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Developing a microalgal-bacterial consortium for the removal of organic pollutants from petrochemical industry

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ABSTRACT

Organic pollutants are one of the most hazardous contaminants due to their toxicity and ubiquity. The presence of organic compounds, such as phenol or BTEXs (benzene, toluene, ethylbenzene and xylene), in petrochemical wastewater causes severe damage in aquatic environments. Different bioremediation technologies have been tested, but with limited efficiencies. This work assessed the biodegradation potential of a consortium composed of the microalga *Chlorella vulgaris* and the bacterium *Rhodococcus opacus* at different levels of phenol and BTEXs in batch assays, which could be a first step to solve the limitations of previous technologies, especially at low concentrations of pollutants. The results showed that *C. vulgaris* tolerated and metabolized high concentrations of these contaminants: 100 mg L^{-1} of phenol, 15, 8, 3 and 1 mg L^{-1} of benzene, toluene, ethylbenzene and *o*-xylene, respectively. Moreover, the co-cultivation of *R. opacus* and *C. vulgaris* enhanced organic pollutant biodegradation when using NO_3^- and NH_4^+ as nitrogen sources. Finally, cultivation of the consortium under N_2/CO_2 (70/30 %) atmosphere resulted in a significant enhancement in phenol and BTEX biodegradation. Under optimized conditions, the consortium *C. vulgaris-R. opacus* metabolized 100 % of a mixture of phenol, benzene, toluene, ethylbenzene and *o*-xylene at 25, 3, 3, 3 and 1 mg L^{-1}, respectively, within 6 days of cultivation. These results highlight the potential of algal-bacterial consortia to biodegrade organic pollutants from petrochemical industries, which can be a low-cost and sustainable alternative for the biodegradation of petrochemical effluents.

1. Introduction

Industrial pollution is nowadays one of the main environmental problems worldwide. Over the last years, the increasing demand for energy and industrial products has caused water quality degradation in different environments. This environmental impact is produced by several inorganic (ammonium, phosphate, heavy metals) and organic (polycyclic aromatic hydrocarbons, pesticides, phenol derivates) pollutants [1]. The industrial relevance of diesel, petroleum and their derivates in society during the last century has rendered petrochemical industries one of the most extended and polluting industries [2]. Petrochemical effluents contain high concentrations of aliphatic hydrocarbons, phenolic compounds, or mono and polycyclic aromatic hydrocarbons. Short-term exposure to these pollutants can cause respiratory problems, eye irritation, skin blisters in humans and animals, and metabolic and DNA damage in microorganisms, even at low concentrations [3].

Despite the composition of the petrochemical effluents is quite diverse, BTEX, phenol, ammonium or phosphates are typically present in these wastewaters. The concentrations of these compounds depend on the effluents, being in the range of 0.08–34.36 mg L^{-1} for benzene, 0.34-41.08 mg L^{-1} for toluene, 0.03–1.90 mg L^{-1} for ethylbenzene, 0.01–33 mg L^{-1} for xylenes, 0.01–23 mg L^{-1} for phenols, or 10–300 mg L^{-1} for NH_4^+ [4–7]. The removal of these compounds has been carried out using physical and chemical methods such as adsorption, flocculation, or oxidation [8,9]. However, these technologies entail high operational costs with limited effectiveness at low pollutant concentrations [10].

In this context, the degradation of these pollutants using microorganisms has emerged as a promising solution to prevent the deterioration of aquatic environments. Different bacteria have been widely investigated in the past years due to their capacity to biodegrade these pollutants [11,12]. However, recent studies have proposed the co-cultivation of microalgae as a promising platform for removing petrochemical pollutants [13]. Microalgae are a group of oxygenic

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photosynthetic organisms that grow under different stress conditions including heavy metals contamination and organic and inorganic pollutants [14,15]. Different studies have reported that green algae and, more specifically, *Trebouxiophyceae* species, such as *Chlorella* or *Coccomyxa* ones, developed different mechanisms to tolerate high concentrations of organic pollutants and obtained excellent results during the biodegradation of these compounds [16,17]. Thus, *Chlorella vulgaris* emerged as a promising option for the biodegradation of organic pollutants present in the petrochemical industry.

Moreover, due to their capacity to produce O_2, microalgae can be cultivated in symbiosis with different oxygenic bacteria to develop a symbiotic relationship based on the exchange of O_2 and CO_2. In addition, they can also support an exchange of carbon sources, vitamins, minerals and trace elements [10]. Microalgae-bacteria systems can be a sustainable alternative to mechanically aerated bacterial systems for petrochemical wastewater treatment due to their capacity to simultaneously remove organic and inorganic pollutants without stripping. Moreover, the absence of mechanical aeration leads to lower operational costs and carbon footprint than their bacterial counterparts [18]. Different microalgae have been grown in symbiosis with bacteria such as *Rhizobium* or *Azospirilium*, resulting in higher biomass productivities or capacities to degrade ammonium, phosphorous or phenol [19–21]. In addition, some microalgae can biotransform organic pollutant into intermediates that can be further biotransformed by bacteria [22]. One of the most promising bacteria for organic pollutants biodegradation is *Rhodococcus opacus*. This microorganism is able to biodegrade compounds that appear in petrochemical wastes, such as phenol, toluene or *o*-xylene [23,24], and it also tolerates high concentrations of these organic compounds [25]. All these characteristics point out *Rhodococcus opacus* as an outstanding candidate for biotechnological applications related to organic pollutants biodegradation. Thus, the combination of *R. opacus*, which is a bacterium that tolerates and biodegrade high concentrations of BTEXs and phenol, and *C. vulgaris*, which is a microalga with a high tolerance to multiple organic pollutants and fast growth rates, would result in an excellent symbiosis between both microorganisms, with a balanced CO_2/O_2 exchange. This novel system can be studied as a promising tool for the bioremediation of petrochemical effluents, which contains high concentrations of organic pollutants [4].

In this work, the tolerance and biodegradation capacity of the microalga *Chlorella vulgaris* cultivated in the presence of different concentrations of phenol, benzene, toluene, ethylbenzene and *o*-xylene were tested. Moreover, the consortium *Chlorella vulgaris-Rhodococcus opacus* was optimized to enhance the biodegradation of these pollutants, using ammonium chloride as a supplementary nitrogen source. Finally, the influence of the headspace composition (air versus a N_2/CO_2 (70/30 %) mixture) on pollutant biodegradation was investigated.

2. Materials and methods

2.1. Microalgal/bacterial strains

The microorganisms used in this work were the freshwater microalga *Chlorella vulgaris* SAG 211-11b (SAG Culture Collection of Algae, Germany) and the bacterium *Rhodococcus opacus* DSM 43205 (DSMZ, Germany). The bacterial inoculum was cultured in ammonium mineral salt medium (AMS), as described by Huang-Lin et al., [26], containing (in g L^{-1}):0.49 Mg_2SO_4, 5 NaCl, 0.5 NH_4Cl, 1 KNO_3 and supplemented with trace elements (mg L^{-1}): 0.01 $CuCl_2$, 0.9 $FeCl_2$, 0.06 $ZnCl_2$, 0.01 $NiCl_2$, 0.06 $CoCl_2$, 0.03 Na_2MoO_4, 0.06 $MnCl_2$, 0.06 H_3BO_3, 0.4 Na_2SeO_3, 0.01 Na_2WO_4, and vitamins (mg L^{-1}): 0.02 biotin, 0.2 nicotinamid, 0.1 *p*-aminobenzoic acid, 0.2 thiamin, 0.1 panthotenic acid, 0.5 pyridoxamine, 0.1 cyanocobalamine and 0.1 riboflavine. 2 g L^{-1} of glucose were added as a carbon and energy source in 2 L closed bottles. Three days before inoculation, the cultures were washed and re-inoculated in AMS medium with 3 mg L^{-1} of the corresponding organic pollutant as the only carbon and energy source for bacterial acclimatization. Microalgal inocula were cultivated in SK medium enriched with peptone (0.0625 g L^{-1}), glucose (3.125 g L^{-1}) and yeast extract (0.0625 g L^{-1}), as described in Vargas-Estrada et al., [27] containing (in g L^{-1}):0.125 Mg_2SO_4, 1.25 KNO_3, 0.11 $CaCl_2{\cdot}2H_2O$, 0.114 H_3BO_3, 0.05 $FeSO_4{\cdot}7H_2O$, 0.088 $ZnSO_4{\cdot}7H_2O$, 0.0144 $MnCl_2{\cdot}4H_2O$, 0.0071 MoO_3, 0.016 $CuSO_4{\cdot}5H_2O$, 0.005 $Co(NO_3)_2$, 0.5 EDTA, 0.624 KH_2PO_4 and 1.325 K_2HPO_4.

2.2. Biodegradation experiments

Microalgal-bacterial biodegradation experiments were performed in 250 mL gas-tight glass bottles, containing 100 mL of SK medium under different gas atmospheres and nitrogen sources, as described in Table 1, which were closed with butyl septa and aluminium caps. The bottles were inoculated with an initial optical density (OD) of 0.2 at 600 nm. For microalgal-bacterial experiments, the OD proportion of microalgae/bacteria was 1:1. The microalga or the algal-bacterial consortium were cultivated at 25 °C and pH initially adjusted to 6.8, under continuous agitation (300 rpm) and light irradiation (300 μmol m^{-2} s^{-1}).

C. vulgaris or the microbial consortium were grown for 5–6 days in SK medium in the microalgal tolerance or alga-bacterial biodegradation tests (Table 1). The pollutants tested were phenol (at aqueous concentrations 25, 50 and 100 mg L^{-1}) and benzene, toluene, ethylbenzene or *o*-xylene (at aqueous concentrations of 3, 8 and 15 mg L^{-1} for single experiments). Moreover, a mixture of these pollutants was tested at a concentration of 25 mg L^{-1} of phenol and increasing concentrations of benzene (3, 3 and 8 mg L^{-1}), toluene (3, 3 and 8 mg L^{-1}), ethylbenzene (1, 3 and 3 mg L^{-1}) and o-xylene (0.5, 1 and 1 mg L^{-1}) in assays Mix 1, 2 and 3, respectively. These concentrations were selected to simulate those of petrochemical effluents [4]. The fate of pollutant concentrations and the accumulation of biodegradation intermediates (i.e. catechol) was monitored by taking aliquots of 10 μL of liquid phase for phenol and 100 μL of gas phase for BTEX every 24 h. A negative control culture of each pollutant without microorganisms was included, showing that the concentrations of the pollutants after 6 days of culture did not show significant variations ($p < 0.05$). Additionally, the OD of the culture broth was measured at 600 nm to determine microbial growth.

BTEX biodegradation by *R. opacus* was performed in 250 mL closed bottles, containing 100 mL of AMS medium and benzene, toluene, ethylbenzene or *o*-xylene as carbon and energy source, under an air atmosphere. The bottles were inoculated at an initial optical density (OD) of 0.2 at 600 nm, at 25 °C and pH initially adjusted to 6.8, under continuous agitation (300 rpm). The initial concentration of BTEXs was 8 mg L^{-1} and 100 μL of the headspace gas phase were taken each 2 h during the first stage of the cultivation for BTEXs and CO_2 determination.

2.3. Analytical procedures

BTEX concentrations were determined by Gas Chromatography (GC)

Table 1
Culture conditions of the experiments carried out in this work.

Microorganisms	Cultivation time (d)	Atmosphere	Nitrogen source	Related figures
C. vulgaris	5	CO_2	NO_3^-	Fig. 1; Fig. 2; Fig. S1
C. vulgaris *R. opacus* *C. vulgaris-R. opacus*	6	Air	NO_3^- and $NO_3^- + NH_4^+$	Fig. 3
C. vulgaris-R. opacus	6	Air	$NO_3^- + NH_4^+$	Fig. 4; Fig. 5; Fig. S2
C. vulgaris-R. opacus	6	N_2/CO_2 (70/30 %)	$NO_3^- + NH_4^+$	Fig. 6; Fig. 7; Fig. S3

coupled to a flame ionization (FID) detector, using an Agilent 8860 GC equipment (Agilent, USA). These organic compounds were separated on an Agilent HP-5 GC column (30 m × 0.320 mm × 0.25 μm) (Agilent, CA, USA). The injector was maintained at 150 °C, and the column was held at 50 °C for 3.5 min and finally ramped at 25 °C min^{-1} up to 110 °C, with a helium flux of 2.5 mL min^{-1}, and held for 0.5 min. The temperature of the FID detector was 250 °C. Retention time for the compounds was 2.05 min for benzene, 3.07 min for toluene, 4.63 min for ethylbenzene and 5.10 min for *o*-xylene. The estimation of BTEX concentrations in the aqueous phase was based on the dimensionless Henry's Law constants ($H_v^{cc} = C_L/C_G$) of each pollutant at 25 °C and the pollutant headspace concentration [28]. ($H_v^{cc} = 4.457$ for benzene, 3.962 for toluene, 3.219 for ethylbenzene, and 4.953 for *o*-xylene).

Phenol was determined by High-Pressure Liquid Chromatography (HPLC) using an Alliance 2695 series system (Waters, USA) equipped with a binary pump system, vacuum degasser, a thermostated column and a diode array detector (DAD). Separation of phenol and catechol was performed in an Intersil ODS-3 C18 (4.6 mm × 150 mm, 3 μm) analytical column. For the successful resolution of the compounds, the elution program was performed using an isocratic flow with two mobile phases at a flow rate of 1.0 mL min^{-1} and a temperature of 30 °C. Solvents were acetonitrile (A) and water (B) in a proportion of 40:60. The injection volume was 10 μL, and the programme recorded the absorbance for 7 min until the two peaks were detected with retention times of 1.25 min for phenol and 3.54 min for catechol. Chromatograms were recorded at 271 and 290 nm.

CO_2 was determined by GC coupled to a thermal conductivity detector (TCD) using an Agilent 8860 GC equipment (Agilent, USA). The compounds were separated in a CP-PoraBOND (15 m × 0.53 μm × 10 μm) column. The injector was maintained at 150 °C, and the column was held at 45 °C for 6.5 min, with a helium flux of 3.5 mL min^{-1}. The temperature of the TCD detector was 200 °C.

2.4. Statistical analysis

All the experiments were carried out using biological triplicates and

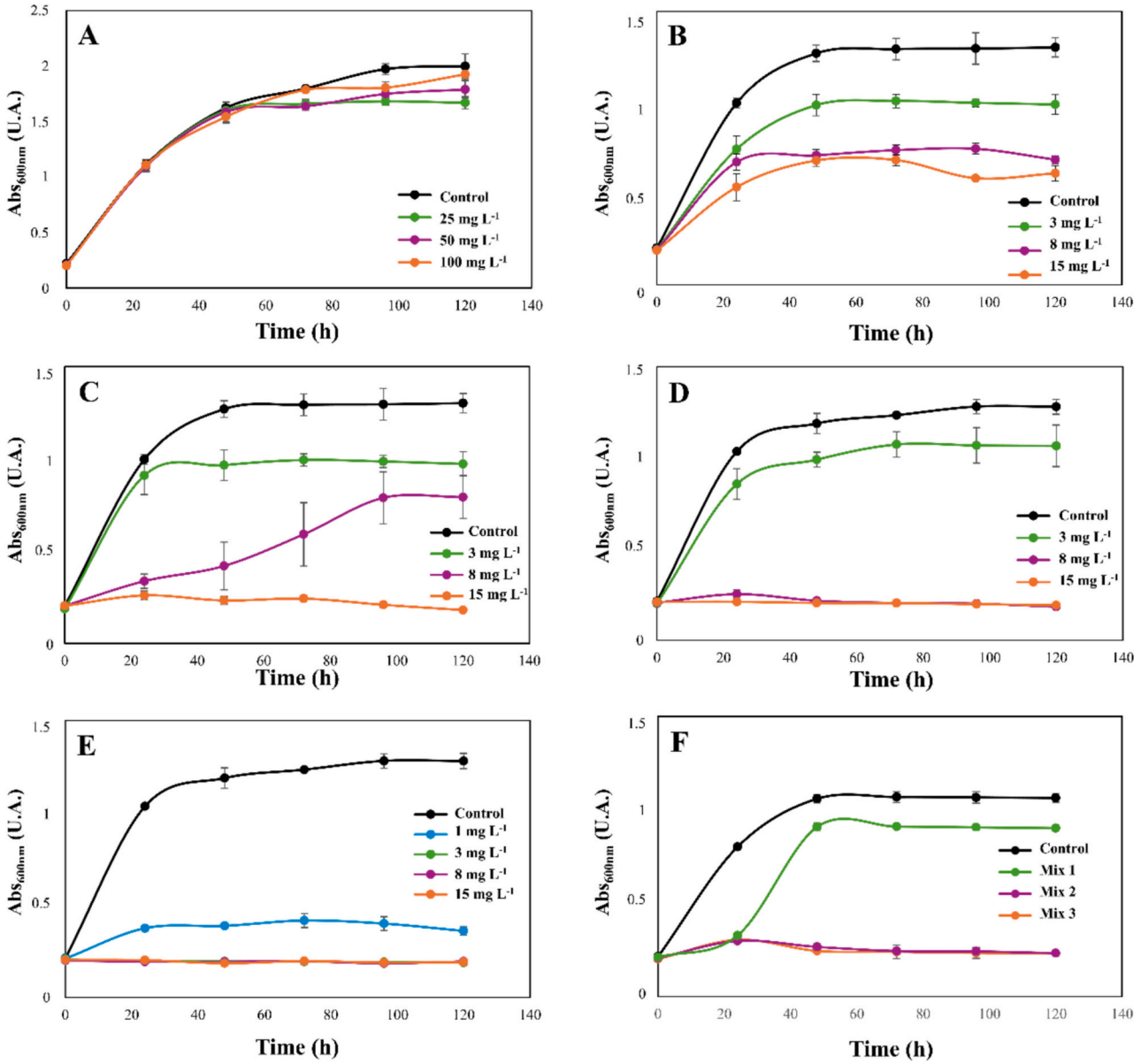

Fig. 1. Tolerance of the microalga *C. vulgaris* to increasing concentrations of (A) phenol, (B) benzene, (C) toluene, (D) ethylbenzene, (E) o-xylene and (F) a mixture of thereof. Initial concentrations of the pollutants in the mixtures were 25 mg L^{-1} of phenol, and 3, 3, 8 mg L^{-1} for benzene and toluene, 1, 3, 3 mg L^{-1} for ethylbenzene; and 0.5, 1, 1 mg L^{-1} for *o*-xylene in Mix 1, 2 and 3, respectively.

represented as the mean value ± standard deviation. Technical replicates were also performed when biological replicates were exposed to Dixon's Q test and an outlier ($Q_{95\%}$) was detected. One-way analysis of variance (ANOVA) was applied to identify significant differences between conditions, which were considered for values with $p < 0.05$. Statistical analyses were performed using IBM SPSS Statistics v29.0 software (Armonk, NY, USA).

3. Results and discussion

3.1. Tolerance of C. vulgaris to phenol and BTEX

The results showed that *C. vulgaris* was able to grow in the presence of phenol at all concentrations tested (Fig. 1A). However, the presence of BTEX in the culture medium influenced the growth kinetics of microorganisms, causing a significant decrease ($p < 0.05$) in the time course of the optical density of *C. vulgaris* at the concentrations tested (Fig. 1B to F). Thus, while the microalga could tolerate up to 15 mg L^{-1} of benzene (Fig. 1B), *C. vulgaris* could not grow at concentrations of 15 mg L^{-1} of toluene (Fig. 1C), 8 mg L^{-1} of ethylbenzene (Fig. 1D) and 3 mg L^{-1} of *o*-xylene (Fig. 1E). These results revealed that ethylbenzene and *o*-xylene were the most toxic pollutants for *C. vulgaris*. Thus, the concentration of these two pollutants was lower than that of the others in the tested pollutants mixture. The tolerance of *C. vulgaris* to a mixture of pollutants was lower than to the individual pollutants as the microalga was only able to grow in Mix 1, although the growth curve showed significant differences ($p < 0.05$) with the control cultures (Fig. 1F). Previous studies indicated that different seawater microalgae, such as *Rhodomonas* sp. JZB-2 and *Thalassiosra* sp. OUC2 can tolerate *p*-xylene concentrations up to 20 and 40 mg L^{-1}, respectively [29,30]. However, to the best of our knowledge, there is no reported tolerance data at concentrations up to 1 mg L^{-1} in mixtures of BTEX compounds in freshwater microalgae. Takácová et al. [31] demonstrated that the microalga *Parachlorella kessleri* could tolerate, with significant differences in grow curves, concentrations up to 0.1 mg L^{-1} of these compounds. On the other hand, Pérez Romero et al. [32] showed that *Chlorella vulgaris* GVG0001 was not able to tolerate concentrations higher than 1 mg L^{-1} of benzene, toluene or xylenes, which confirmed the high tolerance and the potential of *C, vulgaris* strain to develop novel microalgal-bacterial systems able to biodegrade BTEXs compounds.

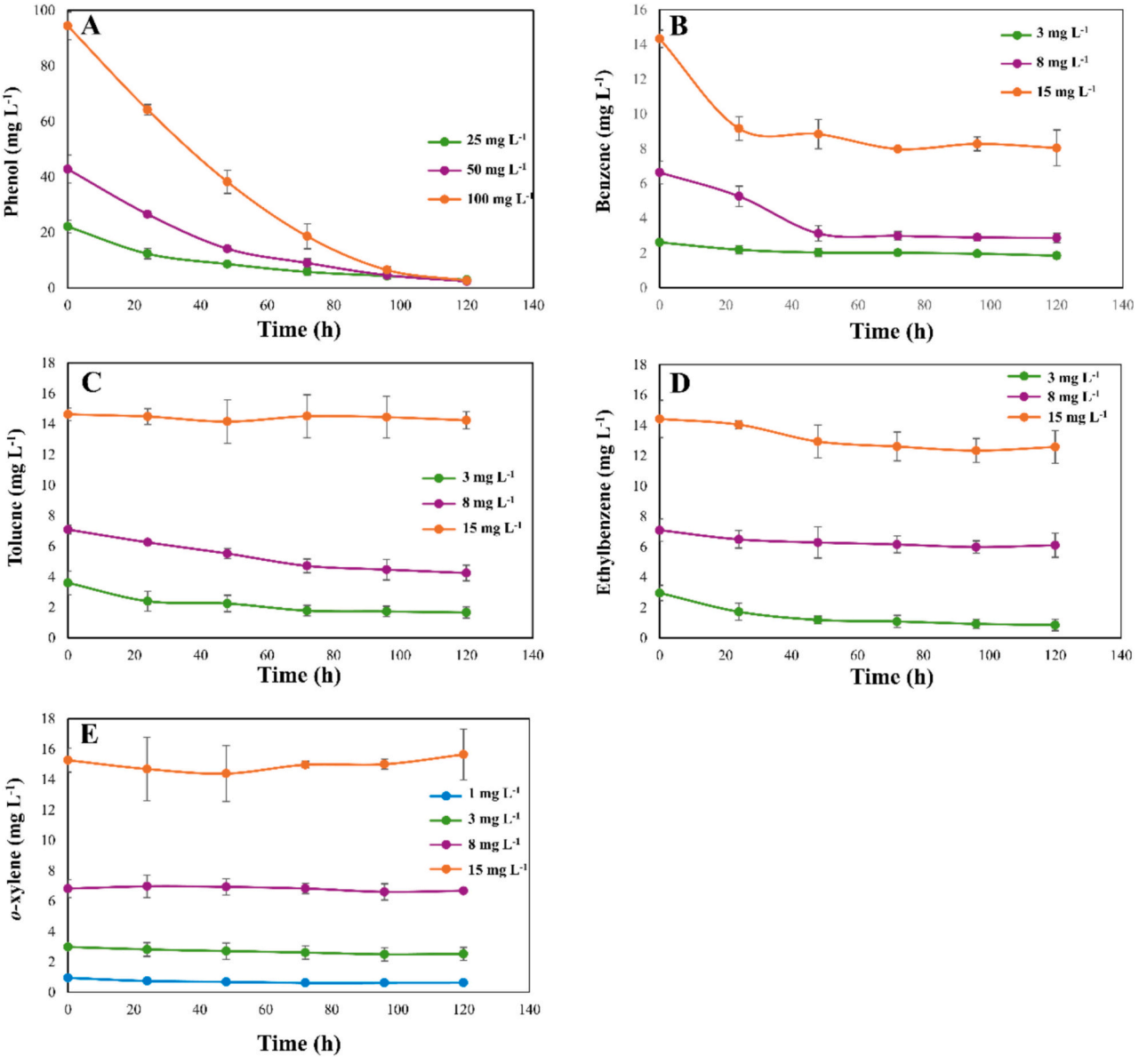

Fig. 2. Time course of the concentration of (A) phenol, (B) benzene, (C) toluene, (D) ethylbenzene and (E) *o*-xylene during *C. vulgaris* cultivation.

The pollutant biodegradation capacity of *C. vulgaris* was also determined in the same experiment. Fig. 2 shows the capacity of *C. vulgaris* to biodegrade each pollutant tested. This microalga exhibited a high capacity to metabolize phenol, removing 98 % of the initial phenol after 5 days when cultivated at an initial concentration of 100 mg L^{-1} (Fig. 2A), and producing catechol as a first step of phenol metabolic biodegradation (Table S1). The biodegradation of benzene was also significant, being able to remove 40–60 % of the initial benzene in the aqueous phase after 5 days of cultivation at the different concentrations tested (Fig. 2B). Nevertheless, the biodegradation capacity of toluene, ethylbenzene and *o*-xylene was lower than that of benzene and phenol likely due to their higher toxicity in *C. vulgaris*. Indeed, the toluene biodegradation of *C. vulgaris* was 55 and 40 % after 5 days at initial concentrations of 3 and 8 mg L^{-1}, respectively. However, no toluene biodegradation was recorded in the microalgal cultures at an initial concentration of 15 mg L^{-1} toluene (Fig. 2B). Similar results were reported for ethylbenzene and *o*-xylene, where pollutant biodegradation in the cultures without cell growth was unsignificant (Fig. 2D and E). At non-toxic concentrations, *C. vulgaris* was able to biodegrade 80 % of the initial ethylbenzene (3 mg L^{-1}) and 33 % of the initial *o*-xylene (1 mg L^{-1}), which means that *C. vulgaris* has a higher affinity to biodegrade some of these compounds, such as phenol, benzene or ethylbenzene, than for others whose biodegradation efficiencies are lower, such as toluene and *o*-xylene. Previous literature studies have shown that freshwater microalgae can biodegrade BTEX compounds with medium-high efficiency. Another strain of *C. vulgaris* could degrade 97 % of the initial benzene concentration (10 g L^{-1} crude oil) along with other hydrocarbons from crude oil such as naphthalene, undecane, and eicosane [33]. Likewise, *Parachlorella kessleri* was able to remove 63 % of toluene in a BTEX mixture at initial concentrations of 0.1 mg L^{-1} of each compound [31]. However, although similar pollutant removals have been reported in the literature, the concentrations of BTEX tested in these studies were lower than those tested in our work. At this point, it should also be noted that the literature assessing microalgae-mediated biodegradation of BTEX is scarce, with most of the studies focusing on the biodegradation of polycyclic aromatic hydrocarbons [10].

The biodegradation capacity of *C. vulgaris* cultured in a mixture of the pollutants was also reported (Fig. S1). The results demonstrated that there is no significant ($p < 0.05$) biodegradation of pollutants in Mix 2 and 3 along the time, in which the microalga could not grow (Fig. S1B and C). However, Mix 1 showed an adequate biodegradation rate of BTEX with efficiencies ranging from 65 % of toluene to 79 % of *o*-xylene (Fig. S1A). Moreover, *C. vulgaris* was able to degrade 70 % of the initial phenol in this mixture (Fig. S1A). The degradation efficiencies were higher than those reported for *Parachlorella kessleri*, which degraded from 30 to 63 % of a mixture of BTEX with an initial concentration of 0.1 mg L^{-1} [31]. However, the bioremediation affinity of *P. kessleri* was toluene > benzene/xylene > ethylbenzene, whereas the affinity of *C. vulgaris* was *o*-xylene > benzene > ethylbenzene/toluene (Fig. S1B), which suggests that the bioremediation affinity during pollutant biodegradation was species specific. This preference can be attributed to the toxicity of the different pollutants. BTEXs metabolic degradation in microalgae has been little investigated. However, the few studies carried out suggest that the first step occurs by an oxidation in the CH_3 carbon (s), followed by a ring cleavage [10], which seems to be similar in all the compounds. In this case, with similar degradation pathways of the tested pollutants, *C. vulgaris* could have higher affinity for the most toxic compound, which is *o*-xylene. This explains the results obtained in Fig. S1A against the reported for *Parachlorella kesslery* by [31]. Although *C. vulgaris* exhibits a high tolerance to these compounds, its biodegradation capacity of BTEXs in a mixture is limited (Fig. S1). A novel approach to solve this problem could be based on combining this microorganism with an oxygenic bacteria able to biodegrade these compounds, such as *R. opacus* [23,24], which also possesses a high tolerance to these compounds [25,34].

3.2. Optimization of the nitrogen source during toluene biodegradation

Toluene biodegradation by *C. vulgaris*, *R. opacus* and a combination of both was investigated using NH_4^+ and NO_3^- as nitrogen sources in an air atmosphere (Fig. 3).

R. opacus supported higher biodegradation efficiencies (75 %) compared to *C. vulgaris* cultures (55 %) in the presence of nitrate (Fig. 3). On the other hand, the algal-bacterial consortium supported final degradation efficiencies of 70 %. Interestingly, the addition of ammonium as an additional nitrogen source resulted in the complete biodegradation of toluene in cultures of *R. opacus* or the microbial consortium within 6 days of cultivation (Fig. 3). Ammonium is typically the preferred nitrogen source for bacterial and green algae as a result of the Redox state of the nitrogen (−3) compared to more oxidized forms of nitrogen such as nitrate (+5) [35]. The lower energy demand during nitrogen assimilation could help bacteria to metabolize different compounds such as BTEX [36]. Furthermore, ammonium concentrations are present in petrochemical wastewaters along with nitrate [4]. The consumption of ammonium during microalgal cultivation produces acidification due to the accumulation of H^+ ions in the culture medium. On the other hand, the development of microalgae cultures produces an alkalinization due to CO_2 sequestration. These two effects also showed that the presence of ammonium can help mitigate pH variations in microalgal cultures [37]. Thus, the subsequent experiments were carried out using SK medium supplemented with an initial concentration of 0.5 g L^{-1} of NH_4Cl (Figs. 4, 5, 6, 7, S2 and S3).

3.3. Influence of the headspace concentration on the biodegradation of pollutants by C. vulgaris and R. opacus consortium

The biodegradation of the tested pollutants by the microalga-bacterium consortium was studied using an air atmosphere, which favoured the activity of *R. opacus*, and an N_2/CO_2 (70 %/30 %) atmosphere, which improved the activity of *C. vulgaris* and ultimately of *R. opacus*. Moreover, this ratio N_2/CO_2 also maintains a similar percentage of N_2 than in the air atmosphere.

The initial presence of air in the headspace guaranteed the absence of oxygen limitation during BTEX and phenol biodegradation by *R. opacus*. However, *C. vulgaris* growth was probably limited by CO_2 availability, which depended on bacterial respiration of organic pollutants. Under these conditions, the consortium microalga-bacterium was able to biodegrade 100 % of the phenol at initial concentrations of 25 and 50 mg L^{-1}, and 85 % at an initial concentration of 100 mg L^{-1}, after 6 days of cultivation (Fig. 4A), concomitantly with the production catechol as a subproduct of phenol biodegradation (Table S1). The extent of phenol

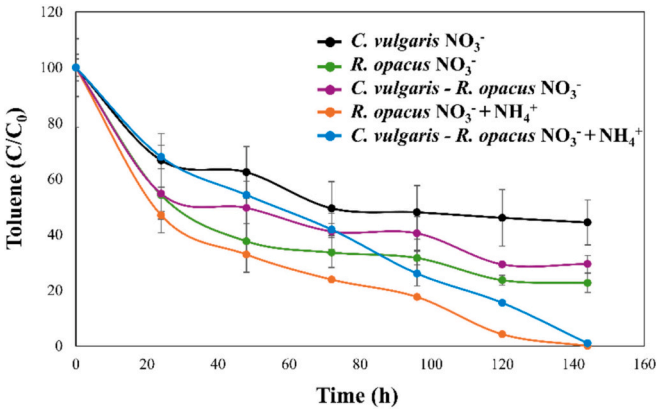

Fig. 3. Time course of toluene concentrations in assays conducted with *C. vulgaris*, *R. opacus* or a consortium of these microorganisms with nitrate or a combination of ammonium and nitrate as nitrogen sources. Initial concentration of toluene was 3 mg L^{-1} in all the conditions.

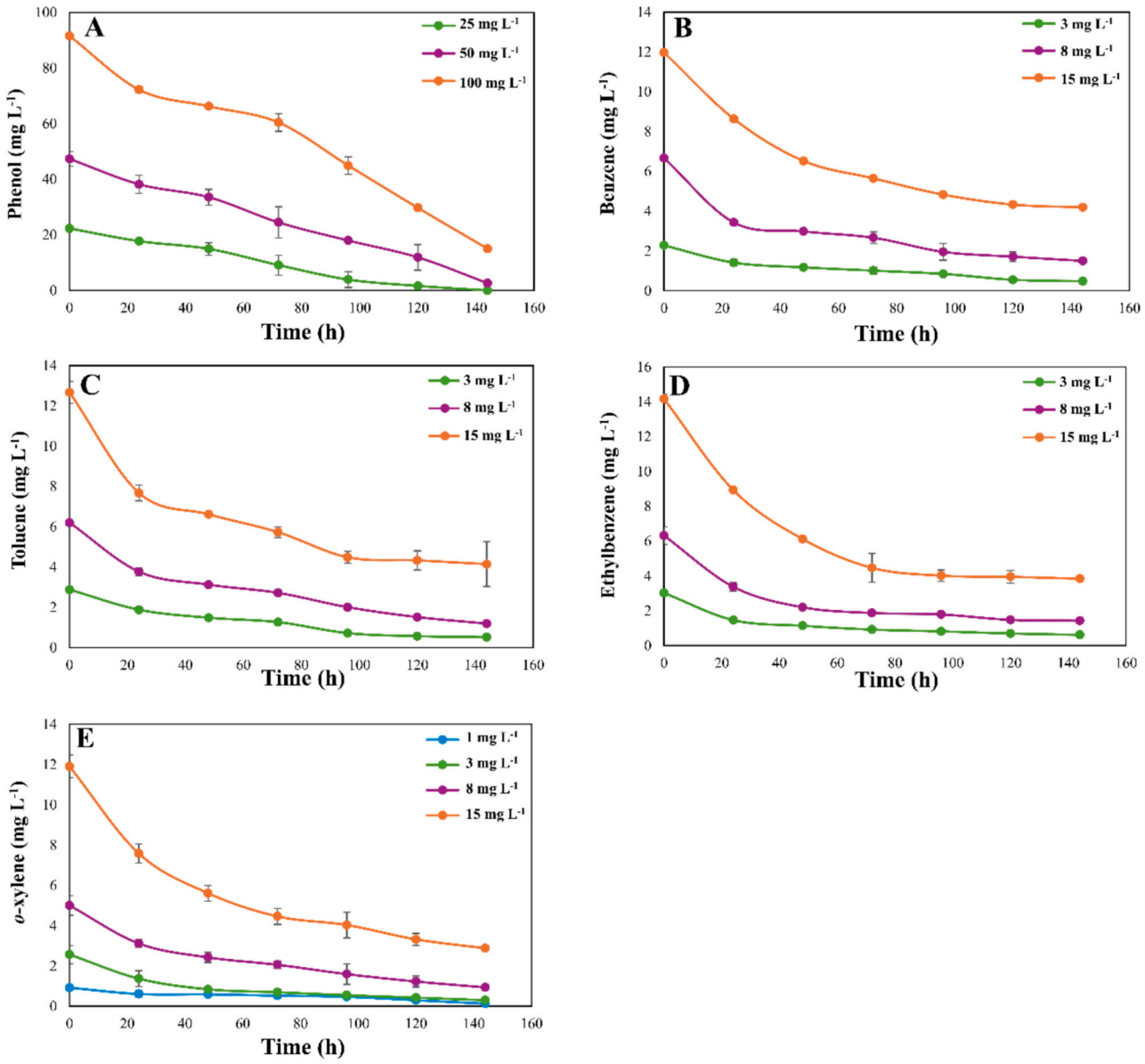

Fig. 4. Time course of (A) phenol, (B) benzene, (C) toluene, (D) ethylbenzene and (E) *o*-xylene concentrations in *C. vulgaris*-*R. opacus* co-cultures under an air atmosphere.

biodegradation correlated with culture absorbance, where increasing initial concentrations of phenol implied higher values of OD (Fig. S2A). Biodegradation efficiencies at an initial phenol concentration of 100 mg L^{-1} were lower than in *C. vulgaris* assays (Fig. 2A). However, the optical density values in the consortium (Fig. S2A) were much lower than in *C. vulgaris* experiments (Fig. 1A), pointing that biodegradation efficiency of unit cell in the microalgal-bacterial consortium was higher than in the microalgal-based treatment. It has been described that there is a synergetic relation between microalgae and bacteria that improves the biodegradation of phenol, with an increase of 85 % of the removal rate, per unit cell in the consortium, due to the presence of the bacteria [38], which is in agreement with the results obtained in Fig. 4 per unit cell in the consortium.

BTEX experienced higher degradation efficiencies by the microalga-bacterium consortium compared to *C. vulgaris*. The consortium of *C. vulgaris* and *R. opacus* biodegraded 80 % of initial benzene at 3 and 8 mg L^{-1}, and 65 % at 15 mg L^{-1} of initial concentration (Fig. 4B). This improvement in biodegradation efficiencies compared to microalgae (Fig. 2) entailed no significant differences ($p < 0.05$) in the biomass growth curves at the different concentrations of benzene tested (Fig. S2B), which was probably due to the decrease in the concentration of this pollutant after the first 24 h of cultivation (Fig. 4B). Similarly, while toluene biodegradation was 80 % after 6 days at initial concentrations of 3 and 8 mg L^{-1}, removals of 67 % were observed with an initial concentration of 15 mg L^{-1} (Fig. 4C). This improvement was caused by co-culture of *R. opacus* with *C. vulgaris*. Bacteria from *Rhodococcus* genera can biodegrade, with an initial concentration of 15 mg L^{-1}, 62 % of total benzene and 50 % of total toluene after 72 h at the same pH and temperature conditions than our experiments [39]. These results of toluene degradation were similar to those obtained by the alga-bacterium system in the same period of time (Fig. 4C). The algal-bacterial growth curves using toluene (Fig. S2C) were similar to those of *C. vulgaris* assays (Fig. 2C), showing that the microalga tolerated this pollutant at concentrations lower than 8 mg L^{-1}. Previous studies reported high biodegradation rates of benzene and toluene using photobioreactors with microalgae-bacteria consortia [40–42]. However, the concentrations tested in these studies were lower than in our study, showing the potential of this consortium formed by the microalga

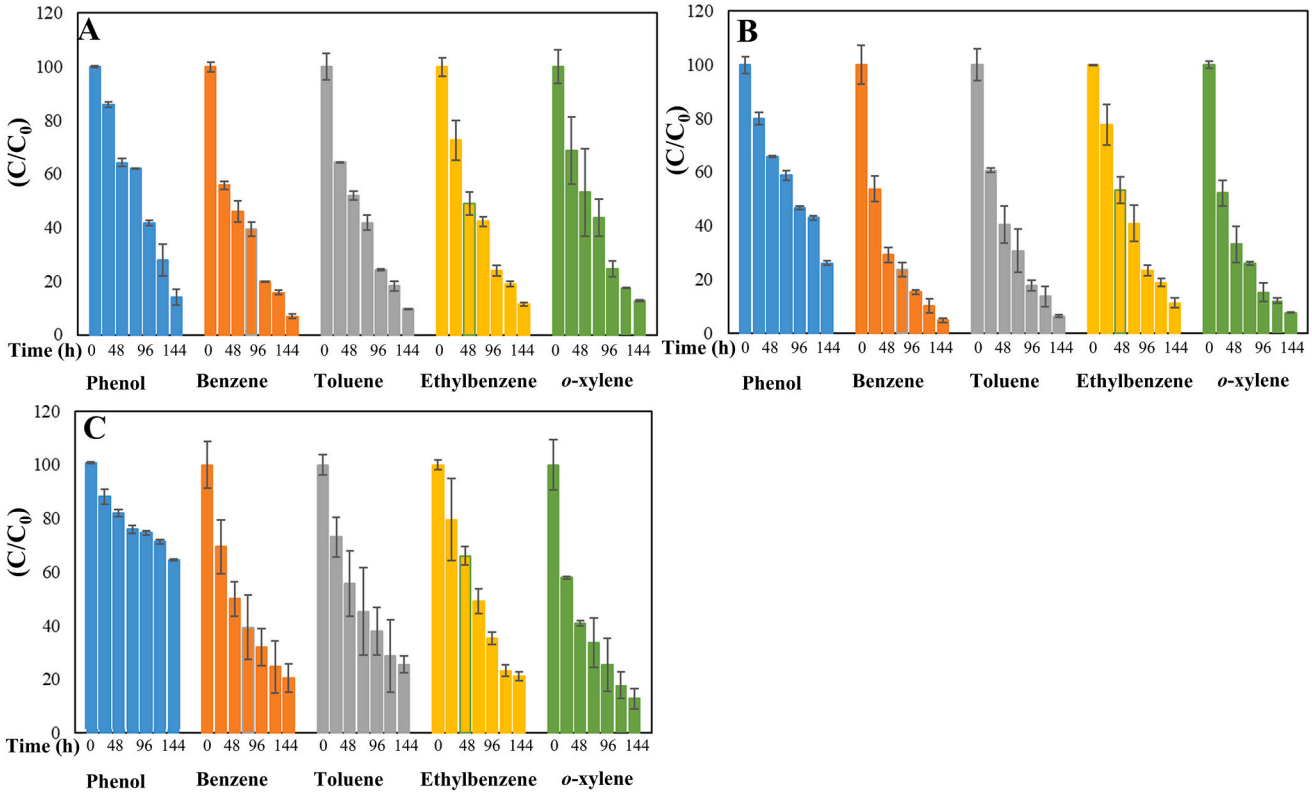

Fig. 5. Time course of phenol and BTEX concentrations in (A) Mix 1, (B) Mix 2 and (C) Mix 3 in *C. vulgaris-R. opacus* cultures cultivated with in a mixture of the five pollutants under air atmosphere. Initial concentrations of the pollutants (100 %) in the mixture were 25 mg L^{-1} of phenol, and 3, 3, 8 mg L^{-1} for benzene and toluene, 1, 3, 3 mg L^{-1} for ethylbenzene; and 0.5, 1, 1 mg L^{-1} for *o*-xylene in Mix 1, 2 and 3, respectively.

C. vulgaris and the bacterium *R. opacus*.

Biodegradation efficiencies of ethylbenzene by the algal-bacterial consortium ranged from 73 to 80 % at all the concentrations tested (Fig. 4D). Additionally, *C. vulgaris* grew in cultures at an initial concentration of 8 mg L^{-1}, and there were no significant differences ($p < 0.05$) between control and 3 mg L^{-1} cultures growth curves (Fig. S2D). This increase in *C. vulgaris* tolerance to ethylbenzene could be also explained by the biodegradation efficiency. Indeed, in the cultures with initial concentrations of 8 mg L^{-1}, the concentration of ethylbenzene after 24 h was 3.37 ± 0.25 mg L^{-1}, which is a concentration much lower than the initial one and seems to be non-toxic for the microalga. On the other hand, Fig. 4E shows that the algal-bacterial consortium presented an excellent capacity to biodegrade *o*-xylene. Removal efficiencies of 86, 89, 82 and 76 % at initial *o*-xylene concentrations of 1, 3, 8 and 15 mg L^{-1}, respectively, were recorded (Fig. 4E). Furthermore, there were no significant differences ($p < 0.05$) in the growth of *C. vulgaris* at 3 mg L^{-1} compared to the control cultures (Fig. S2E). To the best of the authors' knowledge, this work represents the first study on ethylbenzene or xylene removal by a microalga-bacterium consortium. In this context, most BTEX studies were performed using benzene and toluene as model pollutants. Li et al. [29] reported a high biodegradation of *p*-xylene by a newly isolated microalga *Rhodomonas* sp., which could biodegrade until 30 mg L^{-1} of this pollutant within 6 days of cultivation. However, this microalga was screened using increasing concentrations of the pollutant, which suggests that microalgal cells were adapted to such high *p*-xylene concentrations.

Additionally, the biodegradation of mixtures of all pollutants by the microalga-bacterium consortium was also tested under an air atmosphere. Under these conditions, *C. vulgaris* tolerance increased and was able to grow in Mix 1 and 2. However, no growth was observed in Mix 3 (Fig. S2F). The consortium *C. vulgaris-R. opacus* was not able to completely metabolize all phenol in any of the mixtures tested, obtaining phenol removals of 86, 75 and 42 % in Mix 1, 2 and 3, respectively, after 6 days of cultivation (Fig. 5). These results agree with the reported for phenol single experiments (Fig. 4A), demonstrating that air atmosphere is not the best option for phenol biodegradation using the consortium *C. vulgaris-R. opacus*. El-Gendy and Nassar, [43] reported that the presence of CO_2 can increase phenol biodegradation in microalgae, just as the results of Figs. 2A and 4A reported.

The co-culture of *C. vulgaris* and *R. opacus* enhanced BTEX biodegradation compared with *C. vulgaris* assays (Fig. S1). For Mix 1 and 2 removals of 95 % for benzene, 92–95 % for toluene, 89 % for ethylbenzene, and 88–93 % for *o*-xylene were reached (Fig. 5A and B), while Mix 3 underwent biodegradation efficiencies of 80, 75, 79 and 87 % for benzene, toluene, ethylbenzene and *o*-xylene, respectively (Fig. 5C). In this context, removal efficiencies of 90–95 % for benzene, toluene and phenol, at initial concentrations of 10 mg L^{-1} after 7 days, were reported by Cai et al., [44] using a consortium composed of *Coelastrella terrestris* and different bacteria. Although these biodegradation efficiencies were higher than the reported for our system, it is important to note that *C. vulgaris-R. opacus* consortium included other pollutants that were also biodegraded, such as ethylbenzene and *o*-xylene. In addition, Parsy et al. [13] also engineered a consortium with different seawater microalgae and bacteria capable of supporting high biodegradation rates of a mixture of BTEX (60 % of COD), nitrogen (32 % of TN) and phosphorous (98 % of TP), although there is not data of individual BTEX degradation in this work.

On the other hand, a N_2/CO_2 (70/30 %) headspace mediated an enhancement in the biodegradation of phenol, where the removals from culture medium were 100 % at 25 mg L^{-1} cultures and 97 % in 50 and 100 mg L^{-1}, after 6 days of cultivation (Fig. 6A). This improvement was also demonstrated in the volumetric biodegradation rate of phenol during the first 72 h, which was significantly ($p < 0.05$) higher under a N_2/CO_2 atmosphere at the 3 concentrations tested than under an air

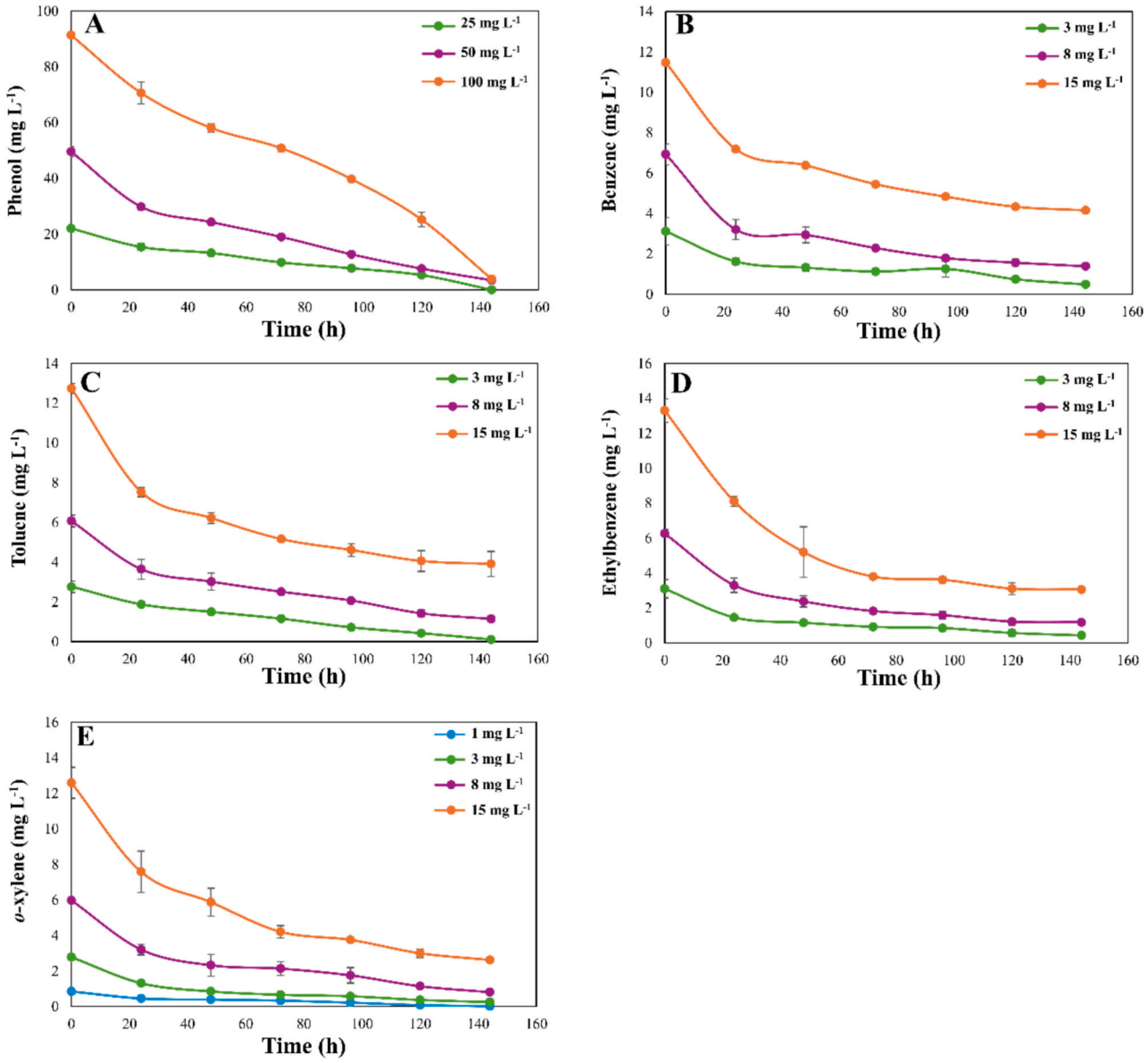

Fig. 6. Time course of (A) phenol, (B) benzene, (C) toluene, (D) ethylbenzene and (E) *o*-xylene concentration in *C. vulgaris-R. opacus* cultures under a N_2/CO_2 (70/30 %) atmosphere.

atmosphere (Table S2). Moreover, an increase in biomass concentrations was observed when the microalga-bacteria consortium was cultivated with phenol, compared to the control (Fig. S3A). These results confirmed the hypothesis that CO_2 plays a key role on phenol biodegradation in the consortium *C. vulgaris-R. opacus*, which also points out the importance of microalgae in this biodegradation process. Papazi and Kotzabasis [45] also reported the importance of an exogenous inorganic carbon source for phenol biodegradation by the microalga *Scenedesmus obliquus*.

Benzene biodegradation efficiencies accounted for 85, 80 and 65 % at 3, 8 and 15 mg L^{-1} cultures, respectively, after 6 days of cultivation (Fig. 6B). These results were similar to the efficiencies recorded under an air atmosphere (Fig. 4B). However, there was a significant improvement in benzene biodegradation rates under a N_2/CO_2 atmosphere (0.0262, 0.0466 and 0.0787 mg L^{-1} h^{-1}, at concentrations of 3, 8 and 15 mg L^{-1}, respectively) compared to the rates under an air atmosphere (0.0136, 0.0425 and 0.0720 mg L^{-1} h^{-1}, at concentrations of 3, 8 and 15 mg L^{-1}, respectively); (Table S2). This suggest that *R. opacus* was the main responsible of benzene biodegradation in the system. The growth of the consortium cultivated with benzene and supplemented with N_2/CO_2 was similar to that recorded in the air atmosphere (Fig. S3B), which correlates with the results of growth and biodegradation of this pollutant. Toluene biodegradation efficiencies reached 97 % at 3 mg L^{-1}, and 82 and 70 % at 8 and 15 mg L^{-1}, respectively (Fig. 6C). Furthermore, the consumption rate of toluene also significantly ($p < 0.05$) increased at 15 mg toluene L^{-1} under a N_2/CO_2 atmosphere (0.1002 vs. 0.0912 mg L^{-1} h^{-1} under an air atmosphere; Table S2). Thus, there was an improvement in toluene biodegradation at low concentrations as a result of CO_2 supply to the consortium (i.e. 97 % in the presence of CO_2 and 82 % in the presence of air) (Fig. 4C). It is also remarkable that, under CO_2 supplementation, *C. vulgaris* tolerated all the concentrations of toluene tested (Fig. S3C), while under an air atmosphere *C. vulgaris* was not able to grow at concentrations higher than 8 mg L^{-1}. There was also an improvement of *C. vulgaris* tolerance to toluene compared with its cultivation alone under a CO_2 atmosphere (Fig. 1), which points out to *R. opacus* as the main responsible of this improvement. The results obtained improved the toluene degradation from the consortium used by Lv et al., [39] at 72 h under the same conditions (68 % vs. 55 %), however, the degradation of benzene was

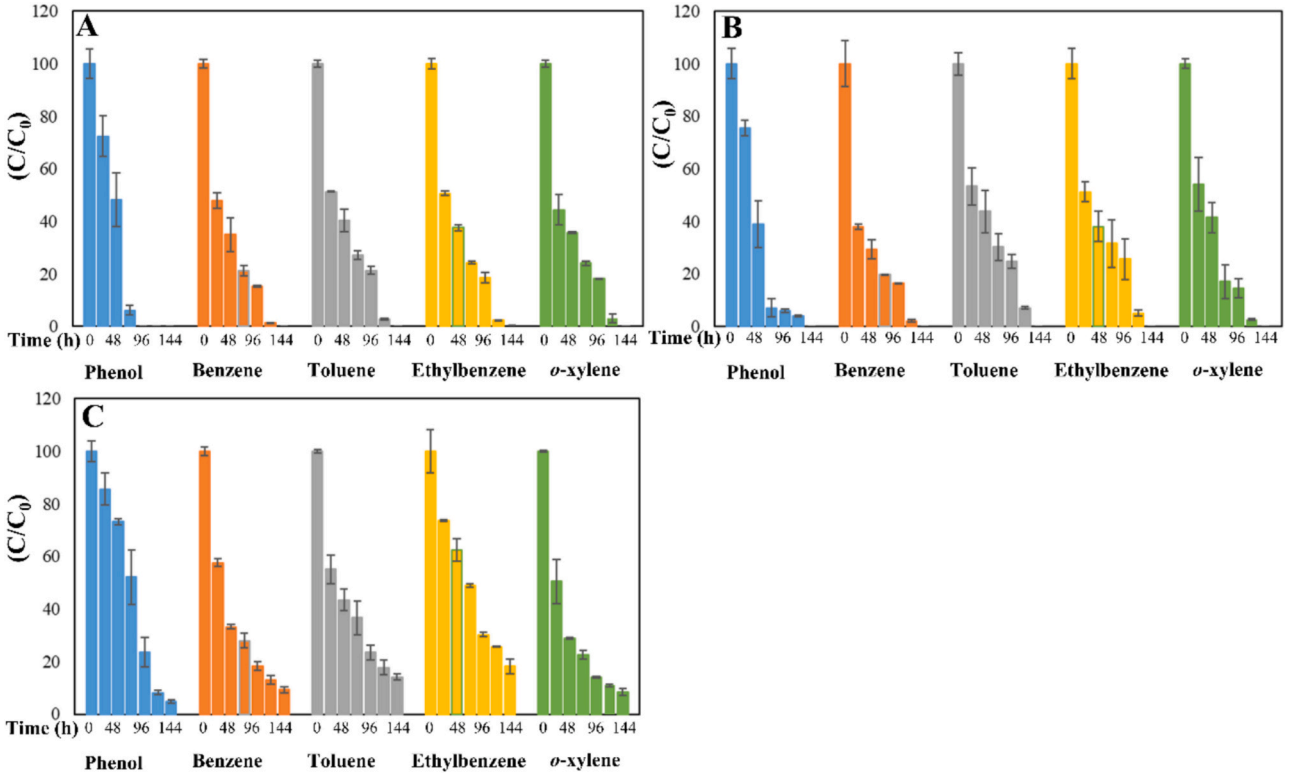

Fig. 7. Time course of phenol and BTEX concentration in (A) Mix 1, (B) Mix 2 and (C) Mix 3 in *C. vulgaris-R. opacus* cultures incubated with a mixture of the five pollutants under a N_2/CO_2 (70/30 %) atmosphere. Initial concentrations of the pollutants (100 %) in the mixture were 25 mg L^{-1} of phenol, and 3, 3, 8 mg L^{-1} for benzene and toluene, 1, 3, 3 mg L^{-1} for ethylbenzene; and 0.5, 1, 1 mg L^{-1} for *o*-xylene in Mix 1, 2 and 3, respectively.

similar in both cases (50 % of initial benzene).

The supplementation of N_2/CO_2 to the consortium also fostered ethylbenzene biodegradation efficiencies. While ethylbenzene biodegradation after 6 days accounted for 80 % at 3 and 8 mg L^{-1}, and 73 % at 15 mg L^{-1} (Fig. 4D), CO_2 supplementation increased ethylbenzene biodegradation up to 86 %, 81 % and 78 % at 3, 8 and 15 mg L^{-1} respectively (Fig. 6D). However, there was no significant ($p < 0.05$) differences in the consumption rate of this pollutant during the first 72 h of cultivation (Table S2). This enhancement in pollutant biodegradation was even more remarkable in *o*-xylene tests, where biodegradation efficiencies of 100, 92, 87 and 80 % were recorded at 1, 3, 8 and 15 mg L^{-1}, respectively (Fig. 6E), compared to 86, 88, 81 and 75 % in the presence of air (Fig. 4E). This increase in biodegradation rates was also showed in the *o*-xylene consumption speed during the first 72 h, which was significantly ($p < 0.05$) higher under a N_2/CO_2 atmosphere (0.0059, 0.0287, 0.0517 and 0.1149 mg L^{-1} h^{-1}, at concentrations of 1, 3, 8 and 15 mg L^{-1}, respectively) compared to the rates under an air atmosphere (0.0049, 0.0256, 0.0397 and 0.1013 mg L^{-1} h^{-1}, at concentrations of 1, 3, 8 and 15 mg L^{-1}, respectively); (Table S2 and 3). Interestingly, similar growth curves were observed during the biodegradation of these two pollutants in the presence of N_2/CO_2 (Fig. S3D and E) and air (Fig. S2D and E). In this context, the higher availability of CO_2 probably promoted the growth of *C. vulgaris*, which increased O_2 availability for *R. opacus* during BTEX biodegradation [46]. Additionally, ethylbenzene biodegradation at 72 h accounted for 73 %, which improved previous results reported by *Rhodococcus* sp. and bacteria consortium [39,47].

In the presence of a N_2/CO_2 atmosphere, the co-culture *C. vulgaris-R. opacus* could tolerate the concentrations of pollutants in Mix 1 and 2 (Fig. S3F), showing higher tolerance to the mixture of this compounds than the cultures of *C. vulgaris* (Fig. 1). However, while there were significant differences ($p < 0.05$) between biomass growth in Mix 2 and control assays under an air atmosphere (Fig. S2F), no significant differences were recorded under N_2/CO_2 atmosphere (Fig. S3F), demonstrating that cellular growth was less affected by the pollutants due to the presence of CO_2 in the headspace. On the other hand, phenol biodegradation efficiencies were significantly higher than those attained under an air atmosphere. Indeed, the consortium removed >95 % of the initial phenol after 3 days and 100 % after 6 days in Mix 1 and 2 (Fig. 7A and B). Additionally, 95 % of the initial phenol in Mix 3 was biodegraded after 6 days of cultivation (Fig. 7C), demonstrating that the presence of CO_2 in the atmosphere boosted phenol biodegradation by the consortium *C. vulgaris-R. opacus*.

The presence of CO_2 in the headspace also entailed an improvement in BTEX biodegradation in mixtures. Hence, the consortium *C. vulgaris-R. opacus* could biodegrade 100 % of the initial BTEX concentrations in Mix 1 and 2 after 5 and 6 days of cultivation, respectively (Fig. 7A and B). In addition, biodegradation efficiencies of 91, 86, 82 and 94 % were also observed for benzene, toluene, ethylbenzene and *o*-xylene after 6 days of cultivation in Mix 3 (Fig. 7C). In comparison, the biodegradation efficiencies without CO_2 in Mix 3 were 80, 75, 79 and 87 % (Fig. 5C). Moreover, this improvement led to obtain similar biodegradation rates of phenol, benzene and toluene than the reported by Cai et al., [44] in less time (6 vs. 7 days). In addition to a total biodegradation, a significant ($p < 0.05$) increase in the pollutants consumption rate during the first 72 h of the experiment under a N_2/CO_2 atmosphere in comparison with an air atmosphere, with the exception of benzene and *o*-xylene in Mix 2 and ethylbenzene in Mix 3 (Table S3). This demonstrates that under a N_2/CO_2 atmosphere the efficiency of the system was much higher than under an air atmosphere, both in terms of pollutant biodegradation efficiencies and rates. Additionally, biodegradation of BTEXs compounds was demonstrated, which confirmed that *R. opacus* could use these compounds as a carbon and energy source while CO_2 is produced in the headspace of closed bottles, being able to duplicate the amount of CO_2 in the headspace in the first 6 h of cultivation (Table S4). These results confirmed that BTEXs compounds can be metabolized by this bacterium if there is not availability of another carbon and energy

source in the culture medium. Thus, the microalgal-bacterial system solves the problem of aeration in closed bioreactors due to the symbiotic CO_2/O_2 exchange in the system. These promising results represent a first step for the development microalgae-based technologies capable of cost-effectively bioremediating petrochemical effluents. However, further research is needed to validate the technology at industrial scale. This research should be focused on developing a continuous bioreactor system with higher working volumes under outdoor conditions.

4. Conclusions

This study demonstrated the high tolerance of the green microalga *Chlorella vulgaris* to phenol and BTEX compounds and a mixture of thereof, along with a significant biodegradation capacity. However, the microalgal-bacterial consortium with *Rhodococcus opacus* supported the most effective pollutant biodegradation. The use of NH_4Cl in the culture medium and the supplementation of N_2/CO_2 (70/30 %) in the head space enhanced phenol and BTEX biodegradation, with complete removals of 3 mg L^{-1} of benzene, toluene and ethylbenzene and 1 mg L^{-1} of *o*-xylene within 6 days of cultivation. These results confirmed that the co-culture *C. vulgaris-R. opacus* is a promising tool for the biodegradation of pollutants present in petrochemical industry wastewaters, avoiding the environmental problems that these effluents cause to aquatic ecosystems.

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CRediT authorship contribution statement

Antonio León-Vaz: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Andrés Felipe Torres-Franco:** Writing – review & editing, Methodology. **Pedro Antonio García-Encina:** Writing – review & editing, Resources, Conceptualization. **Raúl Muñoz:** Writing – review & editing, Supervision, Resources, Conceptualization.

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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.

Data availability

Data will be made available on request.

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