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Different microbial functioning in natural versus man-made Mediterranean coastal lagoons in relation to season

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ABSTRACT

Coastal marsh lagoons are of high ecological relevance playing a key role in the carbon cycle but are threatened to disappear due to global change effects. Restoration practices can counteract this process. This study compares the microbial heterotrophic functioning in three "new" man-made lagoons (created in 2016) to that in three "old" lagoons (two natural plus one created in 2002) from a Mediterranean coastal marsh. The activity of a range of microbial extracellular enzymes, the functional diversity, and the dissolved organic matter (DOM) quality, together with microbial biomass and water physic-chemistry were analysed seasonally in the water column of the six lagoons during 2017. Seasonality was a key driver for the lagoons microbial functioning mainly characterized by lowest microbial activities in winter when DOM was highly aromatic after storm events and an increase in organic matter degradation capabilities from winter to summer probably due to a rise in temperature and DOM input from primary production. Significant differences among lagoons appeared when they were less connected (summer and autumn), and old lagoons showed a greater utilization of proteinaceous and polysaccharidic compounds than new lagoons probably linked to their greater algal biomass (chlorophyll content), which may be supported by their larger phosphorus content. In autumn, there was also a greater use of allochthonous plant material in the old lagoons (higher XYL, and XYL/GLU ratio) probably related to their greater development of riparian vegetation. The functional diversity was the lowest in autumn when the lagoons showed distinct functional fingerprints and the lagoon created in 2002 was grouped with the new ones and distinguished from the natural ones, suggesting that it did not achieve complete restoration. Results indicate that microbial functional parameters related to organic matter use are a relevant and sensitive tool to study lagoon restoration processes, reflecting whole ecosystem nutrient and carbon cycling.

1. Introduction

Coastal marshes are humid environments developed in coastal areas that usually contain salty or brackish lagoons. Such coastal lagoons are of high ecological relevance, especially due to their support for rich biodiversity, the ecosystem services they provide, and their key role in carbon cycling (Millennium Ecosystem Assessement, 2005; Newton et al., 2018). The location of these environments, near the coast, where large population is usually concentrated (Neumann et al., 2015) makes these environments to be exposed to high anthropogenic pressures, resulting, in many cases, in their disappearance (United Nations Environment Programme, 2006). Pressures are very diverse, including fishery, heavy metal and/or nutrient pollution, as well as transformation of the habitat to agricultural or urbanization uses (Gedan et al., 2009). Recently, the awareness of the threats to these ecosystems has led to the development of management and restoration procedures in order to improve their conservation (Gedan et al., 2009). Even though there are different cases of restoration, it is still not clear how much these coastal ecosystems are similar to the natural ones, and, since most of them have been restored in recent years, long term studies are scarce (Mossman et al., 2012). Also, most studies on lagoon ecological restoration focus on the recovery of individual species or habitats while ecosystem functions recovery could be more critical for the ecosystem services these systems provide. While a recovery of specific species or habitats would restore biodiversity and cultural ecosystem services, it would not restore services such as the nutrients and carbon cycling or global climate

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regulation, only recovered if the functions are restored. Thus, not only species composition has to be taken into account in the moment of evaluating up to what extent an ecosystem is recovered, but also if the expected natural functions occur there (Zedler et al., 2001). A restored ecosystem may have a similar species composition as its reference but behave in a different way (Hobbs and Harris, 2001). Nowadays, new methods are needed to study the evolution of restored lagoons, by including functions such as those related to carbon cycling, being most of the previous studies related to nutrients and eutrophication processes (Søndergaard et al., 2007).

Coastal marsh ecosystems usually behave as sinks of carbon due to their high productivity and high accumulation of organic matter in soils and sediments. Additionally, such accumulation takes place in lagoon benthic zones where anoxia conditions may occur, suppressing allochthonous organic matter decomposition and thus slowing down the carbon cycling (Tranvik et al., 2009; Chmura et al., 2003). Microbial communities are implicated in the ecological functioning of these lagoons, being the major responsible for carbon mineralization processes and organic matter dynamics (Kayranli et al., 2010). The microbial community composition and its decomposition activity depend on the nature or quality of the dissolved organic matter (DOM) (Judd et al., 2006; Tranvik et al., 2009). Changes in DOM availability may reflect changes in the major sources of carbon, being either from autochthonous primary production or from allochthonous origin (such as plant material from the riparian area). Therefore, the study of the microbial metabolism and its link to the DOM quality and utilization is the key to understand lagoons' ecosystem functioning. As highlighted for marine ecosystems, the functioning of prokaryotes together with trophic measurements are relevant tools to define environmental status (Caruso et al., 2016). Microbial communities have a great changing potential (Garland, 1997) which allows short-term studies based on their dynamic of responses to environmental changes. This is especially relevant when studying seasonally variable environments such as coastal lagoons in the Mediterranean climate area.

This study analyses Mediterranean coastal lagoons that have a flooding-confinement hydrological cycle. These coastal lagoons receive most of their water from the sea mainly during storms, while entrances of freshwater (surface river water and rains) tend to be minimal and unpredictable. Coastal Mediterranean lagoons' hydrology tends to be very variable and, for instance, long periods of lack of water entrance and high evaporation occur, determining a reduction of water level and even a dry out of the lagoons (Menció et al., 2017; Casamitjana et al., 2019). During these confinement periods, a series of chemical changes to the lagoons take place such as increased salinity, phosphorus accumulation and nitrogen loses through denitrification (Quintana et al., 1998a; Badosa et al., 2006; López-Flores et al., 2014). The dynamics of the ecosystem processes, such as production/respiration, nutrient cycling, and changes in the community composition in Mediterranean lagoons are highly linked to seasonality (especially due to temperature changes) and also significantly affected by unpredictable hydrology changes (Quintana et al., 1998b; Brucet et al., 2006; López-Flores et al., 2006; Gascón et al., 2007). Also, the variability of the physical and chemical conditions might be responsible for the high biological diversity measured in these lagoons (López-Flores et al., 2014).

The studied lagoons are located at La Pletera salt marsh in the Baix Ter wetlands (Girona, NE Spain). They have suffered from high anthropogenic pressure mainly derived from tourism activities as do most coastal Mediterranean ecosystems (Sardá and Fluviá, 1999), especially during the last century. Through an EU Life Project, a restoration and conservation procedure of La Pletera salt marsh has been performed including the creation of new lagoons and the monitoring of both created and already existing ones. The present study focuses on the microbial functioning of pelagic microbiota of newly created lagoons in comparison with that of older lagoons, and to relate it to the availability and use of organic matter. Due to the environmental variability observed in these systems, which could mask restoration efficacy results, this study describes its seasonal variation along one year. Specifically, this study aims to determine: 1) whether the microbial capacity to degrade organic matter in new lagoons after one year of their creation is similar as in the old ones and 2) whether seasonality is differentially affecting the microbial heterotrophic functioning of old and new lagoons. To this aim, microbial organic matter decomposition capacities (by means of a range of extracellular enzymes) and heterotrophic functional diversity and fingerprint (by means of carbon substrate utilization profiles measurement) were analysed in different seasons during the year 2017 (one year after restoration) in three recently created (new lagoons) and three existing lagoons (old lagoons). Complementarily, the quantity and spectroscopic properties of DOM together with chlorophyll (Chl), bacterial density and physicochemical parameters were monitored.

Previous studies in these lagoons performed during the same year showed no differences in the zooplankton communities between the old and the new ones, which has been related to the proximity between lagoons and the high connectivity they have during flooding episodes (Cabrera et al., 2019). Similarly, aquatic microbial communities might be affected by their dispersion capacity and by the proximity and connectivity between the environments (Heino et al., 2015). Also, lake microbial communities have a high resistance to environmental perturbations (Shade et al., 2012). Therefore little differences in the heterotrophic functioning of the microbial communities between these lagoons would be expected. However, in some specific conditions –such as during strong confinement–, environmental pressure may cause a significant effect on microbes, limiting dispersion, and then differences between old and new lagoons in terms of organic matter cycling and microbial functioning are expected to occur.

2. Methods

2.1. Study site

For this study six coastal brackish lagoons located in La Pletera salt marsh, at the north of the Ter river estuary (NE of the Iberian Peninsula, Fig. 1) were analysed. The study area is a natural marsh which selects vegetation and fauna adapted to high salinity, including endangered species such as the Spanish toothcarp (*Aphanius iberus*) (Gesti et al., 2005; Badosa et al., 2007; López-Flores et al., 2010). This area has a great conservation interest but, at the same time, has a strong pressure due to urbanization and tourism. For instance, in 1986 the area suffered from a high anthropogenic pressure resulting in the disappearance of many coastal lagoons. To recover the marsh habitat, several EU-LIFE projects have been developed. In 2002, in the framework of the first Life Project (LIFE99NAT/E/006386) two lagoons of natural origin (Fra Ramon – FRA, and Bassa Pí – BPI) were monitored and G02 was created (old lagoons) (Fig. 1). Within the recent LIFE project ("Life Pletera", 2014–2018, LIFE13 NAT/ES/001001) the marsh area was deurbanized and 3 new lagoons were created in early 2016: L01, L04 and M03 (new lagoons) (Fig. 1). From the beginning of the LIFE Pletera Project all six lagoons have been monitored for physical, chemical and biological parameters in order to control the restoration process. For the present study, data collected in 2017 (one year after restoration) were used.

2.2. Sampling design

The six lagoons were sampled seasonally during 2017 (four sampling times: January 10th, April 19th, July 11th and October 9th) for the study of microbial capacities to decompose organic matter (by means of extracellular enzyme activities), microbial carbon substrate utilization profiles, and DOM quality parameters. On each sampling date, a water sample from each lagoon was collected consisting of a 10 L sample integrating the surface water (between the surface and 30 cm depth approximately) from different points of every lagoon (to this, the sample was collected from a canoe travelling as a circle in the middle of the lagoon). The collected samples were filtered in situ by 50 μm mesh to

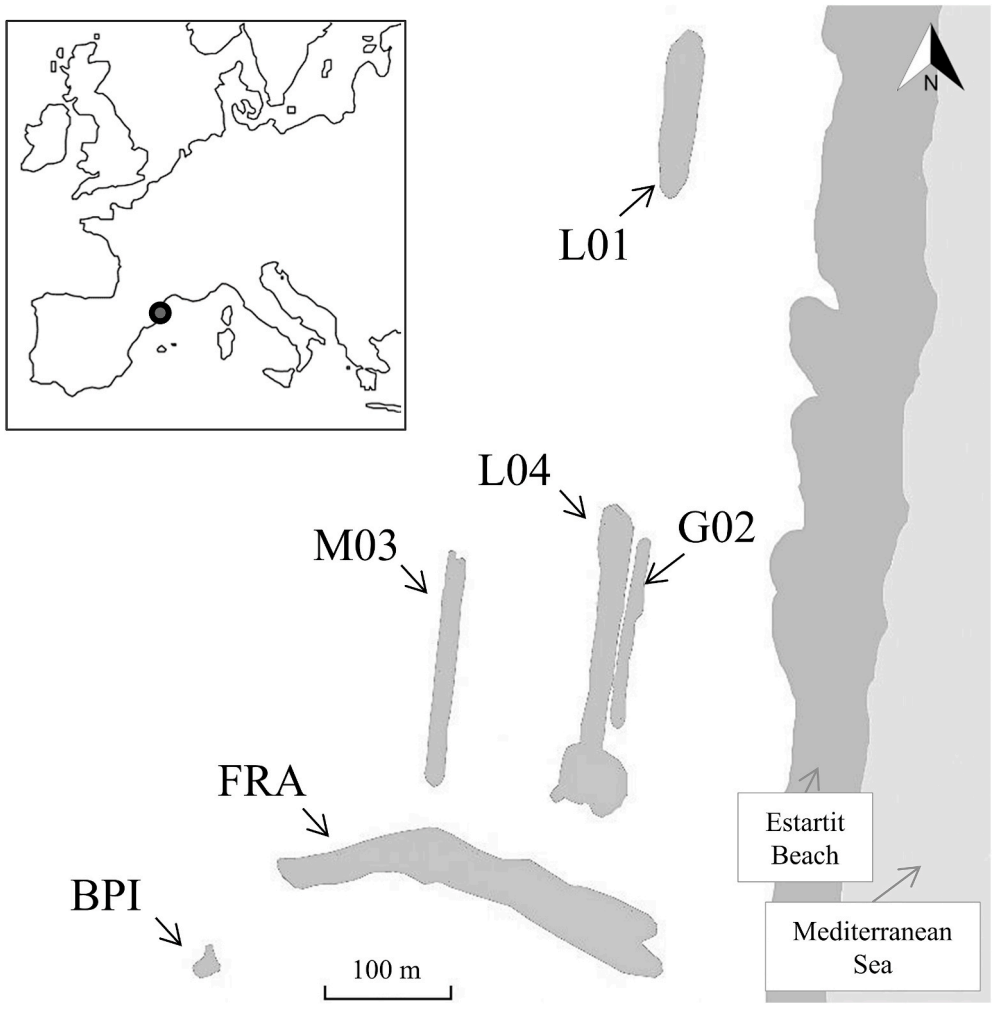

Fig. 1. Map of the study site indicating the location of the six studied lagoons: FRA, BPI, G02 (old lagoons: FRA and BPI natural lagoons, G02 created in 2002), L01, L04 and M03 (new lagoons, created in 2016).

eliminate zooplankton and large sized particulate materials. For the DOM quantity and quality parameters, one sample from each lagoon was kept and preserved in previously burnt glass bottles transported at 4 °C to the laboratory and kept frozen (−20 °C) until its analysis. For the extracellular enzyme activities and carbon substrate utilization profiles analysis three subsamples from each lagoon were collected in situ and transported at 4 °C until their analysis that was performed on the same sampling day. The physicochemical characteristics of each lagoon (temperature, conductivity, pH, dissolved oxygen) were measured in the center of each lagoon by using a Hach HQ30d portable probe.

Complementarily to the seasonal sampling, monthly monitoring data of physical and chemical parameters and bacterial density and Chl were also collected for the entire 2017 for the six lagoons. To this, water samples were collected as described above (10L of an integrated sample per lagoon), filtered in situ (50 μm mesh) and preserved frozen (for inorganic nutrients, carbon content, Chl content) or fixed with formaldehyde (for bacterial density).

Also, a monitoring of water level (cm above sea level) in all lagoons was performed every 15 days, and the topography of the area (monitored in 2018 at the end of the restoration project), allowed to calculate water depth of each lagoon (Martinoy and Pascual, 2018).

Results from the ECELS index (Conservation State of Shallow Lenitic Ecosystems index) were collected annually to describe the vegetation cover in the lagoons (Agència Catalana de l'Aigua, 2010; Compte et al., 2017).

2.3. Water physicochemical analysis, chl-a and bacterial density

Water samples for inorganic nutrient and DOC analyses were filtered in the field through a precombusted (450 °C for 4 h) Whatman GF/F filters (0.7 μm pore). Samples for inorganic nutrients were kept frozen until analysis. Ammonium, nitrite and nitrate were analysed following American Public Health Association, American Water Works Association and Water Environment Federation (2005) and soluble reactive phosphate following UNE-EN-ISO6878. Unfiltered water samples were also frozen for the analysis of Total Nitrogen (TN), Total Phosphorus (TP) and Total Organic Carbon (TOC). TOC, DOC and TN were measured using a TOC analyser (TOC-V CSH SHIMADZU). TP analyses were carried out as described in Hansen and Koroleff (1999). Chl-a concentrations were estimated using a high-performance liquid chromatography (HPLC) following Zapata and Garrido (1991) and López-Flores et al. (2006). Bacterial density was measured with a flow cytometer (FACScalibur from Becton & Dickinson, USA) following López-Flores et al. (2006) and Gasol and Del Giorgio (2000).

2.4. Dissolved organic matter quality

The composition of DOM in natural waters is very complex since it includes a great variety of chemical compounds. Spectrophotometric and fluorometric methods are commonly used to approximate DOM composition since a large proportion of its compounds have color and fluorescence (McKnight et al., 2001; Weishaar et al., 2003). Here we

calculated several DOM quality indicators through absorbance and fluorescence measurements. Samples were first defrost and filtered by 0.2 μm nylon filters. Absorbance scans (200–800 nm) were performed (spectrophotometer UV-2401PC, Shimadzu). Prior to absorbance measurements, samples were acidified with HCl 2N till they reached pH 2 and diluted with distilled MilliQ water when necessary (till their absorbance at 254 nm was approximately 0.15 AU). Dilution was especially needed for BPI and FRA samples (1:3 to 1:8 dilution) while all other samples where 1:2 diluted or not diluted. Then, the same filtered acidified (and diluted when necessary) samples were measured for their fluorescence with a fluorimeter (Shidmadzu, RF-5391PC). The excitation was measured between 230 nm and 460 nm and the emission was measured between 310 nm and 600 nm.

Six DOM quality indicators were calculated from the absorbance and fluorescence data. Following Weishaar et al. (2003) the Specific Ultraviolet Absorbance ($SUVA_{254}$) was calculated, giving information on the aromaticity of DOM. Spectral slope ratio (S_R) provided information on the DOM being subjected to irradiation (Helms et al., 2008). Fluorescence Index (FI) was also calculated following McKnight et al. (2001), which gives information on fulvic acids origin (microbial or terrestrial), and Biological Index (BIX) was calculated following Huguet et al. (2009), also giving information on the recently produced autochthonous DOM. Following the example of Wilson and Xenopoulos (2009), information on the recently produced DOM was obtained through Freshness Index (β/α). Finally, Absorbance slope (E_2:E_3) gives information on the aromaticity and molecular weight of humic substances (Peuravuori and Pihlaja, 1997).

2.5. Extracellular enzyme activities

Five extracellular enzyme activities were measured: β-glucosidase (GLU) (EC 3.2.1.21), β-xylosidase (XYL) (EC 3.2.1.37), leucine-aminopeptidase (LAP) (EC 3.4.11.1), phosphatase (AP) (EC 3.1.3.1–2) and phenol oxidase (PO) (EC 1.14.18.1); responsible for degradation of simple polysaccharides (cellobiose), vegetal origin polysaccharides (hemicellulose, xylobiose), peptides, organic phosphorus compounds, and lignin, respectively (Romaní et al., 2012).

The measurement of the hydrolytic enzymes GLU, XYL, LAP and AP activities was performed with the incubation of the fresh samples with an artificial substrate (4-Methylumbelliferone(MUF)-β-D-glucoside, MUF-β-D-xyloside, L-leucine-(4-methyl-7-coumarinylamide) AMC and MUF-phosphate respectively) at a final concentration of 0.3 mM, which was determined as the saturation concentration through saturation curves (Freixa et al., 2016). The incubation was performed in dark conditions at 20 °C in agitation for each subsample (3 per lagoon) during 1 h. Two controls were prepared and treated like the rest of the samples, one with just the artificial substrate to control its potential abiotic degradation and another with just the sample to determine the fluorescence of the water itself. The reactions were stopped with a glycine buffer (pH 10.4). The fluorescence was measured with a microplate reader (Tecan, Infinite M200 PRO) at 365/455 nm (for MUF-based artificial substrates) and 364/445 nm (for AMC-based artificial substrate, i.e. LAP). The concentration of the degraded artificial substrate was determined by preparing MUF and AMC standards of concentrations 0–50 μM (Freixa et al., 2016).

The PO activity was measured through the L-dihydroxyphenylalanine (L-DOPA) substrate. Samples were incubated at a final concentration of 5 mM with acetate buffer (Sinsabaugh et al., 1994). The incubation of the samples (3 subsamples per lagoon) lasted 2 h at 20 °C on agitation and in darkness. A control of every sample without the substrate was further incubated. The absorbance was measured with a microplate reader (Tecan, Infinite M200 PRO) (Freixa et al., 2016).

The enzyme ratios XYL/GLU and LAP/AP were calculated as indicators of the origin of the polysaccharides (allochthonous/autochthonous) and N versus P acquisition respectively (Romaní and Sabater, 2000; Hill et al., 2012).

2.6. Carbon substrate utilization profiles as a functional fingerprint

The samples were incubated in Biolog EcoPlates™ microplates (Biolog Inc., Hayward, CA, USA) to measure the utilization of a range of carbon substrates by each lagoon community. A classification of the carbon substrates (i.e. amines, amino acids, carboxylic acid, carbohydrates, phenolic compounds, and polymers) was made following Sala et al. (2006). Prior to the incubation, samples were diluted (1:100 dilution, with synthetic water in order to avoid an osmotic shock, filtered by 0.2 μm pore nylon filters) and 130 μL of diluted sample were inoculated to each microplate well. Ecoplates were incubated at 20 °C in the darkness for 8 days. Absorbance at 590 nm was measured every 24 h during the incubation period using a microplate reader (Tecan, Infinite M200 PRO). Values obtained at 190h (absorbance saturation) were used to calculate Shannon Index (*H'*, as a measure of the functional diversity), Richness (*S*, as the number of positive wells), Evenness Index (as a measure of the similarity of the coloured wells in each plate) (Goberna et al., 2005; Sofo and Ricciuti, 2019), and NUSE Index (nitrogen use index, as a measure of the use of substrates that contain nitrogen) (Sala et al., 2006).

2.7. Data analysis

For the analysis of physicochemical and biological parameters, two-way ANOVAs were used considering the *season* (4 levels) and the *lagoon age* (old vs. new) factors. When differences were observed among seasons, a post-hoc Tukey test was applied. When the interaction between the two analysed factors was significant one-way ANOVAs testing the factor *lagoon age* for each season were performed separately. The *season* factor was considered by including 3 monthly values per season for the physical-chemistry properties, Chl and bacterial density data. For the seasonal measurements of the six extracellular enzymes and the four functional diversity/fingerprinting index (Shannon, Richness, Evenness, and NUSE) the results from the 3 subsamples from each lagoon and each sampling period were used. The applied method takes into account the variability within the integrated 10 L sample by using 3 subsamples although they are not strictly replicates but sub-replicates. In the case of DOM quality parameters, different one-way ANOVAs were performed considering the *season* factor (the four sampling periods) and *lagoon age* factor. To improve normality and homoscedasticity, physicochemical parameters (Dissolved organic carbon –DOC–, temperature, NO_3^-, PO_4^{-3}, TP and conductivity), $SUVA_{254}$ index, Chl, bacterial density and enzyme activities were logarithmically transformed before the ANOVA analysis.

In order to visualize potential differences in the functional fingerprint between lagoons, a Metric Multidimensional Scaling (MDS) analysis with Bray-Curtis distance of the absorbance data obtained through the Biolog EcoPlates (measurements after 190h incubation) was performed. Afterwards the ANOSIM test confirmed whether the data were ordered in groups according to the *season* or the *lagoon age* factor.

To visualize the main environmental parameters being responsible for differences between new and old lagoons a principal component analysis (PCA) was performed using the environmental data (conductivity, pH, temperature, O_2, NO_3^-, PO_4^{-3}, TP and DOC) measured monthly for each of the six lagoons from November 2016 to October 2017.

The relationship between water physicochemical parameters (conductivity, pH, temperature, O_2, water level, NO_3^-, PO_4^{-3} and DOC), DOM quality indexes ($SUVA_{254}$, S_R, FI, Freshness and E2:E3) and the biological parameters (extracellular enzyme activities, Shannon Index, NUSE Index, Chl and bacterial density) of the lagoons was assessed through a redundancy analysis (RDA), using the data from the four sampling periods. BIX index was not included due to its high correlation with the Freshness Index, while Richness and Evenness were not included due to their high correlation with the Shannon Index. PO was also excluded from this analysis because of the high number of values obtained below the quantification limit.

3. Results

3.1. Physicochemical characteristics and DOM quality

Annual means of lagoons water physicochemical parameters are shown in Table 1. All parameters, excepting NO_3^-, showed seasonal significant differences (Suppl. Table 1). Conductivity was highest in June and October, almost coinciding with the minimum water level (July and October), which indicates the confinement period. Oxygen values in July were significantly lower than values in January and April (Tukey's test, $p < 0.05$), and also pH values in July were lower than April ones (Tukey's test, $p < 0.05$), while DOC was lower in January in comparison to July and October (Tukey's test, $p < 0.05$). Temperature was minimum in January and maximum during summer from May to August (Tukey's test, $p < 0.05$). October showed higher values of PO_4^{-3} and TP than April (Tukey's test, $p < 0.05$), being the lowest values of TP during January.

Significant differences between old and new lagoons were observed for several physicochemical parameters (Fig. 5, Suppl. Table 1). New lagoons were characterized by having lower conductivity, lower PO_4^{-3} and TP content, and lower DOC than the old ones.

DOM quality indexes showed differences between old vs new lagoons only for $SUVA_{254}$ index and S_R being both higher in old ones, while seasonal differences were found for more quality indexes (Table 2, Suppl. Table 1). Briefly, in January DOM spectroscopic characteristics got significantly separated from all other periods, reporting higher $SUVA_{254}$ index and lower FI, freshness and BIX indexes (Table 2, Suppl. Table 1).

The vegetation cover results from the ECELS monitoring indicated that all lagoons presented vegetation in all their surroundings in exception of L01 that did not present it. During the monitoring for 2016 and 2017 ECELS index only FRA and G02 presented hydrophytic vegetation covering the benthos and also floating in the surface, while the other lagoons did not present any hydrophytic vegetation (Compte et al., 2017).

Table 1
Physicochemical parameters, chlorophyll-a, and bacterial density of the 6 studied lagoons. Values are the annual means and standard deviation from monthly values from November 2016 to October 2017 (n = 12). TP: total phosphorus, DOC: dissolved organic carbon, Chl-a: chlorophyll-a.

Parameter	BPI	FRA	G02	L04	L01	M03
Cond (mS/cm)	57.4 ± 35.9	59.0 ± 15.0	37.9 ± 11.3	35.9 ± 10.1	29.8 ± 8.8	44.8 ± 14.9
pH	8.5 ± 0.2	8.6 ± 0.3	8.4 ± 0.2	8.6 ± 0.3	8.4 ± 0.3	8.6 ± 0.2
Temperature (°C)	16.4 ± 7.2	17.2 ± 7.2	16.4 ± 7.3	16.7 ± 7.4	16.7 ± 7.2	17.6 ± 7.3
O_2 (mg/L)	9.2 ± 4.9	6.2 ± 3.1	6.8 ± 2.3	8.5 ± 1.8	7.5 ± 3.2	8.8 ± 2.1
Water level (cm above sea level)	36.3 ± 25.7	30.6 ± 20.0	34.8 ± 24.2	39.0 ± 23.5	36.1 ± 24.9	42.6 ± 31.5
Water depth (cm)	99.3 ± 25.7	205.6 ± 20.0	159.8 ± 24.2	102.0 ± 23.5	60.1 ± 24.9	78.6 ± 31.5
NO_3^- (μg/L)	35.3 ± 114.3	4.0 ± 3.0	3.9 ± 2.3	8.1 ± 1.8	12.4 ± 3.2	3.8 ± 2.1
PO_4^{-3} (μg/L)	68.1 ± 98.6	35.8 ± 25.9	25.4 ± 23.2	7.2 ± 8.0	13.8 ± 26.8	9.9 ± 11.6
TP (μg/L)	349.3 ± 288.2	275.5 ± 223.0	180.7 ± 96.0	94.0 ± 49.4	67.5 ± 29.8	172.0 ± 88.3
DOC (mg/L)	76.9 ± 40.6	59.0 ± 15.3	23.7 ± 7.1	20.0 ± 6.6	19.5 ± 12.8	26.6 ± 10.1
Chl-a (μg/L)	34.8 ± 32.9	154.7 ± 251.7	32.8 ± 51.4	11.2 ± 9.9	7.4 ± 7.1	10.8 ± 8.4
Bacterial density (cel·10^8/mL)	5.4 ± 5.1	1.9 ± 1.3	3.5 ± 6.5	31.1 ± 4.5	1.1 ± 1.2	4.4 ± 4.3

Table 2
Dissolved Organic Matter Quality indexes. The values in the season columns correspond to the mean and standard deviation of the 6 lagoons for each season (n = 6). The values in the lagoon age columns correspond to the mean and standard deviation of the 3 old or 3 new lagoons for the four study periods (n = 12). Values marked in bold are significantly different than values of other seasons or higher in old than in new lagoons, respectively for the two factors (Tukey's test < 0.05). See statistics in Suppl. Table 1. $SUVA_{254}$: Specific Ultraviolet Absorbance, S_R: Slope ratio, FI: Fluorescence Index, Freshness: Freshness Index, BIX: Biological Index, E2:E3: absorbance slope.

	Season				Lagoon Age	
	January	April	July	October	Old	New
$SUVA_{254}$	**3.2** ± 2.3	1.3 ± 0.6	1.5 ± 0.3	1.3 ± 0.3	**2.2** ± 1.7	1.4 ± 0.5
S_R	1.2 ± 0.5	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	**1.2** ± 0.3	1.0 ± 0.2
FI	**1.44** ± 0.06	1.59 ± 0.10	1.61 ± 0.04	1.63 ± 0.06	1.58 ± 0.10	1.57 ± 0.10
Freshness	**0.60** ± 0.03	0.76 ± 0.08	0.74 ± 0.01	0.75 ± 0.02	0.71 ± 0.06	0.72 ± 0.89
BIX	**0.62** ± 0.02	0.77 ± 0.09	0.76 ± 0.01	0.76 ± 0.02	0.72 ± 0.06	0.74 ± 0.09
E2:E3	13.0 ± 11.0	11.9 ± 2.9	13.1 ± 1.2	14.1 ± 2.2	14.3 ± 6.6	11.9 ± 3.1

3.2. Chl content and bacterial density

Chl content was significantly higher in old lagoons during the whole year 2017, and also presented seasonal variation (Suppl. Table 1), showing the highest content during the summer months (Tukey's test, $p < 0.001$). Bacterial density did not present significant differences either seasonally or between lagoons (Suppl. Table 1).

3.3. Extracellular enzyme activities

The hydrolytic activities of GLU, XYL and LAP significantly increased during the study year, from January to October (Fig. 2, Suppl. Table 1), while AP activity significantly decreased in July (Fig. 2, Suppl. Table 1). *Lagoon age* and *season* factors determined significant changes in the hydrolytic activities expression, and for most enzymes the differences between old and new lagoons were significant at specific periods (Fig. 2, Suppl. Table 1). For GLU, the activity was higher in old lagoons than in new lagoons in October ($p < 0.001$), while for XYL and LAP activities were higher in old than in newly created lagoons both in July and October ($p < 0.01$ for all cases, Fig. 2). For AP activity different responses were obtained; in July this activity was higher in old lagoons ($p < 0.01$) while in April the tendency was opposite and higher values were measured for new lagoons ($p < 0.01$, Fig. 2b). The ratio between XYL and GLU activities showed no significant effect of lagoon age but the two factors interacted (Suppl. Table 1) indicating that the lagoon effect may be different depending on the season. The one-way ANOVAs for each season independently only showed significant differences for the *lagoon age* factor in October ($p < 0.05$), with higher values of the XYL to GLU ratio in the old lagoons (Fig. 2e). The ratio between LAP and AP showed higher values in July with no differences for the *lagoon age* factor in that period (Fig. 2f). In the case of PO activity there were no differences for the *lagoon age* factor and it showed only differences for the *season* factor due to higher values in October (Fig. 2g).

3.4. Carbon substrate utilization profiles

The four studied indexes showed differences in seasonality (Fig. 3, Suppl. Table 1). The lowest functional diversity values (Shannon index) were measured in October, followed by increasing values in April, and

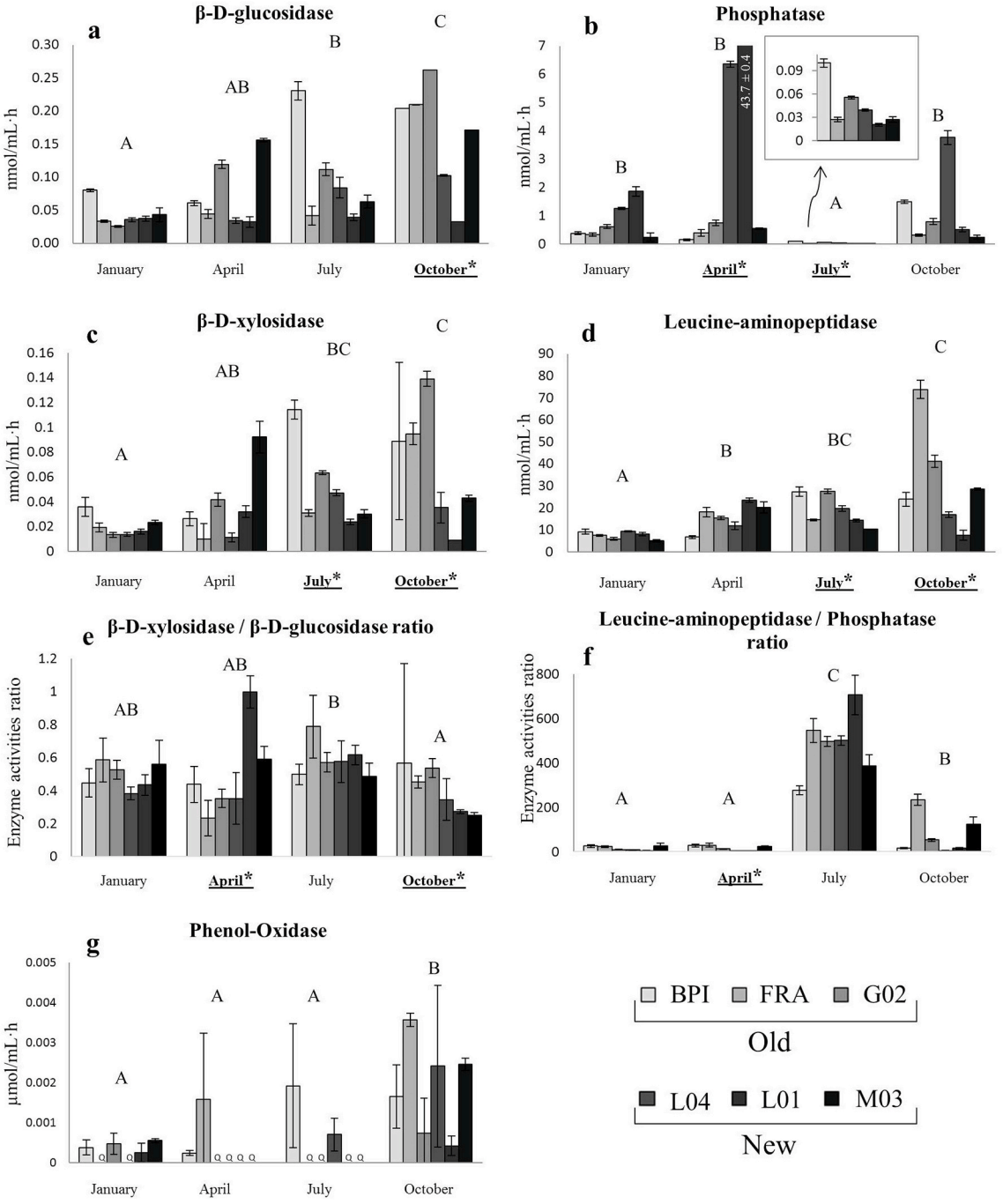

Fig. 2. Extracellular enzyme activities of the six studied lagoons (BPI, FRA, G02, L04, L01 and M03) considering the lagoon age factor and the season factor (four sampling periods). Results from the five analysed enzyme activities and the two enzyme activity ratios are showed. Q: below quantification limit. Sampling periods showing significant differences between old and new lagoons are underlined and bold and identified with an asterisk ($p < 0.05$). Capital letters indicate significant differences among seasons obtained with the Tuckey test ($p < 0.05$). The error bars indicate the standard deviation between the three subsamples analysed from each lagoon integrated sample.

the highest functional diversity being measured in January and July (Fig. 3). Functional richness and evenness followed the same tendency as Shannon functional diversity (data not shown). The NUSE index showed both *season* and *lagoon age* effects (Suppl. Table 1). The highest NUSE index values were measured in April and October (Fig. 3). The *lagoon age* interacted with *season* and it was in October when old lagoons showed a higher NUSE index than the recently created ones ($p < 0.05$).

Results obtained from the microbial heterotrophic fingerprint reflected a distinction between samples based on the *season*, while the *lagoon age* factor did not determine differences in the functional fingerprint when all data were analysed together (Fig. 4a). The main distinction occurred between October and the rest of the sampling periods (ANOSIM, $R = 0.9237$, $p = 0.001$) that also showed a significant higher dispersion between samples than the other study periods (Fig. 4a and c). Considering the substrate guilds adjustment in the MDS spatial distribution, October samples were characterized by a greater utilization of carbohydrates and carboxylic acids. April functional fingerprint was also distinguishable from July and January. The first showed greater ability to degrade amino acids, amines and phenolic compounds, while the latter showed higher ability to degrade polymers (Fig. 4b, ANOSIM

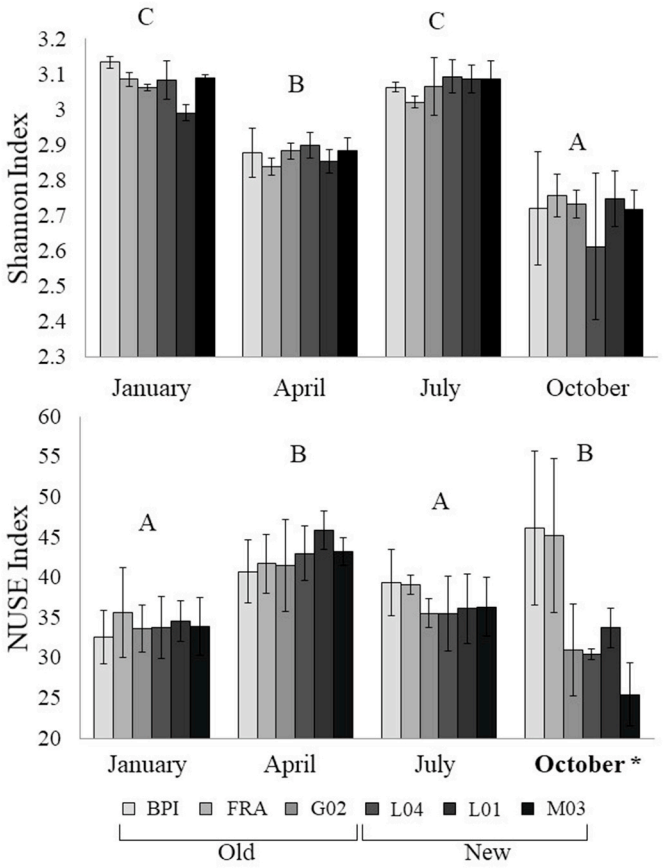

Fig. 3. Functional diversity indexes (Shannon index and NUSE index) of the six studied lagoons (BPI, FRA, G02, L04, L01 and M03) considering the lagoon age factor and the season factor (four sampling periods). Sampling periods showing significant differences between old and new lagoons are identified with an asterisk ($p < 0.05$). Capital letters indicate significant differences among seasons obtained with the Tuckey test ($p < 0.05$). The error bars indicate the standard deviation between the three subsamples analysed from each lagoon integrated sample. NUSE index: nitrogen use index.

$R = 0.7038$, $p = 0.001$).

Differences in the functional fingerprint between old and new lagoons were tested for the different periods separately and only in October differences were found between lagoons where BPI and FRA were significantly separated from the rest of lagoons (Fig. 5c, ANOSIM, $R = 0.56$, $p = 0.001$).

3.5. Relationship between variables

The obtained results from the PCA performed with the environmental data (Fig. 5) showed a strong correlation of the PC1 with DOC, TP, conductivity, PO_4^{-3} and temperature, while PC2 was correlated with O_2 and pH. Samples from January and April (located in the right) were characterized by lower values of temperature, PO_4^{-3}, conductivity, TP and DOC in comparison to samples taken in July or October without any dependence on the *lagoon age*. Within each group of seasons (January–April and July–October) samples from old lagoons were located separately from samples from new lagoons, being the old ones characterized by higher DOC, TP and conductivity (Fig. 5).

Relationships between physical-chemical and biological variables are visualized in the RDA (Fig. 6). At the positive side of RDA1 and positive RDA2, high values of water level and the $SUVA_{254}$ were related to also high functional diversity (Shannon) which occurred in samples from January (all lagoons) and July (new lagoons). At the negative side of RDA1 and negative RDA2, pH, Freshness index and phosphate concentration were related to LAP activity and NUSE index which occurred in samples from October in old lagoons. In contrast samples from October in new lagoons together with most samples from April in new lagoons were related to high oxygen, some of them also showing high AP activity. Finally, at the negative RDA1 and positive RDA2 side, the high GLU activity together with high temperature occurred in July old lagoons samples. Also in this quadrant (negative RDA1-positive RDA2), high conductivity and DOC were related to Chl and XYL activity occurring in some April samples.

4. Discussion

Seasonality was the main factor driving variability of microbial functioning of the studied Mediterranean lagoons, in terms of organic matter use capabilities, as it was expected in this ecosystem (Caruso et al., 2013; Zaccone and Caruso, 2019). However, several differences in microbial functioning between old and newly restored lagoons were evident in specific periods and most of them were significant only during summer and autumn, when lagoons were more isolated between them due to confinement (Menció et al., 2017; Quintana et al., 2018; Casamitjana et al., 2019).

4.1. Lagoons microbial functioning seasonality

The studied Mediterranean coastal lagoons showed several seasonal patterns in the microbial functioning and DOM properties that were observed in all lagoons (including old and new ones). Microbial C and N compounds degradation capacity increased from January to October, as shown by the increase in GLU, XYL, and LAP activities, and this might be related to the increase of temperature during summer and autumn periods. Other studies have found a positive correlation between temperature and some extracellular enzyme activities like GLU, LAP and AP (Christian and Karl, 1995; Caruso, 2010; Ylla et al., 2012; Zaccone et al., 2014). However, shifts in enzyme capabilities in aquatic environments can also be linked to the quality and quantity of organic matter availability (Cunha et al., 2010). For instance, in a study at the Danubian plain, highest enzyme activity was related with reactive DOM content, composed by proteins and polysaccharides, in opposite to what would be expected with much less reactive DOM such as lignin compounds (Sieczko and Peduzzi, 2014). The reduced extracellular enzyme activities measured in winter at the studied Mediterranean coastal lagoons may be related to a low bioavailability of organic compounds during that season. As indicated by DOM quality results, DOM in January was mainly of terrestrial origin (low FI values), being older and with higher aromaticity (lower Freshness index, lower BIX and higher $SUVA_{254}$) than in the other study periods. This different DOM quality in January may be due to the most relevant inundation period produced by heavy storms that occurred just before the January sampling (Weather station from l'Estartit and Torroella de Montgrí, Pascual and Martinoy, 2017) which could be responsible for the input of sea water but also of allochthonous organic matter into the lagoons (Catalán et al., 2013; Sieczko and Peduzzi, 2014).

Internal lagoon processes such as primary production and nutrient balances may further modify organic matter availability and microbial degradation rates (Kormas et al., 2001; Roselli et al., 2009). Increased LAP and GLU enzyme activities in July and October might be related to greater availability of easy degradable peptides and polysaccharides released from primary production (Allison et al., 2012; Ylla et al., 2009), as suggested by greater Chl content measured in the lagoons. As expected, sun radiation and temperature may favour primary production and algal biomass in summer season in lacustrine systems (Liboriussen and Jeppesen, 2009) providing fresh organic compounds for microbial enzymatic degradation (Chróst and Siuda, 2006). The availability of proteinaceous compounds but also the greater needs for N than for P in summer (July) (i.e. a higher availability of P than of N) was further indicated by the highest values of the LAP/AP ratio measured in that

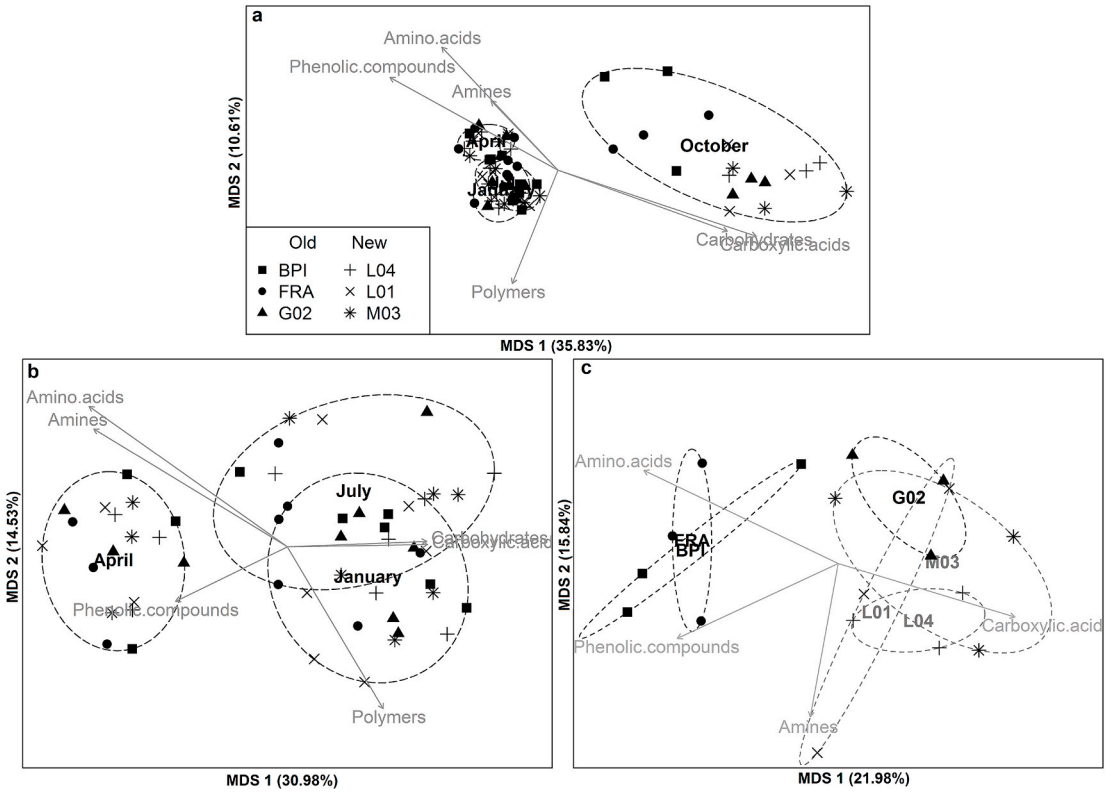

Fig. 4. Metric Multidimensional Scaling obtained from the functional fingerprint data for the six studied lagoons. a: data from the four sampling periods (46.44% of variance explained), b: data from January, April and July 2017 (45.51% of variance explained), c: data from October 2017 (37.82% of variance explained). Symbols indicate different lagoons (the three results from each lagoon and period correspond to the three subsamples analysed from each lagoon integrated sample). In all figures, the groups of substrates (following the grouping described in Sala et al., 2006) mostly correlated with the spatial distribution of samples ($p < 0.05$) are also indicated.

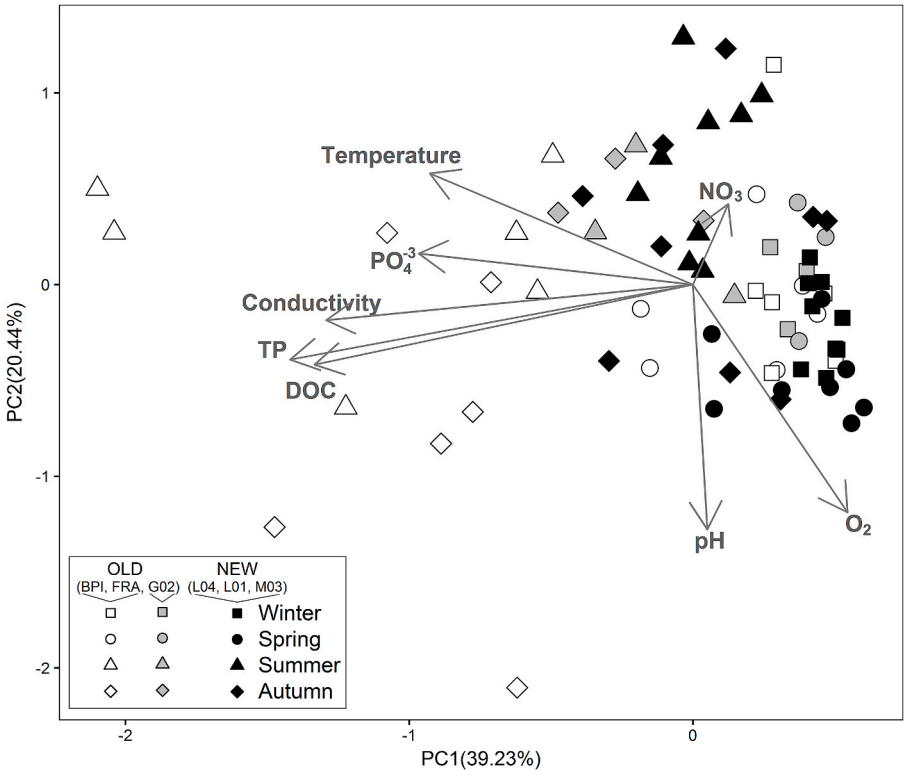

Fig. 5. Principal component analysis (PCA) of samples based on the monthly measured environmental parameters (temperature, conductivity, pH, O_2, dissolved organic carbon (DOC), NO_3^-, PO_4^{-3} and total phosphorus (TP)) at the six lagoons from November 2016 to October 2017. The two first components explain 59.7% of the variance. Symbol shape indicates different seasons (including three monthly values per season) and symbol filling indicates the lagoon type: white and grey for old and black for new lagoons (grey is used to distinguish the G02 lagoon - created in 2002 - amongst the old ones - BPI and FRA being natural ones).

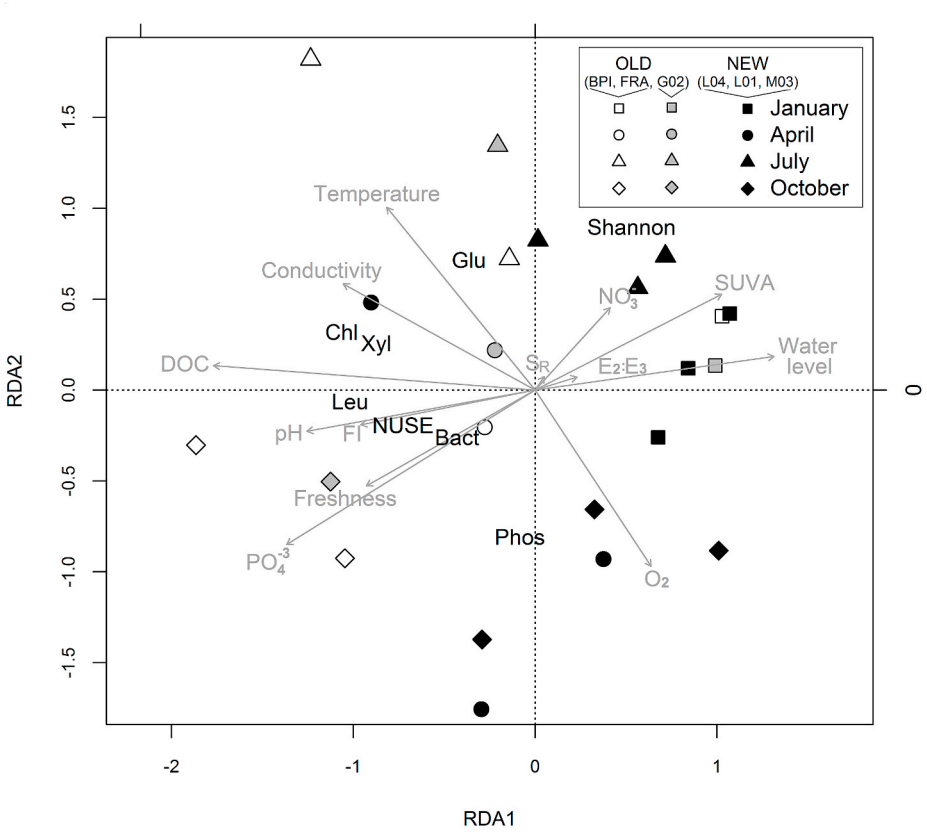

Fig. 6. *Redundancy Analysis results of microbial variables (enzyme activities, bacterial density, Chl-a, and Shannon Index) in relation to physical-chemical parameters and DOM quality indexes for the four studied periods. Symbol shape indicates different periods and symbol filling indicates the lagoon type: white and grey for old and black for new lagoons (grey is used to distinguish the G02 lagoon - created in 2002 - amongst the old ones - BPI and FRA being natural ones). Two points from old lagoons are missing (January and April) since several DOM quality data were missing from them. DOC: dissolved organic carbon, SUVA: Specific Ultraviolet Absorbance, SR: Slope ratio, FI: Fluorescence Index, Freshness: Freshness Index, E_2:E_3: absorbance slope, Chl: chlorophyll-a, Bact: bacterial density, Glu: β-glucosidase, Xyl: β-xylosidase, Leu: leucine-aminopeptidase, Phos: phosphatase, Shannon: Shannon Index, NUSE: nitrogen use index.*

period (Hill et al., 2006, 2012). Previous studies have also detected higher values of inorganic P than N during confinement periods (summer, autumn) in these lagoons (Quintana et al., 2018).

Similarly to the enzyme activity, the functional fingerprinting results also showed differences among seasons. The period which was more different from the others was October, when the fingerprint was characterized by using carbohydrates and carboxylic acids and the separation among lagoons was the largest. The lowest functional diversity (Shannon Index and richness) measured in October together with the highest dispersion in the functional fingerprints between sites (Fig. 4c) suggest the selection of specific functional species adapted to the environmental conditions of each specific lagoon. This would be similar to what is described in Cottenie and De Meester (2003), where the local environmental conditions and chase events of different shallow lakes selected the species of zooplankton present from the same species pool. Probably in this period, the confinement conditions imply that such lagoon has a specific microbial functional fingerprinting determined by a limited organic matter availability quality range and thus reducing the functional diversity. Besides the DOM quality range possibly being limited during confinement, also the highest conductivity during summer and autumn, as a result of high salt content, can also affect microbial diversity and in consequence cause a reduction on functional diversity (Benlloch et al., 2002; Teng et al., 2014); even though, for the zooplankton communities this salinity variations effect was not detected (Cabrera et al., 2019).

In contrast, in the other periods, the greater connectivity among lagoons as well as the effects of the flooding episodes (especially in January, but also in July due to small rain) may provide the microbial community with a greater range of DOM sources increasing the functional diversity (Fig. 3a) as well as homogenizing the functional fingerprinting and making it more similar among lagoons.

4.2. Microbial functioning in old vs newly restored lagoons

Within the seasonal variability framework, new lagoons showed distinct extracellular enzyme activities from old lagoons mainly during the most confined period (summer and autumn). When differences were evident, degradation capabilities were highest for old than for newly restored lagoons suggesting a greater and distinct input of materials in old than in new ones. Higher LAP activity in July and October as well as higher GLU activity and greater use of nitrogen compounds (NUSE index) in October in old than in new lagoons suggest higher availability of polysaccharidic and peptidic compounds in the old ones. As discussed above, these enzyme activities have been mostly related to actual or recently released peptides and polysaccharides from primary production (Caruso, 2010; Mühlenbruch et al., 2018) which matches with the greater Chl-a concentration measured in old than in new lagoons. It is suspected that greater algal biomass is produced in old lagoons, which also had greater P content (Dillion and Rigler, 1974).

Results also suggest that old lagoons had a greater input of plant material from the lagoons shore and riparian area or from aquatic plants than the new ones since higher microbial utilization of plant origin organic compounds (XYL, involved in the degradation of hemicellulose; Ylla et al., 2012) was measured in July and October. The higher $SUVA_{254}$ index measured in the old than in new lagoons may further indicate greater aromatic carbon content which may originate from plant material (Weishaar et al., 2003). In this sense, in the 2016 2nd Life-project monitoring report of the Pletera marsh system (Compte et al., 2017), the ECELS index, evaluating the ecological state and highly focusing on vegetation (ECELS Index, Agència Catalana de l'Aigua, 2010) considered that all lagoons had a good conservation state except for the newly created L01 that had a mediocre state. However, it was emphasized that L04, L01 and M03 did not present hydrophytic vegetation, while old lagoons (BPI, FRA and G02) did. During the 2017 samplings, the vegetation coverage was also visibly low and less dense in new lagoons surroundings. This difference in the vegetation cover of the lagoons

habitat (including their riparian area) can be the cause for the differences between old and new lagoons microbial functioning observed in October. In that period, the higher XYL activity and also higher XYL/-GLU ratio in old lagoons than in the recently created ones may indicate a much greater utilization, availability and inputs of allochthonous plant material in the former (Ylla et al., 2012). Higher availability of labile organic matter such as proteins in old lagoons than in new lagoons could have also increased the priming effect (Steen et al., 2016) in the old lagoons. Thus, labile DOM availability may have enhanced the capacity of recalcitrant plant organic matter degradation in old lagoons (i.e. XYL activity).

As indicated above, the major functional fingerprint differences between lagoons were those observed in autumn (October) (Fig. 4). In this period lagoons were grouped into two groups according to their functional fingerprinting, the natural ones (BPI and FRA) on one side, and the man-made ones (G02, L04, L01 and M03) on the other one (Fig. 4c). It is interesting to observe the fingerprinting of G02. This lagoon was created in 2002 and its fingerprinting places this lagoon closer to the recently created lagoons (created in 2016) than to the natural ones (Fig. 4c), while enzyme activities of G02 are more similar to BPI and FRA ones (Fig. 2). The extracellular enzymes are more linked to in situ organic matter cycling and biogeochemical processes while the functional fingerprinting, resulting from a culture method, provides insights about the potential use of diverse substrates by the microbial community, being a measure of its potential functional richness and diversity (Ylla et al., 2014). It is known that the studied extracellular enzyme activities depend on the availability of C, N, and P in the environment (Hill et al., 2006). Thus, the obtained results may indicate that the organic matter degradation and nutrient cycling processes are recovered in the lagoon created in 2002 (after 15 years of being constructed) while the potential functional diversity it is not. On one hand, this result for the functional fingerprinting in G02 lagoon might be related to its specific physical-chemical conditions which, although being in general similar to the natural ones, showed slightly lower values for DOC and TP, being located between natural and new lagoons (Fig. 5). Changes in the physical-chemical parameters such as changes in DOM availability during the confinement period in the G02 lagoon may affect the functional diversity, as similarly shown in Ruiz-González et al. (2015), these authors noted that in several boreal lakes and rivers the ordination of bacterial communities according to their traits mostly depended on DOM quantity and quality. On the other hand, although the functional fingerprinting based on the utilization of a set of substrates is not strictly a structural community property being not a taxonomical fingerprinting but an index of metabolic diversity, yet it is expected to have some linking to microbial diversity (Ruiz-González et al., 2015). Then our results somehow indicate a faster recovery of the microbial function (enzyme activities) than its "structure" (functional diversity). This is in accordance with the expected easier functional recovery in comparison to structural or composition recovery in restoration practices (Hobbs and Harris, 2001).

Although existing differences between old and new lagoons during the confinement period prevented us to conclude that the restoration was completed in 2017 (one year after lagoons being created), the results from G02 allow us to hypothesize that, at least 15 years after their creation, the functional recovery of the restored lagoons in terms of organic matter and nutrient cycling could be achieved.

4.3. Microbial functioning for ecosystem restoration

It is known that the microbial degradation of organic matter plays a key role in the functioning of aquatic systems (Logue et al., 2016; Zaccone and Caruso, 2019). In our investigation, studying the microbial organic matter degradation capabilities in restored aquatic systems is an original approach to determine the whole ecosystem functioning, including primary production, nutrient and C cycling in a quantitative and qualitative level. On one hand, the present study highlights the relevance of using a set of extracellular enzyme activities related to the use of different organic matter resources and that reflects not only the water column functioning but also considers the whole lagoon habitat state (including the recovery of the vegetation). On the other hand, the analysis of the functional fingerprinting appears as also being sensitive to the restoration process and showed a delayed response compared to that of enzymes as being probably more linked to changes in the microbial community composition. We propose the combination of both functional approaches as a useful and practical tool for assessing and monitoring ecosystem restoration.

It is also important to emphasize that in such a fluctuating ecosystem mainly marked by seasonality plus hydrological changes, as typically found in Mediterranean coastal marshes, the diversity in microbial functioning observed among lagoons, as those between old and new ones, can be masked in periods when large connectivity between lagoons occur such as during sea storms or flooding events. The obtained results showed that the differences between old and new lagoons were only highlighted when enough time after the last homogenization occurred. In this sense, results from summer and autumn clearly indicated that organic matter resources and cycling were still different between old and recently created lagoons (1 year after creation). Also, autumn (October) results indicated that still some differences in the microbial functional fingerprint existed between the lagoon created on 2002 and the natural ones. These results allow us to recommend that if the analysed restored system is affected by seasonality it is important to include this factor in the evaluation of coastal lagoons state.

Authorship statement

JC, GG, NP, XDQ, AMR designed the study. All the authors contributed to the data collection and interpretation. JB, AMR wrote the initial draft of the manuscript. All the authors revised the manuscript and approved the final manuscript for publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2021.107434.

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