software and then converted to a presence–absence matrix.

Bacterial Responses to DOC Source Combinations

The current work aimed at evaluating whether the combination of different DOC sources trigger mechanisms that affect bacterial DOC utilization compared to the performance in single-DOC source treatments. Because a full-factorial substitute design was chosen, we were allowed to compare the bacterial response in combined DOC source treatments to the bacterial response in single-DOC source treatments using the D_t index [38]. D_t index is useful to assess the interaction between different carbon sources to bacterial growth and DOC removal. This index was calculated as follows (Eq. 1):

$$D_t = \frac{O_t - E_t}{E_t}, \quad (1)$$

where O_t and E_t are the observed (O_t) and expected (E_t) values of the bacterial growth and DOC removal parameters in the combined DOC source treatments [19]. E_t was calculated by the sum of the relative contribution of each DOC source in the combined DOC source treatments (p =0, 33.3, 50 or 100 %) plus the parameter value observed in the single DOC source treatments (x) (Eq. 2).

$$E_t = \sum_{i=1}^{3} (p * x)i \quad (2)$$

When the observed results in the combined DOC source treatments were different from those expected, we concluded that the substrates were interacting synergistically. These effects can be both positive in relation to the expected ($D_t > 0$, overyield) or negative in relation to the expected ($D_t < 0$, underyield). However, this model does not allow differentiation between two possible mechanisms (a selection mechanism in which bacteria would select specifically one carbon source or substrate in the mixture or a complementary mechanism in which bacteria exhibit complementary uptake of carbon sources and substrates) [22]. We analyzed the possible complementary effects in the combined DOC source treatments by the proportional deviation of the observed result in the combined DOC treatment (O_t) from the higher result observed among the three single-DOC source treatment (D_{max}, Eq. 3) or from the lower result observed among the three single-DOC source treatment (D_{min}, Eq. 4) [39].

$$D_{max} = \frac{O_t - Max}{Max} \quad (3)$$

$$D_{min} = \frac{O_t - Min}{Min} \quad (4)$$

Transgressive overyielding ($D_{max} > 0$) and transgressive underyield ($D_{min} < 0$) conditions occur when the value of a parameter in a combined DOC source treatment is higher than the highest result observed among the three single-DOC source treatment (Max) and when the value of a parameter in a combined treatment is lower than the lowest result observed among the three single-DOC source treatment ($D_{min} < 0$), respectively. The occurrence of transgressive overyielding provides unambiguous evidence of a complementary mechanism.

Statistical Analyses

We used one-way ANOVA and Tukey's post-hoc test to evaluate the significant differences among the treatments in relation to BP, BD, BR, DOC_{rem}, BGE, and DOC_L. To test the significance of the D_t, D_{max}, and D_{min} values, we performed one-sample t-tests comparing the D_t, D_{max}, and D_{min} values of each treatment with the hypothetical value of zero, which is expected in the absence of interaction effects [40]. The dissimilarities among the bacterial community compositions (presence and absence matrix) in the different treatments were evaluated through Cluster analysis using $1-r$ of Pearson's distance. For this analysis, we only considered significant dissimilarities up to or equal to 50 %. All statistical analyses were performed with the software STATISTICA 7.0 (StatSoft 2001).

Results

The bacterial cultures in all treatments reached the stationary phase after 24 h of incubation; thus, all the bacterial

parameters were calculated between 0 and 24 h of the growth curve. The lowest BR rates were recorded for Hs and the combined DOC treatments, whereas the Ca and Al treatments presented the highest BR rates (Table 1). All the combined DOC treatments presented significant underyield effects on BR (Fig. 1a). However, we could observe transgressive underyield on BR in the Ca+Hs and Ca+Al treatments (Fig. 1a, dark boxplots). Regarding BP, the Al treatment presented the highest values, similar to those presented by the combined DOC treatments (Table 1), whereas the CA and HS treatments showed the lowest BP values (Table 1). Only Al+Hs+Ca presented an overyield effect (Fig. 1b) and also a transgressive overyield effect on BP (Fig. 1b, dark boxplots).

Similar to BP, the absolute differences between the Al and combined treatments presented the highest values of BGE; the lowest values were in the Ca and Hs treatments (Table 1). However, all the combined treatments showed overyield (Fig. 1c) and transgressive overyield effects on BGE (Fig. 1c, dark boxplots). The lowest values of BD, DOC_L, and DOC_{rem} were recorded for Ca and Hs and its combination (Ca+HS), with Al and the other combined treatments showing the highest values (Table 1). However, we found different synergistic interactions for each parameter. BD presented overyield responses in the Ca+Al and Al+Hs+Ca treatments, whereas the other treatments presented non-significant trends of positive effects (Fig. 1d). DOC_{rem} presented an underyield response in the Ca+Hs treatment and an overyield response in the Al+Hs+Ca treatment (Fig. 1e). Only Ca+Hs presented underyield (Fig. 1f) and transgressive underyield effects on DOC_L (Fig. 1f, dark boxplots).

The bacterial community structure was particularly similar in the treatments that included algal extract in the composition (alone or in combination) (Fig. 2). Another community group was formed by the treatments with humic substances (alone or combined with Ca [Hs and Ca+Hs]); the treatment composed of only the Cabiúnas water samples showed the most ubiquitous bacterial community structure (Fig. 2).

Discussion

In this study, we showed that the combination of diverse DOC sources affected the DOC fate inside bacterial cells, resulting in a more efficient use of carbon molecules for bacterial growth and biomass, but not the amount of DOC removed by bacteria. Two alternative hypotheses may explain this pattern: (1) the combination of DOC sources of different origins results in changes in the bacterial community composition, promoting a more efficient use of DOC [41, 42]; and (2) the co-metabolism (complementarity in degradation) of the organic compounds present in the mixture would allow a more efficient use of DOC by bacteria.

Considering the first hypothesis, i.e., that the organic matter composition can alter the microbial aquatic community, a greater diversity of compounds in the mixture could have supported a bacterial community metabolically more diverse and specialized in the degradation of different carbon substrates than the bacterial communities in the single-DOC treatments [41, 43]. In this case, the higher BGE in the combined DOC treatments would be attributed to changes in the bacterial community structure between the treatments. However, our DGGE results do not support this hypothesis. In one hand, the treatments with algal extract (Al, Hs+Al, Ca+Al and Al+Hs+Ca) presented similar bacterial community structures (Fig. 2), but different bacterial metabolism (e.g., BGE) and bacterial DOC consumption rates (Table 1, Fig. 1). On the other hand, treatments with very different bacterial community structures (e.g., treatments Ca and Hs; Fig. 2) had similar rates of bacterial metabolism and bacterial DOC consumption rates (Table 1). In other words, the

Table 1 Means and standard deviations of bacterial density (BD), bacterial respiration rate (BR), bacterial production rate (BP), bacterial growth efficiency (BGE), DOC bioavailability (DOC_L), and DOC removed (DOC_{rem}) in all single-DOC source treatments (Ca, Hs, Al) and combined DOC source treatments (Ca + Hs, Hs + Al, Ca + Al, and Al + Hs + Ca)

Treatment	BD ($\times 10^9$ cells l^{-1})	BR (μM C l^{-1} h^{-1})	BP (μM C l^{-1} h^{-1})	BGE (%)	DOC_L (%)	DOC_{rem} (μM C l^{-1} day^{-1})
Ca	0.95±0.25 ^{A}	1.54±0.23 ^{A}	0.55±0.11 ^{A}	26.5±7.08 ^{A}	18.9±1.15 ^{A}	50.0±3.05 ^{A}
Hs	0.57±0.46 ^{A}	1.07±0.06 ^{B}	0.48±0.32 ^{A}	28.9±13.4 ^{A}	19.8±3.91 ^{A}	37.0±7.33 ^{A}
Al	2.44±0.40 ^{B}	1.67±0.07 ^{A}	2.15±0.58 ^{B}	55.6±6.79 ^{B}	47.96±7.48 ^{B}	91.7±14.3 ^{B}
Ca+Hs	0.79±0.16 ^{A}	0.85±0.08 ^{BC}	0.78±0.30 ^{AB}	46.8±7.35 ^{B}	11.01±2.55 ^{A}	39.2±9.08 ^{A}
Hs+Al	3.49±1.64 ^{B}	1.12±0.03 ^{BD}	4.37±2.06 ^{B}	77.2±8.51 ^{B}	43.2±16.3 ^{B}	132.0±49.8 ^{B}
Ca+Al	3.56±0.79 ^{B}	1.25±0.03 ^{BD}	3.80±1.60 ^{B}	72.9±10.2 ^{B}	39.2±12.3 ^{B}	121.0±38.0 ^{B}
Al+Hs+Ca	2.85±0.89 ^{B}	1.05±0.03 ^{B}	3.19±1.08 ^{B}	74.0±5.91 ^{B}	42.6±11.0 ^{B}	102.0±26.2 ^{B}

All the rates and parameters were calculated in the logarithmic phase of bacterial growth. Significant differences are represented by distinct letters ($p < 0.05$, Tukey's post-test)

Ca Cabiúnas Lagoon water, *Hs* humic substance extract, *Al* algal extract

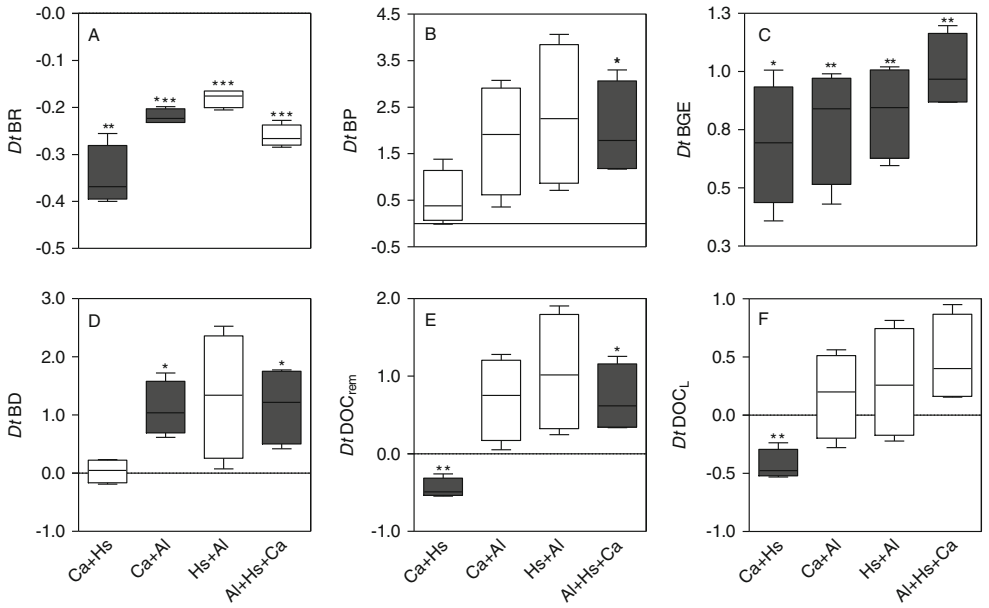

Fig. 1 Proportional deviation of the observed total yield in combined DOC source treatments (Ca + Hs, Hs + Al, Ca + Al, and Al + Hs + Ca) from the expected value calculated from single-DOC source treatments (*Dt*). The *asterisks* indicate that D_t is significantly different from zero (*$0.01<p<0.05$, **$0.001<p<0.01$, ***$0.0001<p<0.001$). The *dark boxplots* indicate when the proportional deviation was lower than the lowest response of a single-DOC source treatment (D_{min} — see the text for details) or higher than the highest response of a single-DOC source treatment (D_{max} — see the text for details). *BD* bacterial density, *BP* bacterial production, *BR* bacterial respiration, *BGE* bacterial growth efficiency, DOC_L DOC bioavailability, DOC_{rem} DOC removed, *Ca* Cabiúnas Lagoon water, *Hs* humic substance extract, *Al* algal extract

dissimilarity of bacterial community composition did not fully explain the differences in the bacterial metabolism encountered among treatments, but changes in the number and type of carbon sources had the strongest effects on bacterial processes and bacterial DOC consumption. Taking into account that we used extreme end-members of DOC quality (i.e., algae extract and humic substances), we could point out that this discrepancy among the quality of DOC sources would drive major changes in the microbial community comparing single and combined treatments, which was not confirmed here (Fig. 2). Thus, if the bacterial community composition somehow influenced the bacterial metabolism in the treatments, it was supplanted by other factors in our study, such as the DOC diversity approach in the alternative hypothesis. We are aware that the DGGE technique might not reveal all taxa present in water samples; groups presented in small abundance are not

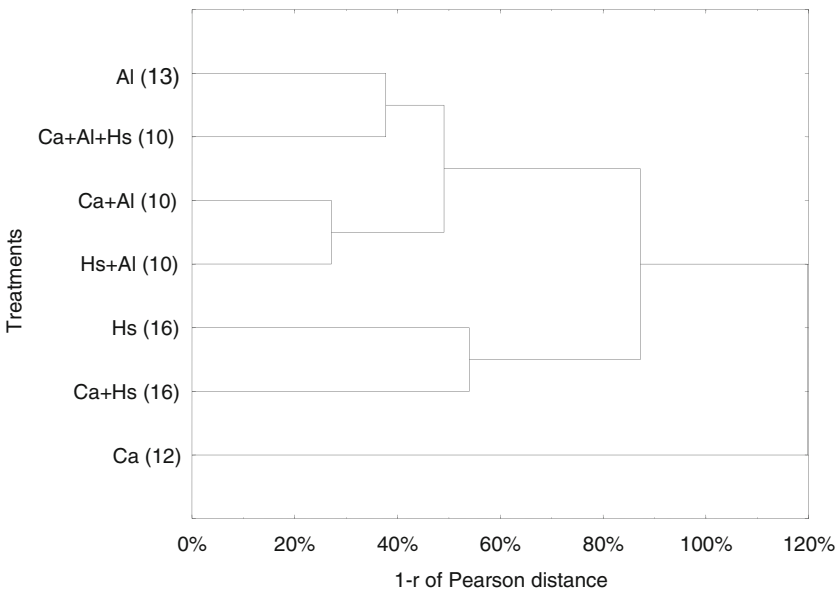

Fig. 2 Dissimilarities between the bacterial communities in the treatments. Cluster analysis of the presence and absence matrix using $1 - r$ of Pearson's distance in which only dissimilarities of ≥50 % were considered significant. *Ca* Cabiúnas Lagoon water, *Hs* humic substance extract, *Al* algal extract. The number of bands recorded based on DGGE is presented between the parentheses next to the treatment identification

detected by the technique and might have had a high impact on the community metabolism. Thus, this hypothesis needs to be further tested with more powerful and modern techniques, such as pyrosequencing.

The alternative hypothesis is that the contrasting quality of the organic compounds present in the mixture would allow a more efficient use of DOC. For instance, a compound of low quality to bacteria would be preferentially catabolized for the maintenance of energy requirements than for growth, resulting in low BGE [3]. Considering that the large molecules and colloids present in the DOC pool must be degraded by exoenzymes before they can be utilized by bacteria [44] and that humic substances are very heterogenic in composition, with each of the components present in low density [45], the high cost of energy for extracellular enzyme synthesis can reduce the metabolism of specific complex compounds [46]. However, the refractory compounds from humic substances present in the Ca+Al, Al+HS, and Al+Hs+Ca treatments might have been degraded by enzymes produced specifically to degrade the labile compounds that would be encountered in higher concentration in the mixed cultures (e.g., the carbohydrates of algal extract) [17] and that can be energetically more profitable [47]. This hypothesis would explain the increase in BGE and the reduction in BR in the combined treatments. De Haan [48] showed that the presence of fulvic acids in a lactate medium increased the *Pseudomonas* sp. (pure cultures) growth rate, suggesting that co-metabolism of refractory organic compounds can occur. Farjalla and colleagues [22] also considered the co-metabolism process as the director of the overyielding and transgressive overyielding found in their mixed treatments (mixture of leachates from different macrophytes species). In this way, a possible explanation for the synergism and transgressive effects encountered, principally with regard to BGE, is the occurrence of co-metabolism of different carbon sources in the combined DOC treatment.

The current study aimed at investigating whether the combination of DOC sources (or increased number of sources) affects microbial metabolism. Our results showed that the combination of DOC sources positively affects BGE, and indicates that this phenomenon occurs as a consequence of complementary degradation pathways and might, thus, be an important mechanism that affects the microbial role in the carbon flux of aquatic ecosystems. In nature, there are several examples of the mixing of different DOC sources that might positively affect the role of microbes in the carbon cycle (for instance, the confluence of the Negro and Solimões Rivers in the Amazon basin; Farjalla submitted). For a better understanding of these mixture effects in nature, it is important that future works include two aspects: (a) the simulation of mixtures of DOC sources at different concentrations for each source (taking the approach of DOC source identity [number of sources and the relative contribution of each source]) because the DOC concentration may directly affect bacterial metabolism [49] and (b) explore different autochthonous DOC sources, such as different algal species, because bacterioplankton may show some preference for DOC produced by a certain phytoplankton species over that produced by another [50].

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