Science of the Total Environment xxx (xxxx) xxx

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

A sustainable approach by using microalgae to minimize the eutrophication process of Mar Menor lagoon

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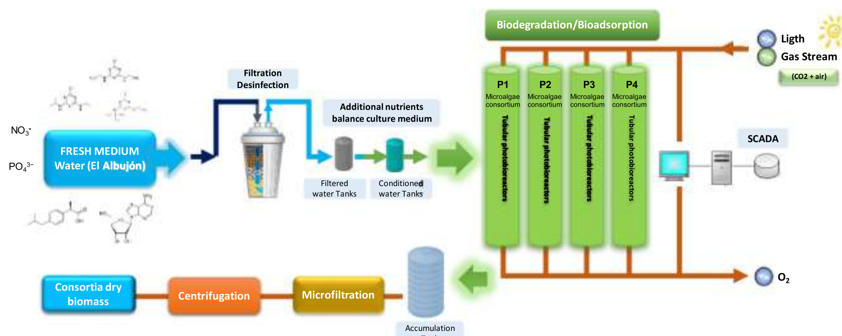
HIGHLIGHTS

- Bioremediation of sewage by autochthonous mixotrophic consortia grown in photobioreactors.
- The four mixotrophic consortia decreased the contaminant burden of the El Albujón wastewater.
- Consortium 1 was effective for removing emerging contaminants (100%, with 78.50% for adenosine).
- Consortium 1 significantly removed nitrates (89.90%) and phosphates (99.70%).
- The contents of carbohydrates, lipids and proteins from the biomass of consortium 1 were 30.51%, 28.09% and 13.59%, respectively.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 11 July 2020
Received in revised form 23 October 2020
Accepted 7 November 2020
Available online xxxx

Editor: Jan Vymazal

Keywords:
Microalgae consortia
Wastewater treatment
Nutrient removal
Emerging contaminants removal

ABSTRACT

The present study evaluates the removal capacity of microalgae photobioreactors of environmental pollutants present in wastewater from the dry riverbed El Albujón, as a way to minimize the eutrophication process of the Mar Menor. Particularly, the capacity of four autochthonous microalgae consortia collected from different locations of the salty lagoon to remove emerging contaminants (simazine, atrazine, terbuthylazine, adenosine and ibuprofen), nitrates, and phosphates, was evaluated.
Among the four microalgae consortia, consortium 1 was the best in terms of biomass productivity (0.11 g L^{-1} d^{-1}) and specific growth rate (0.14 d^{-1}), providing 100% removal of emerging contaminants (simazine, atrazine, terbuthylazine, adenosine and ibuprofen), and a maximal reduction and consumption of macronutrients, especially nitrates and phosphates, reaching levels below 28 mg L^{-1}, that is, a decrease of 89.90 and 99.70% of nitrates and phosphates, respectively. Therefore, this consortium (*Monoraphidium sp., Desmodesmus subspicatus, Nannochloris sp.*) could be selected as a green filter for successful large-scale applications. This study is the first one that combines the successful removal of herbicides, ibuprofen and adenosine as emerging contaminants, and nitrate removal.

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https://doi.org/10.1016/j.scitotenv.2020.143613
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1. Introduction

Large amounts of wastewaters are continuously being produced at present, mainly due to agricultural practices and industrial processes. The regular discharge of wastewaters without adequate treatment has derived in serious trophic imbalances in coastal marine habitats. One of the major problems associated with the disposal of effluents into coastal waters is the enrichment of water resources with nutrients, mainly nitrogen and phosphorus, in a process known as "eutrophication", which causes a dense growth of aquatic plant life (Lam et al., 2020; Liu et al., 2020a, 2020b). Regarding the emerging contaminants (ECs), atrazine, simazine and terbuthylazine are three types of herbicides that are widely used in the agriculture, and their presence in wastewaters ranges from 1.20–190 ng L^{-1}, 20–1600 ng L^{-1} and 2–53 ng L^{-1}, respectively (Cahill et al., 2011; Martin Ruel et al., 2012; Rosal et al., 2010; Köck-Schulmeyer et al., 2013; Benvenuto et al., 2010; Carretta et al., 2019; Singer et al., 2010). Also, drugs have become an increasing problem in water from different origins. For example, ibuprofen, which is a nonsteroidal anti-inflammatory and analgesic that is very frequently used by the population, has been found in high concentrations in wastewaters from a few countries (2–17 mg L^{-1}), dramatically affecting the environment (Thalla and Vannarath, 2020; Pereira et al., 2020); or adenosine (6-amino-9-β-D-ribofuranosyl-9-H-purine, commercial brands, Adenocard or Adenoscan), which is a purine-based drug that acts as a coronary vasodilator for increasing blood flow to the heart muscle and used for the conversion of sinus rhythm of paroxysmal supraventricular tachycardia, has been detected in rivers (Rimayi et al., 2019) and wastewaters (580–610 ng L^{-1}) (Gago-Ferrero et al., 2020).

The Mar Menor is Europe's biggest saltwater lagoon (135 km^2 surface). Intensive runoff along a large agricultural area discharges into this lagoon, which was declared as a sensitive eutrophication area in June 2001 under European Directive 91/271/EEC. Likewise, enriched nutrients (such as nitrates) also confirmed its vulnerability in December 2002 under Directive 91/676/EEC. In addition, the Mar Menor area hosts important human economic activities (Conesa and Jiménez-Cárceles, 2007). It is well-established that the Mar Menor receives between 2500 and 3000 tons of nitrates and phosphates each year, as well as 27.4 kg of ECs, of which 11.4 kg are pharmaceuticals and the rest fertilizers and pesticides, as part of municipal wastewaters, and agricultural drainage, of which 90% falls into the lagoon through this dry riverbed. In fact, the main inputs of nutrients to the lagoon are from the largest dry riverbed, which flows into it: El Albujón. Two eutrophication crises in 2016 and 2019, with the abrupt impairment of the quality of its waters, caused a great political and social concern (Pérez-Ruzafa et al., 2019). Therefore, the Mar Menor lagoon is an especially vulnerable eutrophication ecosystem due to the anthropogenic pressures. In fact, the need for effective treatment methods of effluents that are able to reduce nutrient and pollutant concentrations before their discharge into the marine habitats, was clearly revealed.

For bioremediation purposes, biological treatments of wastewaters using microalgae have been extensively studied for decades, as these microorganisms represent an effective alternative for contaminant removal. In fact, microalgae are unicellular or multicellular autotrophic microorganisms that are typically found in aquatic habitats. The most important nutrients for algae growth are carbon dioxide, nitrogen and phosphorous, well as Ca, Mg, Na and K for photosynthesis (Lam et al., 2020; Fernández et al., 2018). Therefore, waters from different locations (agricultural, industrial, or municipal), are good sources of the required nutrients for microalgae cultivation.

Microalgae can be used for bioremediation processes; however, the selection of the algal strain is of vital importance (Liu et al., 2020b). While most of the biotreatment technologies use bacteria (Liu et al., 2020a; Lu et al., 2019; Cao et al., 2020), microalgae have been used extensively for effluent treatment (Liu et al., 2020b). In fact, single species of *Chlorella* (Pathak et al., 2014), *Scenedesmus* (Oliveira et al., 2019), or *Spirulina* (Zhang et al., 2019), or as mixed consortia (Abdelrazek et al., 2019) to correct nitrogen, phosphorus and chemical oxygen requirements from different types of effluents, has widely sustained this research (Cheng et al., 2020; Arif et al., 2020; Garlapati et al., 2019).

In fact, both monocultures and crops of various microalgae species have been grown in wastewater, showing that they are suitable for the removal of contaminants in wastewater (including waters with a high organic load, whether from livestock or agriculture), and that the effectiveness of the process is very promising (Mark Ibekwe et al., 2017; González-Camejo et al., 2020; Malvis et al., 2019; Seco et al., 2018; Li et al., 2017; Yadavalli and Heggers, 2013). The results have shown elimination percentages of up to100% in some cases, depending on the operating conditions, the species used and the characteristics of the wastewaters (Yadavalli and Heggers, 2013; González-Camejo et al., 2020).

Most studies focus on the selection of a single species, such as the study carried out by Sharma and Khan (2013), which evaluated the potential use of *Chlorella minutissima*, *Scenedesmus* spp. and *Nostoc* spp. for sewage wastewater remediation, selecting *Chlorella minutissima* as the optimal species for these treatments, due to its higher nutrient withholding capability. Others species studied have been *Phormidium*, *Botryococcus*, *Chlamydomonas* and *Spirulina platensis*, which also use different nitrogen compounds as nutrients such as ammonium, nitrate or urea, for their growth (McAllister et al., 2018; Fernandez and Galvan, 2007; Paskuliakova et al., 2018; Almomani, 2019). However, the use of microalgae consortia for the same purposes is scarce in the literature (Gonçalves et al., 2017; Choudhary et al., 2016).

The aim of this study was to evaluate the growth of autochthonous microalgae consortia collected from four different locations in El Albujón dry riverbed, as well as their efficacy in removing emerging contaminants (simazine, atrazine, terbuthylazine, adenosine and ibuprofen), nitrates, and phosphates, as a sustainable approach for the minimization of the eutrophication process of the Mar Menor lagoon. Also, as the microalgae biomass could be useful for further applications such as energy generation or for extracting different high added-value bioactive compounds as ingredients for the manufacturing of functional foods, cosmetics, or nutraceuticals (Chinnasamy et al., 2010; Barbosa et al., 2015; Barbosa et al., 2017; Barbosa et al., 2018; Barbosa et al., 2020; Lopes et al., 2018), in line with the principles of the circular economy; the biomass productivity and composition of the four distinct autochthonous microalgae consortiums were also compared.

2. Experimental work

2.1. Water samples

By using standard sampling techniques (Hunt and Wilson, 1986) and with the authorization of Hydrographic Confederation of the Segura River (Murcia, Spain), untreated water samples (1000*L*) were collected from contaminated areas of the El Albujón dry riverbed for autochthonous microalgae identification as described below (Fig. 1): *i)* effluent from Los Alcázares Waste Water Treatment Plant (WWTP) consortium P1 (coordinates were 37° 44′ 33.58˝**N**; 0° 52′ 27.20˝**O**); *ii)* after the combination with the effluent from the WWTP with El Albujón dry riverbed, consortium P2 (coordinates were 37° 43′ 15.68˝**N**; 0° 52′ 48.34˝ **O**); *iii)* just before the discharge into the Mar Menor, consortium P3 (coordinates were 37° 42′ 57.76˝**N**; 0° 51′ 33.60˝**O**) and *iv)* the entry area into the Mar Menor, consortium P4 (coordinates were 37° 42′ 55.64˝**N**; 0° 51′ 30.46˝**O**).

Then, the samples were transported to the laboratory in 50 L containers within 60 min and stored at 4 °C. Each sample of wastewater was collected in duplicate and monitored under a microscope light to confirm its viability (data not shown). Once the consortia were identified, wastewater taken from coordinates 37°, 43′ 15.68˝**N**; 0° 52′ 48,34˝**O**; was used for subsequent analyses.

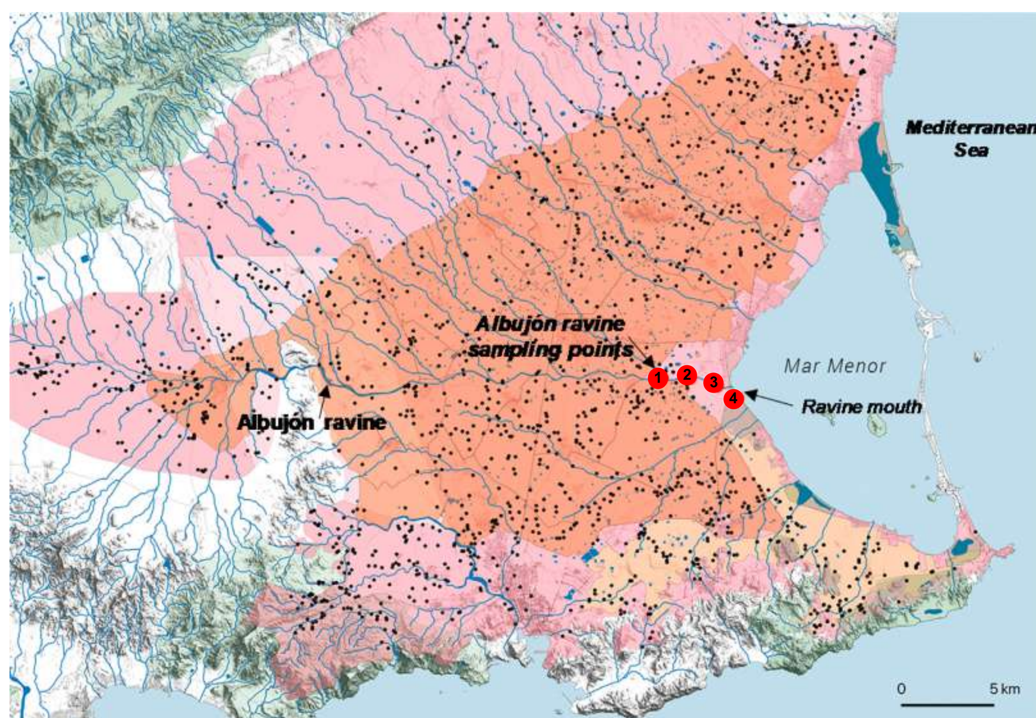

Fig. 1. Location map of the El Albujón dry riverbed and mouth to the Mediterranean Sea. The selected water sampling points are identified as red circles and the number inside is associated with each consortia. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Characterization of water samples

Atrazine, simazine and terbuthylazine were purchased from Dr. Ehrenstorfer (Augsburg, Germany). High purity grade (>99%) of the pharmaceuticals adenosine and ibuprofen were purchased from Sigma-Aldrich (St. Louis, MO. USA). Standard stock solutions of all the compounds were prepared in MeOH (1000 mg L^{-1}), and diluted for determining the aqueous calibration curves, and to perform wastewater fortification. Aqueous standard solutions always contained <0.10% MeOH. Sodium phosphate dibasic dehydrated (98.50% purity), and B, Cu, Fe, Zn were obtained in pure powder form from Sigma Aldrich (St. Louis, MO. USA). All other reagents used were of analytical grade.

2.2.1. Physicochemical analysis

Physicochemical analyses encompassing estimation of major anions (HCO_3^-, Cl^-, SO_4^{2-}, NO_3^-, PO_4^{3-}), aside from the general parameters of pH, electrical conductivity (EC), turbidity, temperature, total nitrogen, total phosphorus, and ammonium, were determined according to analytical procedures recommended by the American Public Health Association (Eaton et al., 2005). The pH, EC, temperature, and salinity were measured at the time of sample collection by a multi probe sensor YSI-Proplus from Xylem (Ohio, USA).

2.2.2. Heavy metal analysis

For heavy metals analysis, water samples were digested in a Mars 6-One Touch microwave (Vertex Technics, Barcelona, Spain) at 180 °C for 20 min at 1000 W using an acid mixture (15 mL HNO_3 and 4 mL $HClO_4$), and were analysed with an Inductively-Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7500 Series) which included ChemStation software (Agilent Technologies, CA, USA) (Radaelli et al., 2019).

2.2.3. Qualitative and quantitative detection of emerging contaminants

The determination of ECs in water samples was carried out with chromatography. Pesticide residues determination was performed with a gas chromatographer coupled to a mass-spectrometer (GC–MS, GC 6890, MS 5973, Agilent Technologies, CA, USA) with an oven temperature program of 60 °C (1 min), 30 °C min^{-1} to 110 °C, 10 °C min^{-1} to 240 °C, 30 °C min^{-1} to 240 °C (10 min). Helium was used as the carrier gas with a flow-rate of 1.20 mL min^{-1} and samples were injected using the splitless mode by an autoinjector (temperature of 250 °C). A Zebron ZB-Multiresidue 1 column (30 m length × 0.25 mm diameter × 0.25 μm thickness; Phenomenex, USA), was used as the stationary phase, and target analytes were detected with selected ion monitoring of three characteristic fragment ions (*m/z* 173, 202, 215 for atrazine), (m/z 173, 158, 186 for simazine) and (m/z 173, 216, 229 for terbuthylazine).

The pharmaceuticals (ibuprofen and adenosine) were analysed by ultrahigh-performance liquid chromatography (UHPLC) coupled to quadrupole time-of-flight mass spectrometry (QTOF), using a methodology adapted from García-Galán et al. (2020).

2.2.4. Measurement of biological indicators of contamination

Classical biological indicators of contamination were included in this study. For the total aerobic plate count (TPC), agar media were prepared according to the manufacturer's instructions (Eaton et al., 2005), poured into Petri dishes and incubated at 37 °C for 18 h to determine sterility. The samples were serially diluted 10-fold in 9 mL of sterilised peptone water contained in each of the tubes by transferring 1 mL of water into the first test tube and mixing. Then 100 μL of two sample dilutions of 10^{-2} and 10^{-4} including the neat (undiluted sample) was plated onto the plate count agar and surface spread using a sterilised glass spreader for uniform inoculation. The plates were incubated at 37 °C for 48 h. After the appropriate incubation time, all visible colonies were counted and multiplied by their corresponding dilution factor, which was reported as colony forming units per mL (cfu/mL).

Total coliforms (TC), faecal coliforms (FC), and faecal streptococci (FS) were all enumerated using the most probable number (MPN) method (APHA, 2005), using a Durham vial in MacConkey broth (BBL, Cockeysville, MD, USA). After 48 h at 37 °C incubation time, those showing gas and acid were confirmed in Levine eosin methylene blue agar

(BBL, Cockeysville, MD, USA) at 37 °C for TC and in MacConkey broth with a Durham vial at 44 °C for 24 h for FC. The FS test was performed with 9 tubes of azide dextrose broth (Oxoid, Basingstoke, Hampshire, UK) to which bromothymol blue (20 mg L^{-1}) was added as the pH indicator. The same amounts of diluted sample were inoculated as for coliforms, and they were incubated at 37 °C for 48 h. Growth was confirmed in ethyl violet azide broth (BBL, Cockeysville, MD, USA).

The investigation of sulphite-reducing *Clostridia* (SRC) spores was performed by anaerobic plate count in reinforced clostridial agar (Difco, Detroit, MI, USA) through the addition of sodium sulphite (2 mg L^{-1}) and ammonium iron (III) citrate (1.25 mg L^{-1}). The vegetative forms were destroyed at 80 °C for 5 min. A heterotrophic plate count (HPC) was performed using the pour plate method in standard methods agar (BBL, Cockeysville, MD, USA), incubated at 37 °C for 48 h (APHA, 2005). The enumeration of *Salmonella* spp. was performed using the MPN procedure (Baudart and Lebaron, 2010).

2.3. Microbial community analysis in water samples

The four water samples were centrifuged to obtain a concentrated pellet of the microalgae and other microorganisms. The pellet was subjected to a DNA extraction protocol and a subsequent analysis via massive sequencing (NGS) of the 16S ribosomal genes for Bacteria, 18S for Eukaryotes (algae) and the internal transcribed spacer (ITS) for fungi. The data obtained were analysed and contrasted with the information available in international databases (GenBank NCBI https://www.ncbi.nlm.nih.gov), to identify the microorganisms in the community at the lowest level possible.

2.3.1. Deoxyribonucleic acid (DNA) extraction

Each sample (15 mL) was centrifuged at 400 ×*g* for 10 min. The supernatant was removed, and the cell pellet was washed with PBS at pH 8.0. This suspension was centrifuged again for 5 min, the supernatant was removed, and the pellet was resuspended in 1 mL PBS. The sample was transferred to a sterilised FastPrep tube, containing glass microspheres (0.10 mm). Afterwards, 0.50 mL of CTAB buffer and 0.50 mL of phenol reagent with chloroform: isoamyl alcohol (24:1, *v*/*v*) were added. Then, 2 homogenization cycles were performed using the FastPrep tool (MP Biomedical, Fisher Scientific, Madrid, Spain) at speed 6.0 for 30 s. The samples were centrifuged for 10 min at 5 °C and 16,000 ×*g*. Then, the supernatant was extracted (aqueous layer) and transferred to a new tube to which 0.50 mL of chloroform:isoamyl alcohol (24:1, *v*/*v*) were added. An emulsion was formed which was centrifuged for 10 min at 5 °C and 16,000 ×*g*, and then the supernatant was extracted again and transferred to another microfuge tube to which 2 volumes of 30% polyethylene glycol (PEG) were added to precipitate the DNA. The samples were incubated at 4 °C overnight. After this, the samples were centrifuged at 16,000 ×*g* for 20 min at 5 °C, and the pellet obtained was washed with 1 mL cold ethanol (70%, *v*/*v*). The same centrifugation process was repeated, and the ethanol was discarded. Lastly, the samples were dried for 20 min in the "Concentrator plus" device from Eppendorf (Hamburg, Germany), at 60 °C. The pellet was resuspended in 30 μL of sterile distilled water and kept in the freezer at −20 °C until use.

2.3.2. Polymerase chain reaction (PCR) and high-performance sequencing

The V4 variable regions of the 16S and 18S rRNA genes were selected and used to study bacteria, archaea and eukaryotes (microalgae), respectively (Stoeck et al., 2010). Finally, the gITS7 5'-GTGARTCAT CGARTCTTTG-3 'ITS4 5′- TCCTCCGCTTATTGATATGC-3′ primers were used to study the ITS2 Internal transcribed spacer (ITS) region for fungi.

The primers contained modifications to the NextEra XT Index Kit in the forward and reverse primers, as well as 12-base random spacers in the forward primer to improve cluster generation and reduce the number of PhiX peaks required for sequencing. The forward primers contained 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGANNNHNN NWNNNH PRIMER SEQUENCE-3 and the reverse primers contained 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGT PRIMER SEQUENCE-3′. The PCR reactions were carried out using GoTaq® G2 Flexi DNA Polymerase (Promega, WI, USA). Reactions contained 5 μL of GoTaq flexi G2 5× buffer, a final concentration of $MgCl_2$ of 2 mM, 0.20 mM of forward and reverse primers, 1.25 Taq polymerase units, 1 μL DNA template containing ~20–50 ng of DNA, and molecular grade water, filling the tube to 25 μL. The PCR products were purified using 0.80× AMPure XP magnetic beads according to the manufacturer's instructions (Beckman Coulter, CA, USA). Products were quantified using a high sensitivity Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific, UK) and diluted to 10 ng μL^{-1}.

The 16S and 18S amplicons were combined into equimolar volumes and indexing was performed by applying the Illumina 16S metagenomic workflow (https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). Indexed libraries were pooled, quantified, and diluted to 4 pM and processed at Illumina Miseq (York University, UK) using the V3 2 × 300 bp Illumina sequencing kit according to the manufacturer's instructions.

2.3.3. Sequence processing and operational taxonomic units (OTU) generation

The sequences were analysed as previously described (Taylor and Cunliffe, 2015), using a combination of USEARCH v7.0.1090 (32Bit) (Edgar, 2010) and QIIME v 1.8.0 (Caporaso et al., 2010). Multiplexed fastq files were stripped of all 13 random base spacers using the USEARCH-fastq_strip_left command. The 16S and 18S libraries were separated based on the sequence using the script split_libraries.py. Fastq files were filtered according to their quality (low quality: expected error > 0.50 and short sequences (<200 bp), truncated length: 250 bp for prokaryotes and fungi and 370 bp for eukaryotes) and converted to FASTA files. FASTA files were first replicated, abundance was sorted, and single sequences were removed, OTUs were grouped using the UPARSE grouping algorithm (Edgar, 2013). The chimeras were filtered using UCHIME (Edgar et al., 2011) and the Gold database for 16S sequences or the SILVA 97% OTU (operational taxonomic unit) database for 18S sequences as the reference (Edgar, 2010). The OTUs were mapped back to the original readings and an OTU table was produced. The taxonomy was assigned to OTUs using the uclust method in QIIME v1.8.0 against the representative 97% OTU SILVA database (Quast et al., 2013) for the 16S sequences of Prokaryotes and 18S of Eukaryotes, and the base of UNITE data for fungi (Kõljalg et al., 2005). To study the similarity between the communities of the different samples, a similarity analysis was made based on the OTU tables for the three groups studied, using the UniFrac distance as an index (Lozupone and Knight, 2005) that incorporates information on the phylogenetic relationship of the different members of the community, as well as information on their relative abundance.

2.4. Microalgae consortia cultivation

Once the waters were characterized and the species identified, microalgae growth was carried out in duplicate in both the adaptation phase for 24 d (inoculum microalgae growth), and then for 29 d in photobioreactors (PBRs) (Fig. 2).

In order to improve the growth and stability of the autochthone's algae species, the consortia were grown outdoors (light and ambient temperature), and in PBRs in a batch (6 L capacity), securely plugged to a timer for the pulsed injection of air and CO_2 every 20 min. The cultures were maintained in axenic conditions and shaken periodically.

2.5. Analytical determinations

2.5.1. Growth parameters: biomass concentration and cell count

The growth of the consortia was recorded in terms of biomass concentration and cell count present in each water sample. Biomass

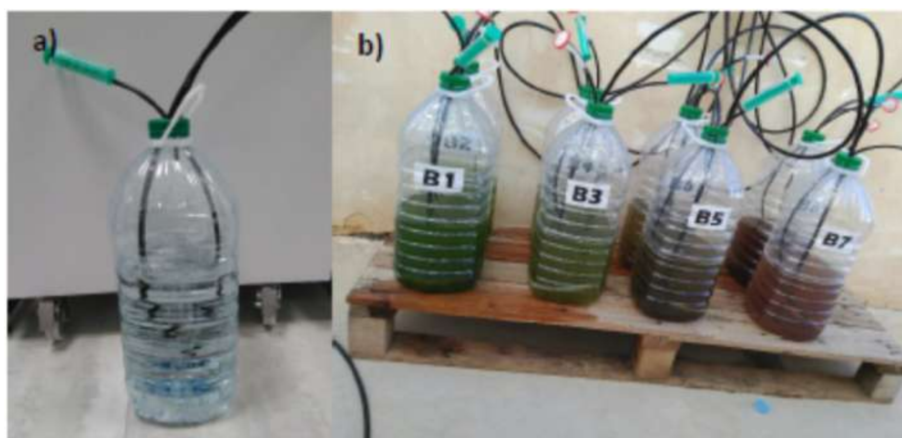

Fig. 2. Image of PBRs in its initial (lag) phase, containing a microalgae inoculum from the end of the exponential phase of the adaptation phase (a); PBRs containing microalgae cultures of each consortium (b). (Sample identification: P1 = B1; P2 = B3; P3 = B5; P4 = B7).

concentration expressed as g dry weight (DW)/L during the microalgae inoculum growth (adaptation phase) and in the PBRs, was estimated by the gravimetric method at lag, exponential (log) and stationary microalgae growth phases, and these data were used to determine biomass productivity in both the adaptation phase and in the PBRs (μ expressed as g L^{-1} d^{-1}), by calculating the slope resulting from the linear regression of the exponential phase, divided by the number of days in this log phase. In addition, the specific growth rate (v, expressed as d^{-1}) in the PBRs was calculated over a period of time where the biomass increases gradually (exponential phase), and it was determined by:

$$v = Ln\ (C/C_0)/t \quad (1)$$

where C_0 is the initial biomass concentration (initial g DW/L), and C is the biomass concentration (g DW/L) at time t (expressed in d). Moreover, temperature and pH were registered daily.

2.5.2. Nutrient removal

The nutrient removal efficiency was monitored every 72 h throughout the entire experiment. Briefly, 10 mL from each sample were collected from the different PBRs and centrifuged (15 min, 1800*g*), followed by filtration using 0.45 μm filters. These samples were used for analysis of nitrates (NO^{3-}), and phosphates (PO_4^{3-}), as described in Section 2, as well as the rest of the analytical parameters (Eaton et al., 2005). The removal efficiency as a percentage was determined by using the following equation:

$$Removal\ (\%) = [Initial\ concentration\ (mg/L) - Final\ concentration\ (mg/L)] / [Initial\ concentration\ (mg/L)] * 100 \quad (2)$$

3. Results and discussion

3.1. Wastewater characterization

The wastewater was taken from a highly contaminated area (Fig. 1), located close to the mouth of the El Albujón dry riverbed, and was used for the subsequent analyses (coordinates 37° 43′15.68˝**N**; 0° 52′48.34˝ **O**). The physicochemical characteristics of the wastewater collected were initially analysed in the non-treated wastewater, and then after treatments with each microalgae consortium (Table 1).

The Hydrographic Confederation of the Segura River (Murcia, Spain) approved the extraction of 1000 L of water for the current study. The wastewater used in the current study showed high concentrations of nitrates (274 mg NO_3^- L^{-1}) and sulphates (3,76 mg SO_4^- L^{-1}), in agreement with a previous study (Alvarez-Rogel et al., 2011).

However, this analysis also reported low amounts of phosphates and total phosphorus (19 mg PO_4^{3-} L^{-1}, equivalent to 6.20 mg P L^{-1}), which are needed for the homeostasis of cell energy, with the right concentration ranges being 6 to 47 mg P L^{-1}, depending on the microalgae species (Fernández et al., 2018; Abd et al., 2018; Zhou et al., 2017; Ruiz et al., 2011; Chinnasamy et al., 2010), which indicates to an unbalanced medium, not suitable for an optimal microalgae growth (for this reason the P added was up to 47 mg P L^{-1} as the final concentration). In addition, some micronutrients were also found to be insufficient for the proper growth of microalgae, especially levels of boron, iron, zinc, and copper (Table 1). These micronutrients are essential and play important roles in microalgae growth (García-Camacho et al., 2016). Iron, zinc, and copper participate in redox reactions and as cofactors of key enzymes involved in important metabolic pathways related to cell growth. In fact, several models have been developed to precisely apply the optimal micronutrient concentrations for each type of microalgae growth (Liyanaarachchi et al., 2020; García-Camacho et al., 2016; Song et al., 2012). In addition, the scarce content of iron, zinc and copper was a crucial issue to be solved for microalgae cultivation, as they have important interactions and competitive inhibition of transport and bioavailability, with copper being the limiting element, as it can be toxic at certain concentrations, decreasing microalgae growth and cell density (i.e. 3–4.50 μM L^{-1} of Cu in *Chlorella vulgaris*) (Kebeish et al., 2014), and influencing the extra- and intracellular content of copper itself, iron and zinc (Kumar and Shin, 2017). On the other hand, boron has a structural role in the cell wall, and in fact, boron deficiency alters the physical properties (extensibility and plasticity) of the cell wall and, therefore, impairs cell elongation (Goldbach and Wimmer, 2007). Moreover, phosphorus absorption is greatly hindered in boron deficiency conditions, with this micronutrient being essential in all metabolic processes where phosphorus intervenes.

Therefore, the wastewater used in this study showed a nitrogen:phosphorus (N:P) ratio that was unsuitable for microalgae growth and thus, phosphorus supplementation (8:1 N:P), together with the micronutrients boron, iron, zinc and copper was required to optimize the growth of microalgae (Kumar and Shin, 2017; García-Camacho et al., 2016).

As for the microbiological parameters, the CFU of non-pathogenic aerobic species was high, but not enough to be toxic to the microalgae, and their presence could be an advantage, as shown by the possible synergistic effect with the microalgae consortia for detoxifying pesticides and heavy metals, as well as increasing nutrient depletion in wastewaters (Fouilland et al., 2018).

3.2. Characteristics of microalgal consortia compositions of water samples

Since there was no previous information on the existing populations, a metagenomic analysis of the major groups of microorganisms

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Table 1
Concentration of different physicochemical and microbiological parameters of non-treated wastewater from El Albujón which was used as the substrate for microalgae growth, and treated wastewater with each microalgae consortium (P1, P2, P3 and P4).

	Non-treated wastewater	P1 treated	P2 treated	P3 treated	P4 treated
Nutrients (macro)					
NH_4^+, mg L^{-1}	2.80	<1.00	<1.00	1.01	<1.00
NO_2^-, mg L^{-1} (nitrites)	<0.25	<0.25	<0.25	<0.250	<0.250
NO_3^-, mg L^{-1} (nitrates)	274	27.70	55.60	56	21.9
SO_4^-, mg L^{-1}	3765	4081	3764	3464	3787
HCO_3^-, mg L^{-1}	968	541	1023	922	520
Total Organic Carbon, mg L^{-1}	4.20	61	66	65	97
Total nitrogen (N), mg L^{-1}*	62	11.40	18.60	15.50	12.60
Total nitrogen (N), mg L^{-1}**	<1.00	9.80	11.60	11.50	10.48
Total phosphorus, mg L^{-1}	19.10	<0.05	2.49	2.74	1.75
Nutrients (micro)					
B, mg L^{-1}	3.01	3.83	3.80	3.75	4.01
Cu, mg L^{-1}	<0.05	0.36	0.39	0.36	0.37
Sr, mg L^{-1}	41.70	41.80	40.40	36.00	36.40
Fe, mg L^{-1}	0.16	1.85	1.06	0.96	1.23
Hg, mg L^{-1}	<0.01	<0.01	<0.01	<0.01	<0.01
Pb, mg L^{-1}	<0.05	<0.05	<0.05	<0.05	<0.05
Zn, mg L^{-1}	0.04	1.83	1.87	1.83	1.94
Microbiological characterization					
Ecotoxicity EC_{50}, *Vibrio fischeri*	<25.0 equitox m^{-3}	<1.00	<1.00	<1.00	<1.00
Escherichia coli, cfu 100 mL^{-1}	55	<1	<1	<1	<1
Clostridium perfrigens, cfu 100 mL^{-1}	4	<1	<1	<1	<1
Gut enterococci, cfu 100 mL^{-1}	32	<1	<1	<1	<1
Salmonella sp.	n.d. in 1 L	n.d. in 1 L	n.d. in 1 L	n.d. in 1 L	n.d. in 1 L
Total aerobic bacteria at 22 °C	12,000	390	200	810	490
Physical parameters					
Conductivity at 25 °C, mS cm^{-1}	12.98	16.67	16.36	15.70	16.50
pH	8.05	8.04	8.17	8.17	7.90
Raw weight at 180 °C, mg L^{-1}	13,313	13,909	13,804	11,825	12,554
Turbidity, mg L^{-1}	6.80	278	393	249	121

n.d.: not detected. *Total nitrogen (Kjeldahl + NO_3 + NO_2); **Total nitrogen Kjeldahl.

(Bacteria, Fungi, and Eukaryotes) belonging to the four microalgal consortia, was carried out using a massive sequencing technique. This provided detailed information on the composition of the communities and the differences between them, despite the level of discrimination and characterization of the different OTUs up to the level of well-defined species, as this depends on the quality of the data found in international databases.

A total of 44,686 sequence reads of the 16S rRNA gene were obtained for the P1 samples after trimming low quality sequences and removing chimeras and single/doubletons, 23,931 of them for Eukaryotes, 8388 for Fungi and 12,367 for Bacteria (Fig. 3). These sequences were further divided into 93 OTUs (99% similarity cut-off), that were assigned to Eukaryotes (5), Fungi (6) and Bacteria (82).

For the P2 samples, a total of 1834 readings were obtained for Eukaryotes, 22 for Fungi and 190 for Bacteria. In addition, a total of 5 OTUs of Eukaryotes and 56 of Bacteria were detected, showing the absence of OTUs of Fungi. In the P3 samples, 24,143 sequence reads were obtained for Eukaryotes, 9584 for Fungi and 384 for Bacteria, identifying 3 OTUs of Eukaryotes, 5 of Fungi and 33 of Bacteria. Regarding the P4 samples, 35,201 readings were obtained for Eukaryotes, 18,858 for Fungi and 8522 for Bacteria, and 15 OTUs of Eukaryotes, 7 of Fungi and 107 of Bacteria were identified. Although samples P2 and P3 had the greatest similarity for both Eukaryotes and Bacteria in terms of the composition of their community, samples P1 and P4 showed a significant difference, with the latter having the greatest diversity of OTUs (Fig. 4).

As shown in Fig. 4, the P1 consortium was mainly composed by Eukaryotes. Between them, 61% of the sequences were identified as belonging to the *Selenastraceae* family of green algae with a high similarity (99%) with *Monoraphidium sp* KMMCC 23 (accession code JQ315545). The next most abundant sequence (23% abundance) was identified as the green algae *Desmodesmus subspicatus* (100%), which belongs to the *Scenedesmaceae* family (accession code KU666441). The

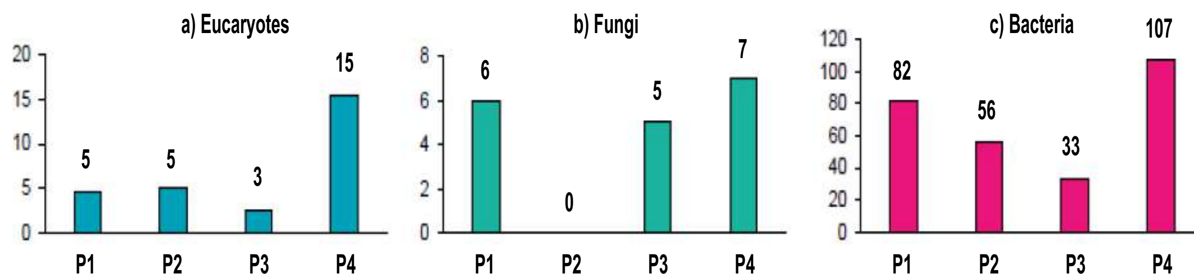

Fig. 3. OTU number obtained per sample in a normalised sequence number by the sequence library for: a) Eukaryotes (1800 sequences), b) Fungi (8000 sequences) and c) Bacteria (2500 sequences).

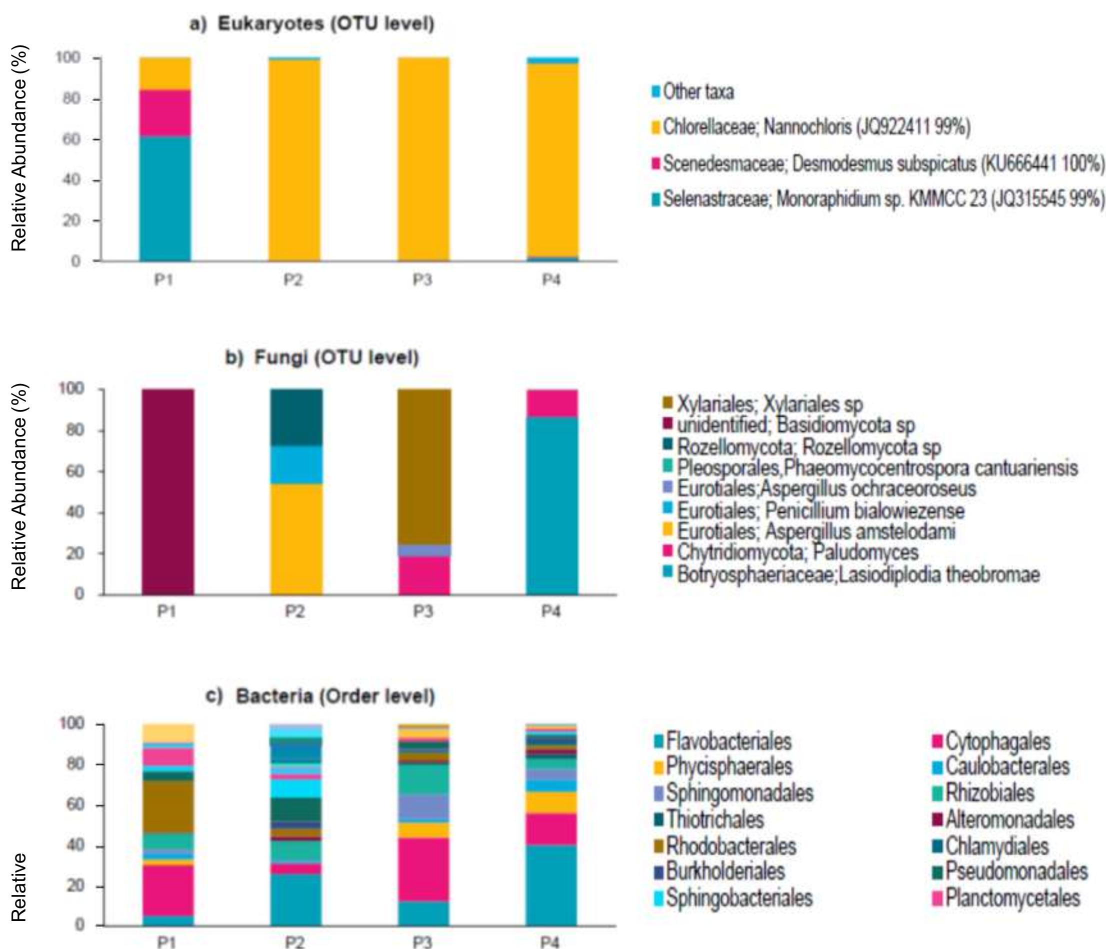

Fig. 4. Relative abundance of OTUs for Eukaryotes (a), Fungi (b) and Bacteria (c) communities from various sampling points.

third most abundant OTU (15.60% abundance) was identified (99%) as the green algae *Nannochloris sp*, from the *Chlorellaceae* family (accession code JQ922411). Only one fungus was found that was classified as a *Basidiomycota* sp. that had not been previously identified. In addition, *Rhodobacterales* (24.90%), *Cytophagales* (24.40%), *Planctomycetales* (8.70%) and *Rhizobiales* (7.90%), dominated the bacterial community, with *Rhodobacterales* and *Cytophagales* being the most abundant (Fig. 4).

Regarding samples P2 and P3, the most abundant OTU (99%) was identified as *Nannochloris sp* at a 99% level of similarity, which belongs to the *Chlorellaceae* family (accession code JQ922411). The rest of the OTUs were found at abundance levels lower than 1%, and were considered unverified data. However, regarding the bacterial community, although a similarity was observed concerning three of the four groups present, they showed significant differences regarding the dominant species. Thus, *Flavobacteriales* (25.30%) were the most abundant in the P2 samples, with *Pseumonodales* (11.60%), *Rhizobiales* (9.50%) and *Sphingobacteriales* (8.90%) appearing in a smaller proportion, whereas in the P3 samples, *Cytophagales* (31.20%) was the most abundant order, three times higher than that found for *Rhizobiales* (14.60%), *Sphingomonadales* (12.0%) and *Flavobacteriales* (12.0%).

The main Eukaryotes present in the P4 samples (96.90%) were a combination of *Nannochloris sp.* (95%) from the *Chlorellaceae* family (accession code JQ922411), and *Monoraphidium sp.* (1.90%) green algae KMMCC 23 (accession code JQ315545), while the abundance of the rest of the OTUs was lower than 1%, corresponding to non-identified taxonomic units. In the bacterial community, the most abundant groups fit within the *Flavobacterial* (39.0%), *Cytophagales* (15.60%), *Phycisphaerales* (10.10%) and *Caulobacterales* (5.80%) orders.

Among the four consortia described (P1-P4), the PCR technique was able to identify mostly only genus and not species owing to the similarity with sp. that had not been previously described. Despite the analysis carried out tentatively but not unequivocally, and the different sp. inside the consortium, the comparison of similarity with sp. collected in the databases was around 99%. P2 and P3 had the most similar profiles (*Nannochloris sp.*) and less variety of microalgae, while P1 and P4 had a greater diversity of OTUs (*Monoraphidium sp.*, *Desmodesmus subspicatus* and *Nannochloris sp.*, 99.6% identified sequences and *Nannochloris* sp. and *Monoraphidium sp.*, 96.9 identified sequences, respectively). Also, there was a general contamination of parasitic fungi and bacteria in all consortia, predominantly from the *Chytridiomycota* group, and especially in P4, which could be associated with an increased metabolic condition for the potential production of interesting metabolites and enzymes. No diatoms were detected in any consortium.

Cooperative and competitive interactions between microalgae and bacteria and/or fungi is a key issue for establishing the higher or lower degree of nutrient and ECs removal according to their different mechanisms of action (Gonçalves et al., 2017). The fungal occurrence in most microalgae consortia is not usually mutualistic, and can provoke the bio-flocculation of the eukaryotes (Egede et al., 2016). In this regard, as described above, P1, P2, P3 and P4 were microalgae-bacteria-fungi consortia (Fig. 4). According to the previous literature, a single microalgae consortium was applied to dairy manure (Wilkie and Mulbry, 2002; Woertz et al., 2009; Qin et al., 2016), and in general, to

sewage wastewater (Renuka et al., 2013), while only microalgae consortia (Koreivienė et al., 2014; Woertz et al., 2009; Silva-Benavides and Torzillo, 2012; Samorì et al., 2013; Su et al., 2012; Tripathi and Shukla, 1991) or microalgae-bacteria consortia (Van Den Hende et al., 2011; He et al., 2013) have been applied to municipal wastewater treatments, depending on its origin and characteristics. Regardless the dairy industry, the agri-food processing, oil, metal and chemical industries have also applied microalgae-bacteria consortia for its wastewater in all cases (Hernández et al., 2013; Raposo et al., 2010; de Godos et al., 2009; Ren et al., 2015; Safonova et al., 2004). Therefore, our consortia were more related to wastewater from municipal and agri-food origin, owing to the common microalgae-bacteria association. Regarding the type of eukaryotes, bacteria or fungi, and according to previous research, P1 was the consortia that showed the highest similarity with the microalgae composition corresponding to *Chlorellaceae* (*Nanochloris sp.*) and *Scenedemaceae* families used in numerous municipal, sewage and agri-food company's (dairy) wastewaters (Qin et al., 2016; Van Den Hende et al., 2011; Woertz et al., 2009; Su et al., 2012; Koreivienė et al., 2014; Tripathi and Shukla, 1991; Renuka et al., 2013). The *Chlorellaceae* family was only used for the treatment of numerous agri-food companies' (plant-based foods) wastewater as it is common for P1, P2, P3 and P4 consortia (de Godos et al., 2009; Raposo et al., 2010; Hernández et al., 2013). Therefore, the *Chlorellaceae* and *Scenedemaceae* family's consortia could be the most suitable microalgae composition for the municipal and agri-food processing wastewater generated in the Mar Menor and the El Albujón area. The fungi and bacteria composition of the four (P1-P4) consortia was not in agreement with the previous wastewater studies, demonstrating that the key is the microalgae composition (Gonçalves et al., 2017).

3.3. Characterization of microalgae growth

Microalgae growth was assessed by measuring biomass concentration (expressed in g DW L^{-1}) and cell count (cell mL^{-1}), throughout the cultivation period, both in the adaptation period (for growing of microalgae inoculum, for 24 d), and in the PBRs (for 29 d).

Prior to the cultivation of microalgae in the PBRs, a characterization was performed of their growth during the adaptation period (for generating the microalgae inoculum) from the four consortia. Fig. 5 shows the time courses of the microalgae growth, in the adaptation period for microalgae inoculum generation (expressed as biomass concentration, g DW L^{-1} and cell count (cells mL^{-1})) of the different consortia. As observed in Fig. 5, microalgae cultures from the four consortia showed a typical S-shaped growth curve starting with an initial cell count of around 1–4.50 × 10^4 cells mL^{-1} (which was equivalent to a DW L^{-1} of about 0.15–0.30 g), that was maintained for the first five days (corresponding to their lag phase). Regarding the growth during this phase of the microalgae cultivation, a slow and small increase in biomass concentration was observed in all consortia, being smaller in consortia P1 and P2 (Fig. 5). It is important to note that in this first phase of

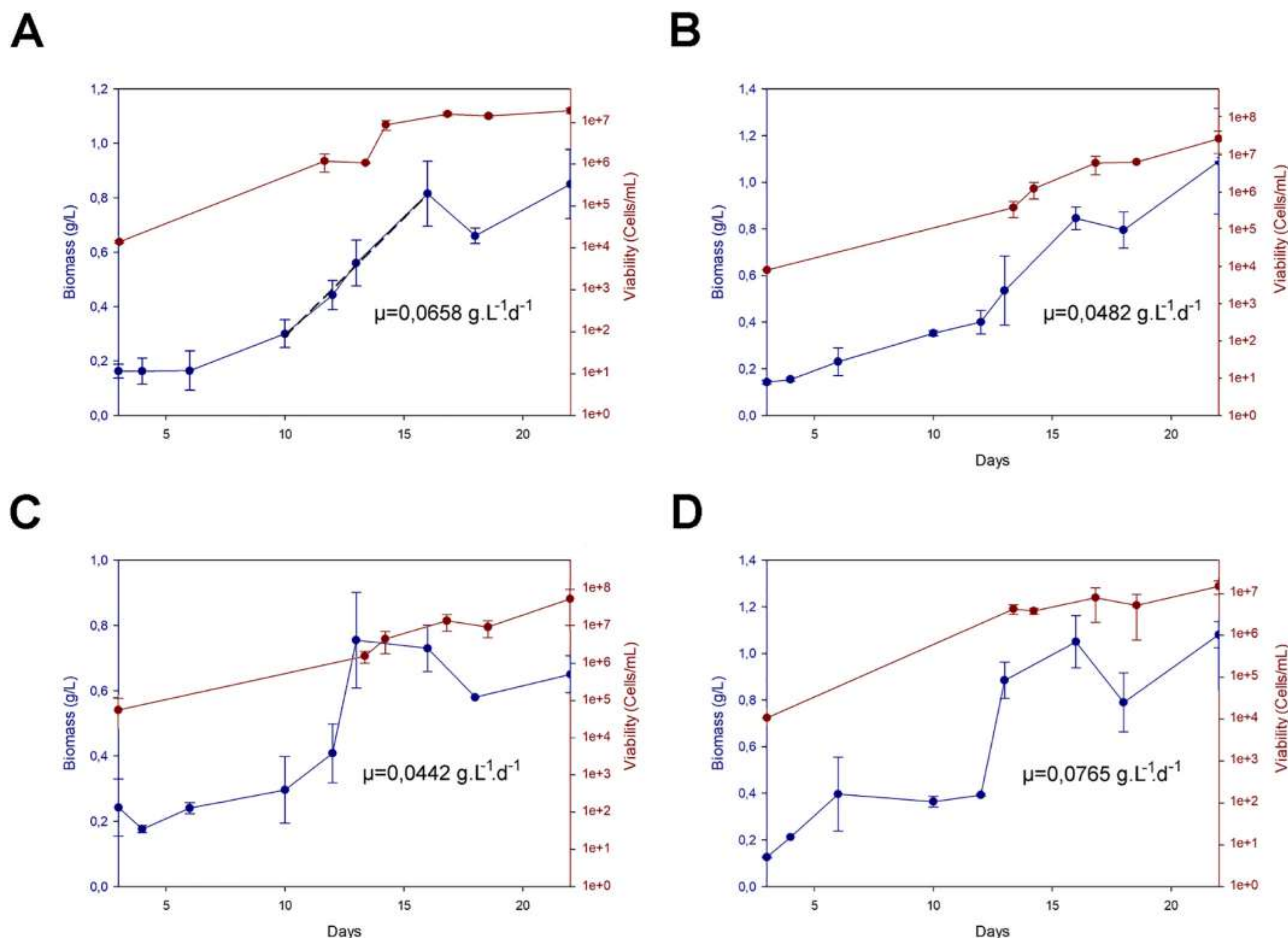

Fig. 5. Time course of the microalgae growth in the adaptation phase for microalgae inoculum generation (expressed as biomass concentration, g DW L^{-1} and cell count (cells mL^{-1}) of the different consortia: A) P1, B) P2, C) P3 and D) P4.

cultivation (lag phase), growth was reduced because the microalgae were carrying out the processes of nutrient uptake they needed to initiate cell division and growth processes.

Then, microalgae cultures showed a marked exponential growth phase which began from approximately day 10 until day 20–21, where cell count increased up to $1.70–5.20 \times 10^7$ cells mL^{-1}, and the maximal biomass concentration was enhanced to around 0.80–1 g DW L^{-1}, depending of microalgae consortia (Fig. 5). In fact, during this phase, cell division and growth processes were activated, observed as a large increase in the number of cells and the highest values of cell biomass. It is important to note that when the microalgae are in this phase of greater metabolic activity (exponential phase), they consume a greater quantity of mineral nutrients, greatly reducing their concentration in the culture medium.

Lastly, cell division slows down due to the depletion of some essential nutrients in the microalgae cultures and growth stops (reaching their stationary phase) with the biomass concentration remaining around these values until 22–24 d of cultivation.

In addition, after 10 d of cultivation (which corresponded to the exponential growth phase), a progressive reduction of green microalgae, which were more abundant at the beginning of the cultivation, was observed, along with a gradual emergence during the exponential phase of different diatoms species in the P1 and P2 consortia, when analysed under the light of the microscope (see Fig. S1, B and D). However, while green microalgae remained in the P1 consortium together with diatoms (see Fig. S1 B), in consortium P2, the latter (microalgae) stopped being majority becoming diatoms (see Fig. S1 D).

Moreover, a clear increase in diatoms and some brown algae was observed in the P3 consortium over time, while green microalgae tended to disappear (see Fig. S2 B). However, in the P4 consortium, the brown microalgae were found to be predominant, with the microalgae cultivation adopting a reddish appearance (see Fig. S2 D), although diatoms also appeared, as in the previous consortia. These results indicated an evolution of the microalgae consortia over time, as diatoms species were no detected by metagenomic analysis as described above.

Furthermore, the viability data (not shown) assessed with vital dyes showed a high number of viable cells at the end of the exponential phase, reaching high viability percentages in all consortia (between 80 and 98%). This high viability indicated the good growth of microalgae cultures.

In addition, the slope, calculated by linear regression of the exponential phase (Fig. 5), provided a biomass productivity in the adaptation period of around 0.04–0.07 g DW L^{-1} d^{-1} under the culture conditions applied, with this biomass generation rate being higher in microalgae consortia P1 and P4 (Fig. 5). These results, obtained from the adaptation period, make feasible the use of microalgae from different consortia as inoculum to initiate microalgae growth in the PBRs.

3.4. Effect of different microalgae consortia on the removal of metals and nutrients in the photobioreactors

To determine the degree of decontamination that each consortium was able to achieve, as well as, to verify if they were able to grow in the presence of mineral nutrients, metals and ECs, microalgae cultures were initiated by inoculating 22-day-old microalgae obtained from the adaptation period, and grown in the PBRs for 29 d. The determination of the growth profile was performed by taking periodic samples from the beginning until 29 d of culture, in which characteristic growth parameters such as DW L^{-1}, cell count, as well as the conductivity and pH of the culture medium were measured. Fig. 6 shows the different growth curves, of sigmoidal type, of the different microalgae consortia, and their dependence on the concentration of nutrients contained in the wastewater (Table 1). The specific growth rate (d^{-1}) was obtained through the changes in the DW, as indicated in Section 2.5.1, and the productivity of microalgae biomass was calculated from the linear regression of the exponential phase. Microalgae consortia responded rapidly, showing a typical growth pattern with an exponential phase starting at day 10 and finishing at day 23 of cultivation (t_a), the exhaustion time in which there was a depletion of some essential nutrients needed for microalgae growth (Fig. 6). At this point, a maximum biomass production was observed in all the microalgae consortia, reaching a value between 1.32 and 1.80 g DW L^{-1} (indicating a cell count value of $9.31–13.65 \times 10^7$ cells mL^{-1}). Also, the variation of the pH of the culture medium was measured during the growth of microalgae in PBRs over time (Fig. 6). In all the microalgae consortia, a decrease in pH to values close to 7 was observed at the beginning of the culture during the lag phase. However, from day 10 on, this trend changed, with a continuous increase in pH observed until reaching values close to 8, coinciding with the exponential growth phase of the microalgae cultures, and subsequently a slight decrease in pH from the entry of the microalgae cultures into the stationary phase. This pH variation is conditioned by cellular metabolism (energy state of microalgae for the synthesis of reduced cofactors) and depends on the availability of nitrogenous sources that modify the pH of the culture medium throughout the increase of the microalgae biomass. At the beginning of the culture, during the lag phase, when the metabolism is reduced, microalgae use the nitrogen source whose absorption is less energy costly. Thus, they begin to use ammonium, causing a slight drop of pH, as the entry of ammonium ions into cells is accompanied by the extrusion of protons. However, when the cells are in the phase of greatest metabolic activity (exponential phase), they consume a greater amount of mineral nutrients, especially decreasing the nitrate compounds in the culture medium, as observed in Fig. 6. This consumption of nitrates contributes to the increase in the pH of the medium due to the consumption of protons that are used for the reduction of nitrates.

It is important to note that the incorporation of nitrogen also depends on the ratio of ammonium and nitrate concentrations in the culture medium (Loulakakis and Roubelakis-Angelakis, 2001) and the use of carbon sources for obtaining energy. Therefore, coinciding with the entry of the microalgae into the stationary phase (from day 23 of the culture), in which the primary metabolism slows down, a decrease in pH was observed once again.

3.5. Biomass productivity, nitrates removal and phosphorus consumption by the microalgae consortia

As observed in Fig. 6, the microalgae inoculum obtained from the adaptation period, from the WWTP effluent from Los Alcázares (consortium P1), showed an exponential growth, with a specific growth rate and biomass productivity of 0.14 d^{-1} and 0.11 g DW L^{-1} d^{-1}, respectively. The combination of *Chlorellaceae*, *Scenedemaceae* and *Selestranaceae* families produced a higher content of biomass than *Chloraceae* itself (Choudhary et al., 2016). Most of the nitrates were removed during the exponential growth (initially being 274 mg L^{-1}) decreasing to values below 27.70 mg L^{-1} in this phase (Fig. 6A). Also, the physicochemical analysis of the treated wastewater confirmed the consumption of other macronutrients such as ammonium (<1.00 mg L^{-1}), bicarbonate (<550 mg L^{-1}) and phosphorus (<0.05 mg L^{-1}) (Table 1). Thus, P1 was considered one of the more interesting candidates for total nitrogen removal and phosphorus consumption. This finding was in agreement with previous studies which described the efficient nitrogen removal and phosphorus consumption of the two main microalgae found in the P1 consortium, *Chlorella* sp. and *Scenedemus sp.* (Koreivienė et al., 2014; Whitton et al., 2016), as well as the presence of diatoms (Marella et al., 2020). The content of the micronutrients increased due to the supplementation performed to maintain microalgae growth. Thus, the concentrations of B, Cu, Fe and Zn, which were initially added to maintain microalgae growth, significantly increased proportionally in all consortia (Table 1).

In addition to nutrient removal, the acute ecotoxicity (EC_{50}) of non-treated wastewater was nearly absent in the wastewater treated with the microalgae consortia, and the microbiological analysis also revealed

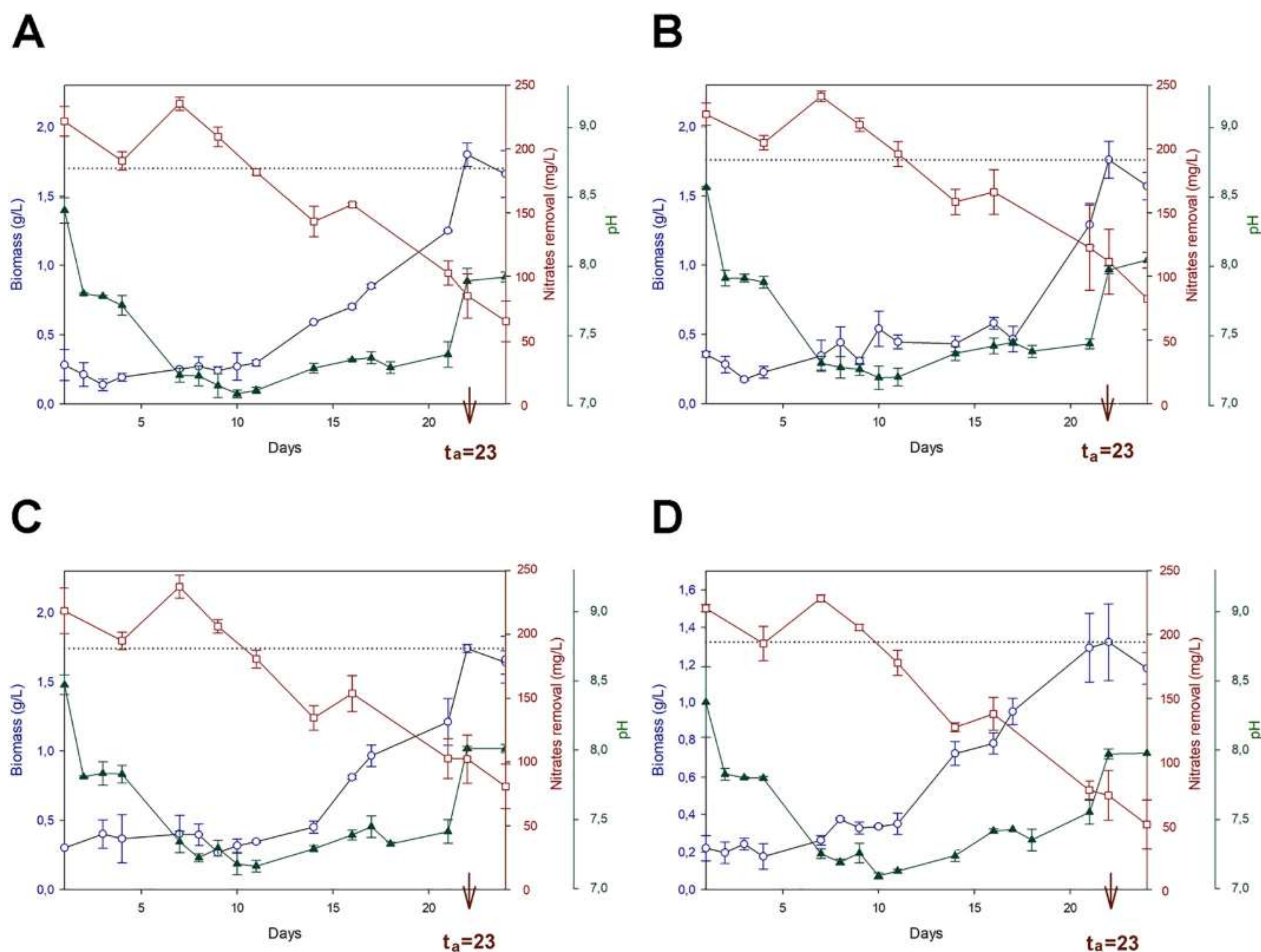

Fig. 6. Biomass concentration and nitrates removal. Exponential phases are shown. A) Consortia P1. B) Consortia P2. C) Consortia P3. D) Consortia P4.

an improvement of the wastewater quality after treatment with the P1 consortium (Table 1). The appearance of bacteria could be mutualistic or independent of the specie (Gonçalves et al., 2017). A similar trend was observed in all microalgae consortia for EC_{50} ecotoxicity, and in the analysis of pathogenic bacteria (not detected at the end of the microalgae cultivation cycle), further improving the microbiological load of total aerobic bacteria with respect to the initial data on untreated wastewater (Table 1).

Regarding the P2 consortium (recovered after the WWTP effluent with El Albujón dry riverbed), the results showed that the microalgae growth was as P1 but slightly lower in terms of biomass production and nutrient removal (Fig. 6B). In fact, the specific growth rate was 0.12 d^{-1} (13% lower than that achieved by P1), while the productivity of microalgae biomass was 0.10 g DW L^{-1} d^{-1} (6.30% lower than that reached by P1). This biomass production was in agreement with the *Chlorellaceae*-dominated consortia (Choudhary et al., 2016). The highest removal of total nitrogen (79.70% of NO_3^-), was achieved 10 days after reaching the stationary phase (at day 29), and was 11.34% lower than that obtained for P1 (89.90%), that is, 55.6 mg L^{-1} for P2 compared to 27.7 mg L^{-1} for P1 (Table 1), demonstrating the synergistic action of *Chlorella* sp. and *Scenedemus* sp. in P1 rather than *Chlorella* sp. alone in P2 (Whitton et al., 2016; Gonçalves et al., 2017).

The phosphorus concentration decreased steadily, reaching a minimum value of 2.49 mg L^{-1} at the end of experiment, but was above P1, which peaked below minus 0.05 mg L^{-1} at the end of the study. However, there were no differences between P1 and P2 for ammonium removal, P2 showed better removal capacity for Fe and Sr than P1, but without changes in the concentration of bicarbonate and other nutrients such as sulphates (Table 1).

Consortium P3 (recovered before drainage into the Mar Menor), showed an exponential shape with a specific growth rate and biomass productivity of 0.13 d^{-1} and 0.10 g DW L^{-1} d^{-1}, respectively in agreement with previous results (Choudhary et al., 2016).

The phosphorus and ammonium concentrations progressively dropped down to 2.74 mg L^{-1} and 1.01 mg L^{-1}, respectively, with the removal capacity being slightly worse than those achieved by P1 and P2, but similar to the latter in the removal of nitrates (Fig. 6C). This could be related to the presence of *Sphingomonodales* or *Cytophagales* bacteria in this P3 consortium, which perhaps hindered the metabolism of microalgae (Gonçalves et al., 2017), as microalgae species present in the P3 were the same as in P2. However, no changes in the concentration of bicarbonate or other nutrients such as sulphates were observed, as in the case of the P2 consortium (Table 1).

In the case of P4 (sampled from Mar Menor muds), the specific growth rate and biomass productivity were 0.10 d^{-1} and 0.07 g DW L^{-1} d^{-1}, respectively. This lower biomass production could be explained by the presence of other, less productive microalgae or by bacteria that hindered the microalgae growth (Gonçalves et al., 2017; Choudhary et al., 2016). The biodegradation ability for nitrates (92% of initial load, 21.90 mg L^{-1}), was similar

to that achieved by P1, being even better than the P2 and P3 consortia (Fig. 6D). Ammonium and bicarbonate were also practically depleted, once more with a similar behaviour to the P1 consortium (Table 1). Phosphorus consumption was nearly complete at up to 1.75 mg L^{-1}. In this case, some minor microalgae (*Monoraphidium sp.*) or bacteria could have been acting in a synergistic manner with *Chlorella sp.*, and the presence of diatoms could have helped achieve similar rates of removal of nitrates and phosphorus consumption as P1 (Gonçalves et al., 2017). No changes were observed in sulphate concentration, and the supplemented micronutrients (Fe, B, Cu and Zn) slightly increased in concentration after the treatment of wastewater with the P4 consortium, in line with the results previously described for the rest of the consortia.

3.6. Effect of different microalgae consortia on the removal of emerging contaminants in the photobioreactors

ECs were also considered for this study, especially some herbicides and drugs. Atrazine and adenosine were initially detected in wastewater at the concentrations described in Table 2, while simazine, terbuthylazine and ibuprofen, which were not found initially, were further added (ng L^{-1}) to the non-treated wastewater using the concentrations found in previous reports (Table 2), due to the current interest in removing trace emerging contaminants from domestic wastewater. Regarding pesticides, atrazine and simazine are two types of herbicides that are widely used in agriculture, and their occurrence in wastewaters ranges from 1.20–190 ng L^{-1} and 20–1600 ng L^{-1}, respectively (Cahill et al., 2011; Martin Ruel et al., 2012; Rosal et al., 2010; Köck-Schulmeyer et al., 2013; Benvenuto et al., 2010). Terbuthylazine is a chloro-triazine characterized by ethylamine and *tert*-buthylamine side chains, which belongs to the family of triazine-type herbicides such as atrazine and simazine, and it has been found in a similar concentration or slightly lower than the other pesticides in wastewaters (2–53 ng L^{-1}) (Carretta et al., 2019; Singer et al., 2010; Köck-Schulmeyer et al., 2013; Benvenuto et al., 2010). Ibuprofen is a nonsteroidal anti-inflammatory and analgesic that is very frequently used by the population in general, and its high concentrations found in wastewaters in some countries (2–17 mg L^{-1}) dramatically affect the environment (Thalla and Vannarath, 2020; Pereira et al., 2020). In fact, this drug causes the inhibition of photosynthesis at doses between 10 and 100 mg L^{-1}, and lysosomal membrane instability at much lower concentrations in frogs (250 ng L^{-1}) (Ding et al., 2017; Falfushynska et al., 2017). It has been found in wastewaters from different sources and in other environmental samples (1900–16,000 ng L^{-1}) (de Oliveira et al., 2020, Carretta et al., 2019, Lizano-Fallas et al., 2017). Adenosine (6-amino-9-β-D-ribofuranosyl-9-H-purine, commercial brands, Adenocard or Adenoscan), a purine-based drug which acts as a coronary vasodilator for increasing blood flow in the heart muscle, and used for the conversion of sinus rhythm of paroxysmal supraventricular tachycardia, is scarcely detected in wastewaters. To the best of our knowledge, only a few previous reports have described the occurrence of this drug in rivers (Rimayi et al., 2019), and wastewaters (580–610 ng L^{-1}) (Gago-Ferrero et al., 2020). In our study, adenosine was detected at a much lower concentration (3.25 ng L^{-1}) (Table 2).

Table 2
Emerging contaminant levels (ng L^{-1}) before and after the wastewater treatments with microalgae consortia.

Water sample	Simazine	Atrazine	Terbuthylazine	Ibuprofen	Adenosine
Non-treated	45.40	8.90	11.40	200.50	3.25
P1 treated	n.d.	n.d.	<LOQ	n.d.	0.70
P2 treated	n.d.	n.d.	3.28	n.d.	0.75
P3 treated	n.d.	n.d.	4.58	n.d.	1.40
P4 treated	n.d.	n.d.	1.31	n.d.	0.90

n.d.: not detected; LOQ: limit of quantitation (simazine, 10 ng L^{-1}; atrazine, 3 ng L^{-1}; terbuthylazine, 0.50 ng L^{-1}, adenosine, 0.50 ng L^{-1} e ibuprofen, 20 ng L^{-1}).

After the wastewater treatments with the different microalgae consortia, simazine, atrazine and ibuprofen were undetectable in all treatments while terbuthylazine was only not detected in P1 (Table 2). The partial reduction of terbuthylazine found after the wastewater incubation with P2, P3 and P4 consortia (71.20, 59.80 and 88.50%, respectively) was in agreement with previous studies (Hultberg et al., 2016; Hultberg and Bodin, 2018). A possible explanation of the effectiveness of the P1 consortium in the total removal of terbuthylazine could be the combined action of microalgae with bacteria, since the joint action of these microalgae with fungi was also inefficient (Sutherland and Ralph, 2019). On the other hand, adenosine was not completely removed from treated wastewaters by any of the microalgae consortia, although P1 and P2 were the most efficient, managing to eliminate more than 75% of this compound (Table 2).

According to previous research, *Chlorella vulgaris*, present in all the microalgae consortia, may be the key factor for the total removal of simazine and atrazine (González-Barreiro et al., 2006; Mishaqa, 2017), while the total removal of ibuprofen required the combined action of this microalgae with bacteria, which were present in the four consortia, to achieve the complete removal of this compound in wastewater (Fig. 4) (Sutherland and Ralph, 2019; Kruglova et al., 2014), as the combination of *Chlorella* with *Scenedemus* was not sufficient (it was achieved only 40% removal) (Matamoros et al., 2016).

Regarding the drug adenosine, this is the first study that describes its partial removal for all the consortia studied, as its total degradation was only achieved by inorganic photocatalysis (Romão et al., 2016).

Similar studies have been carried for the removal of pollutants using microalgae bioremediation in numerous municipal, sewage and agri-food processing, oil, metal and chemical industries wastewaters (Qin et al., 2016; Van Den Hende et al., 2011; Woertz et al., 2009; Su et al., 2012; Koreivienė et al., 2014; Tripathi and Shukla, 1991; Renuka et al., 2013; Hernández et al., 2013; Raposo et al., 2010; de Godos et al., 2009; Ren et al., 2015; Safonova et al., 2004) but the current study is the first one that has combined the successful removal of herbicides, ECs such as ibuprofen and adenosine, and nitrates and phosphates.

Once the capacity of four microalgae-based consortia to reduce water contamination was demonstrated, we evaluated the biomass composition of the P1 consortium (the best in terms of nutrient depletion and emerging contaminant removal), for a further potential valorisation, with special emphasis on nutritional composition, profile of fatty acids and carotenoids. Although the protein content was not too high (usually between 30%–60%), it was still one of the major fractions, along with carbohydrates (see Table S1). Although the lipid fraction was rather low, it was perhaps the fraction with the greatest commercial interest because it usually contained molecules such as pigments, sterols, and polyunsaturated fatty acids. The carotenoid profile (see Table S2), showed a natural pigment content of 47 mg kg^{-1} in the P1 fresh biomass, of which more than 68% was lutein (about 0.90% *w*/w of total biomass), and almost 20% β-carotene, although appreciable amounts of the rest of pigments were no detected. Considering the humidity of the sample (96.70%), it would result in 1424 mg of carotenoids for each kg of dry matter, representing 1.40% w/w of total carotenoids in algal biomass.

These results showed the valorisation potential of the P1 microalgae biomass, either through the extraction of compounds with nutraceutical, cosmetic, alimentary or pharmacological interest such as oxylipins, fatty acid derivatives for cosmetics and functional foods, and proteins, peptides and carbohydrates for organic fertilisation or for energy generation (biofuel or biogas production).

4. Conclusion

The El Albujón watercourse has a high content of inorganic nitrogen and sulphates, with phosphorus levels being the lowest before discharge into the Mar Menor lagoon. The presence of certain pesticides

and drugs also contributes to the increase of water contamination. The aim of this study was to evaluate the growth of autochthonous microalgae consortia collected from four different sites in the El Albujón dry riverbed, as well as their efficacy in removing ECs (simazine, atrazine, terbuthylazine, adenosine and ibuprofen) and nitrates and phosphates, as a sustainable approach for the minimization of the eutrophication process of the Mar Menor lagoon.

Among the four microalgae consortia, consortium P1 was the best in terms of biomass productivity (0.11 g L^{-1} d^{-1}) and specific growth rate (0.14 d^{-1}), providing 100% removal of ECs (simazine, atrazine, terbuthylazine, adenosine and ibuprofen) and a maximal reduction and consumption of macronutrients (especially nitrates and phosphates, reaching levels below 28 mg L^{-1}, that is, a decrease of 89.90 and 99.70% for nitrates and phosphates, respectively). Therefore, this consortium (*Monoraphidium sp., Desmodesmus subspicatus, Nannochloris sp.*) could be selected as a green filter for successful large-scale applications.

CRediT authorship contribution statement

Use this form to specify the contribution of each author of your manuscript. A distinction is made between five types of contributions: Conceived and designed the analysis; Collected the data; Contributed data or analysis tools; Performed the analysis; Wrote the paper.

For each author of your manuscript, please indicate the types of contributions the author has made. An author may have made more than one type of contribution. Optionally, for each contribution type, you may specify the contribution of an author in more detail by providing a one-sentence statement in which the contribution is summarized. In the case of an author who contributed to performing the analysis, the author's contribution for instance could be specified in more detail as 'Performed the computer simulations', 'Performed the statistical analysis', or 'Performed the text mining analysis'.

If an author has made a contribution that is not covered by the five pre-defined contribution types, then please choose 'Other contribution' and provide a one-sentence statement summarizing the author's contribution.

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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.

Acknowledgements

This work was funded by the Instituto de Fomento de la Región de Murcia, Spain (RAMBLA Project 2015.08.ID+I.0003), under research and innovation program.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.143613.

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