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Chlamydomonas-*Methylobacterium oryzae* cooperation leads to increased biomass, nitrogen removal and hydrogen production

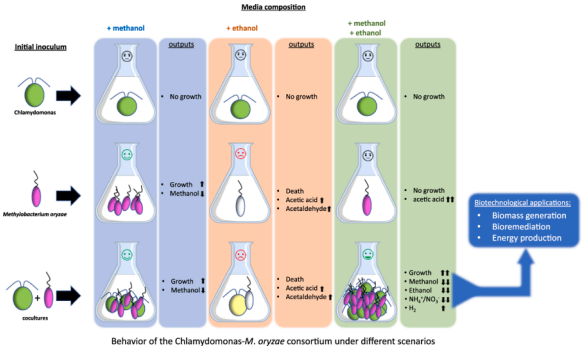
María Jesús Torres, David González-Ballester *, Aitor Gómez-Osuna, Aurora Galván, Emilio Fernández, Alexandra Dubini

Universidad de Córdoba, Departamento de Bioquímica y Biología Molecular, Campus Universitario de Rabanales, Ed. C6, Planta Baja, 14071 Córdoba, Spain

HIGHLIGHTS

- The alga Chlamydomonas and the bacterium *M. oryzae* show metabolic cooperation.
- Monocultures cannot grow in media containing ethanol and methanol.
- Consortium can grow very efficiently in media containing ethanol and methanol.
- Biomass generation can increase up to 700% and hydrogen is produced.
- Nitrogen removal from synthetic media and urban wastewater is almost complete.

GRAPHICAL ABSTRACT



Behavior of the Chlamydomonas-*M. oryzae* consortium under different scenarios

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ABSTRACT

In the context of algal wastewater bioremediation, this study has identified a novel consortium formed by the bacterium *Methylobacterium oryzae* and the microalga *Chlamydomonas reinhardtii* that greatly increase biomass generation (1.22 g L^{-1}·d^{-1}), inorganic nitrogen removal (>99%), and hydrogen production (33 mL·L^{-1}) when incubated in media containing ethanol and methanol. The key metabolic aspect of this relationship relied on the bacterial oxidation of ethanol to acetate, which supported heterotrophic algal growth. However, in the bacterial monocultures the acetate accumulation inhibited bacterial growth. Moreover, in the absence of methanol, ethanol was an unsuitable carbon source and its incomplete oxidation to acetaldehyde had a toxic effect on both the alga and the bacterium. In cocultures, both alcohols were used as carbon sources by the bacteria, the inhibitory effects were overcome and both microorganisms mutually benefited. Potential biotechnological applications in wastewater treatment, biomass generation and hydrogen production are discussed.

* Corresponding author.
E-mail addresses: mjtorres_@msn.com (M.J. Torres), dgballester@uco.es (D. González-Ballester), b12goosa@uco.es (A. Gómez-Osuna), bb1gacea@uco.es (A. Galván), bb1feree@uco.es (E. Fernández), alexandra.dubini@uco.es (A. Dubini).

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1. Introduction

Despite bacterial and algal biotechnology have innumerable applications, the potential of combining both types of microorganisms has received much less attention, and the complex and versatile relationships that they establish in natural ecosystems are just glimpsed. Still, numerous reports point to the beneficial effect of coculturing algae and bacteria, especially to improve biomass yield and algal cell density (Yao et al., 2019), wastewater (WW) treatment (WWT) (Mohsenpour et al., 2021; Saravanan et al., 2021; Sial et al., 2021), biofuel production (Fakhimi et al., 2020; Ray et al., 2022; Yao et al., 2019), biofertilizers (Kang et al., 2021), bioplastics (Chong et al., 2021), and diverse raw materials (Jill et al., 2021). However, further research is needed to consolidate the industrial feasibility of these applications and avoid some associated bottlenecks such as environmental sustainability, culture stability, and the development of more cost-effective technologies for biomass harvesting and product extraction (Jill et al., 2021).

Bacteria and microalgae are very adaptable organisms. Most bacteria are heterotrophs that use different carbon (C) sources, while many microalgae can change their metabolism from autotrophic to mixotrophic or heterotrophic depending on the specific growth conditions (Perez-Garcia et al., 2011). When bacteria and algae are cultivated together, they can establish mutualistic relationships that benefit the growth of both organisms (Saravanan et al., 2021; Sial et al., 2021). On the one hand, the bacteria can solubilize and mineralize sulfur (S), nitrogen (N) and phosphorus (P), which become available to the algae. In addition, bacterial heterotrophic metabolism releases CO_2, which can be used by algae as a C source under autotrophic conditions. Another important possibility, although less studied, is that the fermentative end-products excreted by bacteria, such as succinate, ethanol, glycerol, lactate or acetate, can be used by some algal species for heterotrophic growth (Chong et al., 2021; Perez-Garcia et al., 2011; Saravanan et al., 2021; Sial et al., 2021; Yao et al., 2019). On the other hand, bacteria can obtain photosynthetic O_2 and various organic C sources, such as acetate, formate, ethanol and glycerol, from algae. Other bidirectional exchanges include organic N (e.g., amino acids), vitamins or cofactors (Fakhimi et al., 2020; Saravanan et al., 2021; Sial et al., 2021; Yao et al., 2019). These metabolic collaborations ultimately increase the growth of the microorganisms defining a consortia, and in some cases enable growth on nutrients that are unsuitable when these microorganisms are grown independently (Calatrava et al., 2018).

WWs are ideal for cost-effective cultivation of algal-bacterial consortia, and the resulting biomass can be valorized in many different ways, thus supporting other industries and circular economy models (Chong et al., 2021; Leong et al., 2021; Sial et al., 2021). Moreover, the photosynthetic O_2 provided by algae can decrease the aeration required during WWT for aerobic heterotrophic bacteria, which can account for as much as 50% of the costs of the process (Mohsenpour et al., 2021).

The major via for biological N removal in current biological WWT consist of the sequential bacterial activity of nitrifiers (ammonium to nitrate/nitrite) and denitrifiers (nitrate to N gaseous species, such as NO, N_2O, and N_2) (Lu et al., 2014; Rahimi et al., 2020). As consequence, N_2O gas is released during biological WWT, whose impact on global warming raises some concerns since its greenhouse effect is 300 times more potent than that of CO_2 (Rahimi et al., 2020). Supplementation with methanol during WWT is commonly used to enhance biological denitrification (Lu et al., 2014; Rahimi et al., 2020).

Land plants are considered the larger source of methanol, however, there are also evidences of aquatic ecosystems rivaling with terrestrial sources (Millet et al., 2008). *Methylobacterium* spp. are the dominant colonizers of the phyllosphere since they can utilize one-carbon (C1) compounds, such as methanol, as their sole C and energy sources (Dourado et al., 2015; Yurimoto et al., 2021). *Methylobacterium* spp. have excellent biotechnological potential in agriculture (plant growth promotion through N_2 fixation, phytohormone production, or plant protection against pathogens and pollutants) and also participate in WWT (Dourado et al., 2015). However, there are little evidence showing that *Methylobacterium* spp. can associate with algae in natural ecosystems and promote algal growth (Calatrava et al., 2018; Holland, 2019; Krug et al., 2020). Calatrava et al (2018) first described a mutualist collaboration between *Chlamydomonas reinhardtii* (Chlamydomonas throughout) and *Methylobacterium aquaticum* cocultured in media with peptides/amino acids as the sole C and N sources, in which only the cocultures, but not bacterial or algal monocultures, were able to grow. Under this scenario the bacteria provided inorganic N in the form of ammonium to the alga, and the alga released glycerol (and possibly other organic C forms) used by the bacterium.

Chlamydomonas, a green alga that can grow heterotrophically/mixotrophically on acetic acid as the sole C source, is commonly used as a model system to study numerous processes, including biotechnological applications such as biohydrogen ($bioH_2$) production (Touloupakis et al., 2021). $BioH_2$ is considered as a potential source of green H_2, particularly if inexpensive WWs are used for microbial cultivation (Aydin et al., 2021). Heterotrophic bacteria link H_2 production to their fermentative metabolism (dark H_2) (Dahiya et al., 2021), while microalgae and cyanobacteria are able to produce H_2 linked to their photosynthetic activity ($photobioH_2$) (Dubini and Gonzalez-Ballester, 2016). In recent years, Chlamydomonas-bacterial cocultures have been used as an efficient strategy to improve $bioH_2$ production (Fakhimi et al., 2020). Remarkably, some consortia can develop metabolic relationships based on the exchange of acetate that lead to synergetic $bioH_2$ production (Fakhimi et al., 2019a).

In this study, a consortium of Chlamydomonas-*M. oryzae* was identified with the ability to increase the growth, N removal and $bioH_2$ production when cultivated in ethanol- and methanol-containing medium. The metabolic relationships underlying this mutualistic relationship may aid in the design of future biotechnological applications.

2. Material and methods

2.1. Algal and bacterial strain pre-cultures

Chlamydomonas reinhardtii wild-type strain CC-1960 (21gr Sager, mt^+) was used in all experiments. Algal pre-cultures were grown mixotrophically in Tris Acetate Phosphate (TAP) medium, which contains 17 mM of acetic acid and 8 mM of NH_4Cl (among other mineral nutrients) (Harris et al., 2009). Precultures were incubated at 24 °C, under agitation (140 rpm) and a continuous photosynthetic photon flux density (PPFD) of approximately 80 μmol photons·m^{-2}·s^{-1} provided by LED panels. The bacterial strains used in this work were *Methylobacterium oryzae* CBMB20 (Madhaiyan et al., 2007), *Microbacterium sp.*, *Bacillus sp.* and *Stenotrophomonas* sp. *M. oryzae* was provided by the DSMZ institute (German Collection of Microorganisms and Cell Cultures). The remaining bacteria were isolated and identified during this work from a fortuitously contaminated Chlamydomonas culture. Pre-cultures of *M. oryzae* were grown in *Methylobacterium* Medium (MeM) (Calatrava et al., 2018), and *Microbacterium sp.*, *Bacillus sp.* and *Stenotrophomonas* sp. were grown in TAP medium supplemented with yeast extract (1 g·L^{-1}) and mannitol (10 g·L^{-1}), referred here to as TAP–Yeast extract–Mannitol (TYM) medium. All bacterial pre-cultures were incubated for 2–3 days at 28 °C under agitation (140 rpm).

2.2. Coculture of algae and bacteria

Chlamydomonas cells were cultured for 3–4 days in TAP medium until mid-logarithmic growth phase was reached, then harvested by centrifugation (5,000 rpm for 5 min) and washed twice with fresh minimal mineral medium (MM) without any N source (MM-N). MM contained the following (per liter): 0.4 g NH_4Cl, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.05 g $CaCl_2{\cdot}H_2O$, 1.15 g K_2HPO_4, 0.46 g NaH_2PO_4, 0.05 g EDTA, 5.1 mg $MnCl_2{\cdot}4H_2O$, 22 mg $ZnCl_2$, 11 mg H_3BO_3, 1.6 mg $CoCl_2{\cdot}6H_2O$, 1.6 mg $CuCl_2{\cdot}2H_2O$, 0.214 mg $Na_2MoO_4{\cdot}2H_2O$ and 5 mg $FeSO_4$. MM-N medium

lacked NH_4Cl. Bacterial batch-cultures were incubated in liquid MeM or TYM medium until the optical density at 600 nm (OD_{600}) reached 0.8–1, then were harvested by centrifugation (7,500 rpm for 5 min) and washed twice with fresh MM-N. Algae and bacteria were cocultured in 250 mL flasks containing 100 mL of the corresponding medium or WW. Algal and bacterial mixtures were adjusted to an initial chlorophyll concentration of 5 $\mu g \cdot mL^{-1}$ for the alga (occasionally 15 $\mu g \cdot mL^{-1}$) and an initial OD_{600} of 0.1 for the bacterium. Algal and bacterial monocultures were used as controls. All cultures were incubated at 24 °C with continuous agitation (80–140 rpm), and under continuous illumination (80 PPFD) or photoperiod of 12:12 h light:dark.

The compositions of the media used for the cocultures were as follows: MM-N supplemented with 10 mM of NH_4Cl and 5 mM of potassium acetate (MMA) (which was used as the base for the remaining media); MMA supplemented with yeast extract (1 $g \cdot L^{-1}$) (MMA+Y); MMA supplemented with 10 mM of glucose and 10 $g \cdot L^{-1}$ of mannitol (MMA+S); MMA supplemented with 5 $mL \cdot L^{-1}$ of ethanol and 5 $mL \cdot L^{-1}$ of methanol (MMA+OH); MMA supplemented with 5 $mL \cdot L^{-1}$ of ethanol (MMA+Et); MMA supplemented with 5 $mL \cdot L^{-1}$ of methanol (MMA+Me); and MM-N supplemented with 5 mM of potassium acetate, 10 mM of KNO_3, 5 $mL \cdot L^{-1}$ of methanol and 5 $mL \cdot L^{-1}$ of ethanol (MMnA+OH).

The WW used in this work came from a sewage pond located in the town of Saint-Paul-lez-Durance, France. The WW was filtered with Whatman paper before use to remove solid particles in suspension. After filtering, the measured concentrations for ammonium, nitrate/nitrite and chemical oxygen demand (COD) were 2.85 mM, 1.5 mM and 192 $mg \cdot mL^{-1}$, respectively.

2.3. Determination of algal growth

The algal growth was assessed in terms of chlorophyll content. Chlorophyll measurements were performed by mixing 200 µL of the cultures with 800 µL of ethanol 100%. The mixture was incubated at room temperature for 2–3 min, then centrifuged for 1 min at 12,000 rpm. The supernatant was used to measure chlorophyll (a + b) spectrophotometrically (DU 800, Beckman Coulter) at 665 and 649 nm (Wintermans and de Mots, 1965).

2.4. Microbial growth estimation through a customized selective centrifugal sedimentation approach

When bacteria and microalgae coexist in the same culture, the accurate determination of the bacterial growth can be problematic if no molecular techniques are used. Hence, in this study, a selective centrifugal sedimentation (SCS) approach to estimate *M. oryzae* growth in cocultures was developed. The goal of the approach consisted of determining the centrifugation parameters leading to maximal algal cell sedimentation while minimizing bacterial cell sedimentation. Thus, measuring the O.D. of the supernatant after centrifugation provided an estimate of the bacterial growth in the cocultures. The O.D. before (A_{BC}) and after (A_{AC}) centrifugation were measured at different forces (from 100 × g to 500 × g) and times (1 and 2 min). Then the percentages of precipitated cells from each monoculture were estimated using the A_{BC} and A_{AC} (see e-supplementary materials). Using 200 × g for 1 min led to 90.2% Chlamydomonas sedimentation, whereas only 10.1% of the bacterial cells decreased (meaning that 89.9% of the *M. oryzae* cells remained in the supernatant). This condition was chosen as a good compromise for SCS and was used to evaluate the contribution of the bacteria to the OD in cocultures ($^{SCS}OD_{600}$). Moreover, the pink color of the *M. oryzae* monocultures allowed to visually identify the difference between the bacterial and algal growth (pink and green colors, respectively) in the cocultures (see e-supplementary materials).

2.5. Dry weight biomass determination

Different volumes of cultures (15–50 mL) were centrifuged at 7,500 rpm for 5 min. The pellets were dried at 70 °C for 24 h before weighing.

2.6. Ammonium and nitrate/nitrite determination

Nessler's reagents (MERCK 109011 and 109012) were used to determine the ammonium concentrations in the culture supernatants (Koch and McMeekin, 1924). Equal volumes of freshly prepared Nessler's reagents and cell-free supernatants (diluted 1:5) were mixed and incubated for 5 min. The absorbance at 415 nm was read with a microplate reader (iMark, Bio-Rad). Ammonium calibration curves with known NH_4Cl concentrations were used for each set of independent samples.

The nitrate and nitrite concentrations in the cultures were simultaneously determined according to the method described by Miranda et al. (2001). Cell-free supernatant (40 µL) was mixed with the corresponding reagents, then incubated for 60 min at 37 °C before measurement of the absorbance at 540 nm. Nitrate calibration curves were performed with known concentrations of KNO_3.

2.7. Chemical oxygen demand determination

The COD of the WW samples was determined with a Spectroquant® COD Cell Test 114,541 commercial kit (Merck, measurement range 25–1500 $mg \cdot L^{-1}$) according to the manufacturer's instructions. Previously, the chloride content of each sample was verified with an MQuant® Chloride Test (Merck). Measurements were performed in a Spectroquant® Colorimeter Move100 instrument (Merck).

2.8. Acetic acid, ethanol and methanol quantification

Acetic acid, ethanol and methanol were analyzed by HPLC (Agilent series 1200, Agilent Technologies) with an ion-exchange column (Agilent Hi-Plex H, 300 × 7.7 mm, 6 µm I.D.) and isocratic elution with 5 mM H_2SO_4 at 50 °C. Samples (500 µL) were centrifuged, filtered (0.2 µm) and injected (20 µL) into the HPLC system at a flow rate of 0.6 $mL \cdot min^{-1}$. Retention peaks were observed with refractive index detector.

2.9. Hydrogen production determination

Algae and bacteria were cocultured in hermetically sealed bioreactors (155 mL) with screw caps equipped with butyl septa (WHEATON, cat. No. 240680) and containing 100 mL of the corresponding medium with a final chlorophyll concentration of 10 $\mu g \cdot mL^{-1}$ (estimated 3×10^6 to 4×10^6 $cells \cdot mL^{-1}$) for the alga, and an OD_{600} equal to 0.1 for the bacterium. The bioreactors were placed in a growth chamber equipped with LED panels (AlgaeTron AG 230, Photon System Instruments) at 25 °C with continuous agitation (200 rpm) under an illumination of 80 PPFD. The bioreactors were opened under sterile conditions every 24 h (aeration) to release the H_2 partial pressure and replace the gas composition in the headspace with atmospheric air (Jurado-Oller et al., 2015). Gas samples from the headspaces (250 µL) were collected with a 1 mL Hamilton's SampleLock™ syringe and manually injected into a gas chromatograph (Agilent 7820A, Agilent Technologies). H_2 and O_2 were separated with a packed column (60/80 Molecular Sieve 5A, Ref. 13133-U, Supelco) at 75 °C and detected with a thermal conductivity detector. Argon was used as the carrier gas.

3. Results and discussion

3.1. Growth screening of Chlamydomonas-bacteria consortia

Methylobacterium oryzae, *Microbacterium* sp., *Bacillus* sp. and *Stenotrophomonas* sp. were initially chosen to test their ability to improve Chlamydomonas growth when cocultured in different synthetic media. Members of these genera have been described as plant growth promoting bacteria (Santoyo et al., 2016), and reported to promote algal growth

(Calatrava et al., 2018; Ji et al., 2019; Krug et al., 2020; Watanabe et al., 2005).

All Chlamydomonas-bacterial cocultures were grown on MM supplemented with acetate (MMA) plus other additional C sources, depending on the bacterial strain: *M. oryzae* cocultures were supplemented with alcohols (methanol and ethanol) (MMA+OH); *Microbacterium sp*. and *Stenotrophomonas sp*. cocultures were supplemented with sugars (mannitol and glucose) (MMA+S); and *Microbacterium sp*. and *Bacillus* cocultures were supplemented with yeast extract (MMA+Y) (see e-supplementary materials).

Significantly greater chlorophyl content was observed in cocultures with *Microbacterium* sp. and *Stenotrophomonas* sp. supplemented with sugars (142% and 215%, respectively), and with *M. oryzae* supplemented with alcohols (219%), relative to algal monocultures (see e-supplementary materials), which reveals the suitability of these bacteria to promote Chlamydomonas growth. On the basis of this preliminary screening, the Chlamydomonas-*M. oryzae* consortium was selected to further evaluate the relationships established by these two microorganisms in alcohol containing media.

3.2. Growth of Chlamydomonas-M. Oryzae cocultures incubated in the presence of alcohols: Effects of different nitrogen sources

To study the possible effects of different N sources, two different synthetic media with ammonium (MMA+OH) or nitrate (MMnA+OH) as the sole N source were assayed. Notably, all MMA-derived media

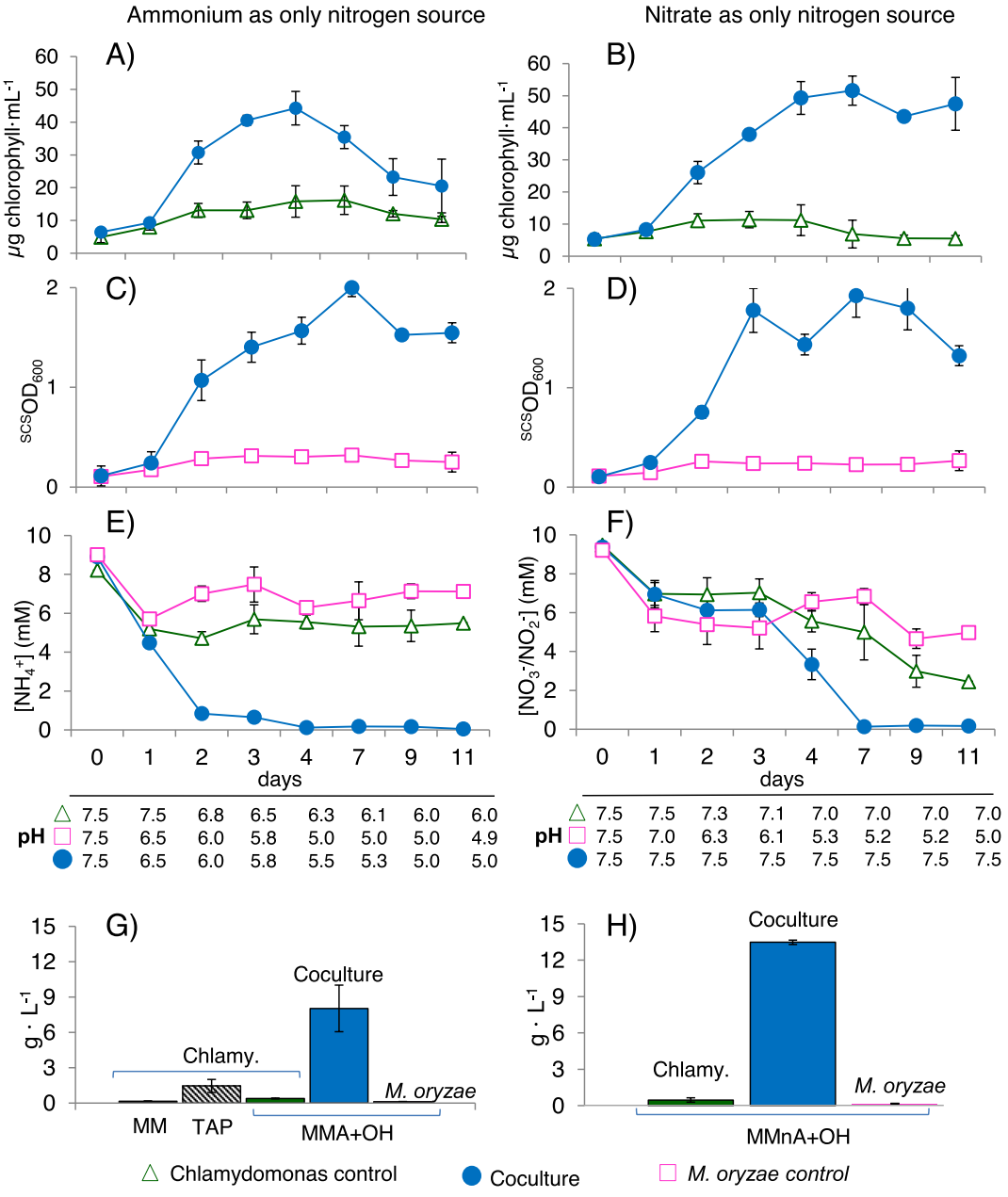

Fig. 1. Growth of the Chlamydomonas-*M. oryzae* consortium in the presence of methanol and ethanol: effect on different nitrogen sources (ammonium and nitrate). The Chlamydomonas-*M. oryzae* and the respective monocultures were incubated in ammonium- (MMA+OH) (A, C, E, and G) or nitrate-containing medium (MMnA+OH) (B, D, F, and H). A and B, total chlorophyll; C, and D, OD for SCS at 600 nm ($^{SCS}OD_{600}$); E and F, Ammonium and nitrate uptake, respectively; G and H, dry weight biomass after 11 days. For panel G, Chlamydomonas monocultures incubated in TAP and MM were also included as controls. The pHs of the respective cultures are indicated under panel E and F. Cultures were incubated under a photoperiod of 12:12 h (80 PPFD). Data correspond to the means of at least three biological replicates. The graphical representation of some small standard deviation values may not be visible in the current graphic scales.

contained only 5 mM of acetate, thus limiting the mixotrophic growth of the algae (the standard mixotrophic medium, TAP, contains 17 mM of acetic acid). The algal and bacterial growth, N uptake, pH evolution and dry weight biomass of the cocultures were evaluated over 11 days (Fig. 1).

In ammonium containing media, the chlorophyll content was 2.6 times higher in the consortium cultures than in the Chlamydomonas monocultures (44.3 μg·mL^{-1} and 16.7 μg·mL-^{1}, respectively) after 4 days (Fig. 1A). This algal bloom in the cocultures was followed by a decline in the chlorophyll content from day 4, descending to 20.5 μg·mL^{-1} after 11 days. The pH of the cocultures and bacterium monocultures underwent acidification (pH 5 and 4.9, respectively), which was probably associated with bacterial metabolism and might explain the decrease in chlorophyll content in the cocultures after day 4. A decrease in chlorophyll content and pH was also observed in the algal monocultures, despite being less intense. Acidification of the media below pH 4 has previously been observed in algal-bacterial cocultures incubated with organic C sources and ammonium (Fakhimi et al., 2019a; Liang et al., 2013), and found to cause loss in the algal cell viability and to require pH control systems (Liang et al., 2013). However, the Chlamydomonas-*M. oryzae* consortium showed some pH compatibility in medium with ammonium, wherein the pH never decreased below 5. The $^{SCS}OD_{600}$ values for *M. oryzae* were also much higher in cocultures than in monocultures (>6-fold on day 7) (Fig. 1C). Indeed, bacterial monocultures showed very poor growth in MMA+OH medium, thus indicating that the bacteria grew much better in the presence of the algae, as also deduced by visual observation of the pink color of the supernatants after SCS (see e-supplementary materials). The dry weight biomass of the cocultures incubated in MMA+OH (8 g·L^{-1}) was much higher than that obtained in the monocultures (0.4 and 0.1 g·L^{-1} for Chlamydomonas and *M. oryzae* monocultures, respectively) and in the Chlamydomonas monocultures incubated in standard TAP and MM media (1.4 and 0.15 g·L^{-1}, respectively) (Fig. 1G). Together, these results indicated that both microorganisms showed greatly improved growth when cultivated together in MMA+OH medium.

The results obtained in nitrate-containing media (MMnA+OH) were very similar to those obtained with ammonium as the only N source, showing greater chlorophyll content, $^{SCS}OD_{600}$ values and biomass accumulation in cocultures than those of the monocultures (4.4-, 8- and 30-fold, respectively) (Fig. 1B, D and H). Hence, Chlamydomonas and *M. oryzae* growth was increased in cocultures regardless of the N source used. Though, when using nitrate, the chlorophyll content did not decrease as much as when using ammonium. Moreover, in contrast to the ammonium cocultures, no pH decrease was observed, and the pH values remained constant in the nitrate cocultures. Importantly, the cocultures incubated in nitrate ultimately had a higher biomass than those incubated in ammonium after 11 days (30 and 20 times, respectively, relative to the corresponding Chlamydomonas control cultures) (Fig. 1G and H). In Chlamydomonas, ammonium uptake contributes to acidification of the media (NH_4^+ is taken up as NH_3 and H^+ is released), whereas nitrate/nitrite uptake lead to basification of the media (NO_3^-/NO_2^- is symported with H^+) (Galván et al., 2006). This fact may explain the different pH values observed among media and supports the possibility that the chlorophyll decrease observed in the MMA+OH cocultures was due to the acidification of the medium. Therefore, incubation of the Chlamydomonas-*M. oryzae* consortium with nitrate can be convenient to avoid acidification and increase the biomass, which may also be an interesting strategy to apply to other algal-bacterial consortia.

The capacity for N uptake was evaluated (Fig. 1E and F). The cocultures grown in MMA+OH medium were able to consume nearly all the initial ammonium (10 mM) after 2 days (Fig. 1E). In contrast, ammonium uptake ceased after the first day in both the bacterial and algal monocultures (Fig. 1E). The contribution of ammonia stripping (NH_3 gas release) appeared negligible in all cultures because the pH never exceeded 7.5. Similarly, in cocultures incubated in MMnA+OH, the initial nitrate concentration (10 mM) was fully consumed after 7 days, whereas the monocultures were unable to fully consume the nitrate after 11 days (Fig. 1F). Of note, the uptake of ammonium in the cocultures was much faster than that of nitrate: 2 days were required to consume nearly all the initial ammonium vs 7 days to consume nearly all the initial nitrate. Nevertheless, the N removal rates were very similar (90 mg·L^{-1}·d^{-1} and 88.6 mg·L^{-1}·d^{-1} for ammonium and nitrate, respectively). These data are comparable to other studies using synthetic media and optimized algal-bacterial cocultures for N removal (Jia and Yuan, 2018).

Overall, all these results indicate that *M. oryzae* and Chlamydomonas show mutual growth benefits when they are cultivated together in the presence of ethanol and methanol, independently of the N source used. However, the use of nitrate can prevent pH acidification and prolong the viability of the cocultures. The high biomass obtained in the cocultures (0.73 and 1.22 g·L^{-1}·d^{-1} in ammonium and nitrate medium, respectively) was accompanied by a high capacity to take up inorganic N: 180 mg·L^{-1} of ammonium and 620 mg·L^{-1} of nitrate were almost fully consumed after 2 and 7 days, respectively, representing > 99% N removal. Note that the ammonium and nitrate concentrations used were higher than those typical of most urban WWs (total N ranging from 4.5 to 260 mg·L^{-1}) (Jill et al., 2021; Mohsenpour et al., 2021), thus indicating the great potential of this consortium for N removal.

3.3. Chlamydomonas-M. Oryzae consortium potential to bioremediate urban wastewater

The *Chlamydomonas-M. oryzae* consortium was tested for its ability to grow, remove inorganic N and decrease the COD in WW from an urban sewage pond (Saint Paul lez Durance, France) (Figs. 2 and 3, and see e-supplementary materials). The WW was not sterilized and consequently contained the endogenous microorganism population.

To evaluate consortium growth, two independent sets of experiments were performed varying the initial chlorophyll concentration of the Chlamydomonas inoculum (5 and 15 μg·mL^{-1}). Both cocultures and Chlamydomonas monocultures showed very little ability to grow on WW, although the acclimation and survival of the alga was slightly higher in the cocultures than in monocultures (Fig. 2A and B). WW without algal inoculation showed very little chlorophyll increase, thus revealing that the endogenous photosynthetic population of this WW was very scarce. The COD decrease after 9 days was 55% and 30% for cocultures and Chlamydomonas monocultures, respectively (see e-supplementary materials). Unexpectedly, the endogenous microorganism population were highly capable of ammonium removal, and the inoculation of the WW with any of the microorganisms had an inhibitory effect over this endogenous ability (Fig. 3A). The effect of the inocula over the endogenous microbial population may be occasioned by an alteration of the medium composition and/or by nutrient competition. In contrast, cocultures showed the highest ability to remove nitrate/nitrite, whereas the rest of the cultures, including non-inoculated cultures, showed little or no nitrate/nitrite removal ability. Moreover, a noticeable excretion of nitrate/nitrite was observed in all cultures from the 4th to the 7th day, being the lowest in the cocultures (Fig. 3B). The high ammonium removal and the excretion of nitrate/nitrite observed in the non-inoculated cultures probably reflected the conversion of ammonium into nitrate/nitrite by the activity of nitrifying bacteria naturally existing in the WW samples. In a different experimental set up, the WW was supplemented with methanol (5 mL·L^{-1}) to evaluate any potential benefit of this alcohol on the performance of the cocultures. The supplementation of WW with methanol is a common practice in biological WWT to foster the denitrification process (Lu et al., 2014; Rahimi et al., 2020). The addition of methanol to the WW greatly increased the coculture performance. The chlorophyll content and dry weight biomass were 5.2- and 2.8-fold higher, respectively, in cocultures than in Chlamydomonas monocultures (Fig. 2C and D). Unfortunately, the $^{SCS}OD_{600}$ measurements to determine the bacterial growth were unreliable

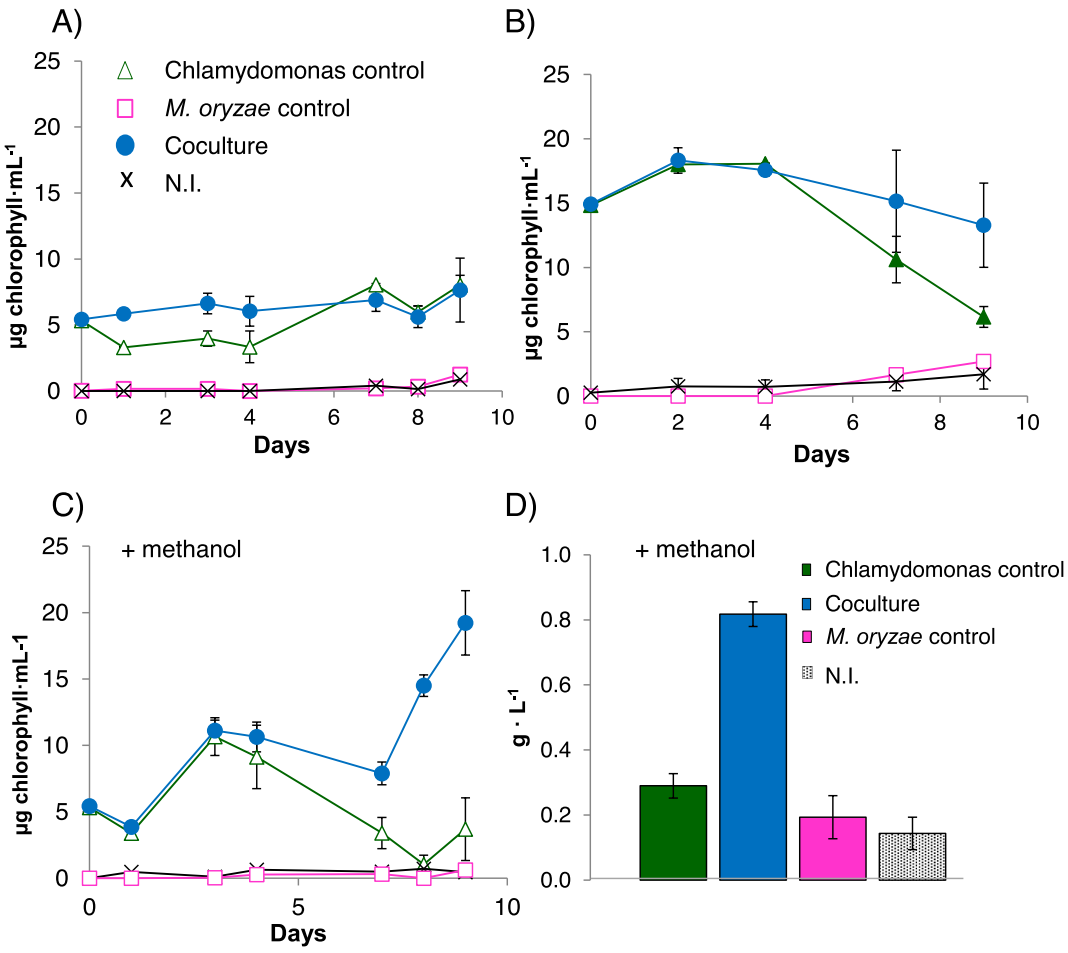

Fig. 2. Growth of the Chlamydomonas-*M. oryzae* consortium incubated in urban WW. The Chlamydomonas-*M. oryzae* consortium and the respective monocultures were incubated in urban WWs (A and B), or urban WWs supplemented with methanol (5 mL·L^{-1}) (C and D). WW without any inoculum were also used as control (N. I). A, B and C total chlorophyll. For panel A and C initial chlorophyll was set to 5 µg·mL^{-1}, whereas in panel B was set to 15 µg·mL^{-1} D, dry weight biomass corresponding with cultures from panel C (day 9). Cultures were incubated under continuous illumination (80 PPFD). Data correspond to the means of at least three biological replicates. The graphical representation of some small standard deviation values may not be visible in the current graphic scales.

because of the presence of suspended particles in the WW. However, a pink color was observed in the pellets of the cocultures and *M. oryzae* monocultures incubated in WW supplemented with methanol, thus indicating very efficient *M. oryzae* growth. This pink color was not observed in WW without *M. oryzae* inoculation or in the absence of methanol supplementation (see e-supplementary materials). The ability to decrease the COD was also enhanced in the cocultures (72% vs 49% reduction for cocultures and monocultures, respectively) (see e-supplementary materials). Methanol supplementation greatly increased ammonium removal in the cocultures and in the monocultures (Fig. 3C). Similarly, nitrate/nitrite removal was also initially enhanced in all cultures (Fig. 3D), probably because of the activity of the denitrifying bacteria naturally present in the WW. However, as in WW non-supplemented with methanol, a prominent nitrate/nitrite excretion was observed after 7 days in all cultures but in the cocultures. This finding indicated that the cocultures had a greater ability to remove nitrate/nitrite from WW. Although both Chlamydomonas and *M. oryzae* are potentially able to participate in denitrification (Lu et al., 2014; Plouviez et al., 2017), their collaboration with the endogenous bacterial population in the denitrification process is unlikely, because algal and bacteria monocultures have low ability to remove nitrate/nitrite in methanol-containing synthetic media (Fig. 1F). More likely, the inorganic N was assimilated by the consortium, which prevented the accumulation of nitrate/nitrite in the medium and probably also partially prevented the denitrification (nitrate to gas) process. This possibility was supported by the increase in biomass observed in the cocultures, which was more compatible with N assimilation than N denitrification. Previously, other algae-bacteria consortia have been proposed to remove N primarily via assimilation into biomass (Su et al., 2011).

Overall, Chlamydomonas-*M. oryzae* consortia showed poor capacity to grow or remove inorganic N when cultivated in urban WW without methanol supplementation. Moreover, the inoculation of the consortium into the WW negatively affected the ability of the in-situ microbial population to remove ammonium. However, when WW were supplemented with methanol a great inorganic N removal was observed in the cocultures, with a > 98% decrease in the initial ammonium (50.4 mg·L^{-1}) and nitrate/nitrite (93 mg·L^{-1}) after 9 and 3 days, respectively, and 73% decrease in COD. As commented before, similar results were obtained in synthetic media (>99% N removal), whose initial concentrations of ammonium and nitrate were 180 and 620 mg·L^{-1}, respectively (Fig. 1). However, the biomass obtained in WW was much smaller (0.8 g·L^{-1} after 9 days) (Fig. 2) than that obtained in synthetic media (8 g·L^{-1} and 13.4 g·L^{-1} after 11 days in ammonium- and nitrate-containing media, respectively) (Fig. 1), which clearly indicates that the consortium performance in WW need to be optimized.

Similar studies using algal-bacterial for WW bioremediation (most of them focused on the algae *Chlorella* and *Scenedesmus*) have reported N removal efficiency ranging from 69% to > 99% and COD decrease around 85–90% (Jill et al., 2021; Mohsenpour et al., 2021; Rahimi et al., 2020; Wang et al., 2016; Wollmann et al., 2019). Compared with

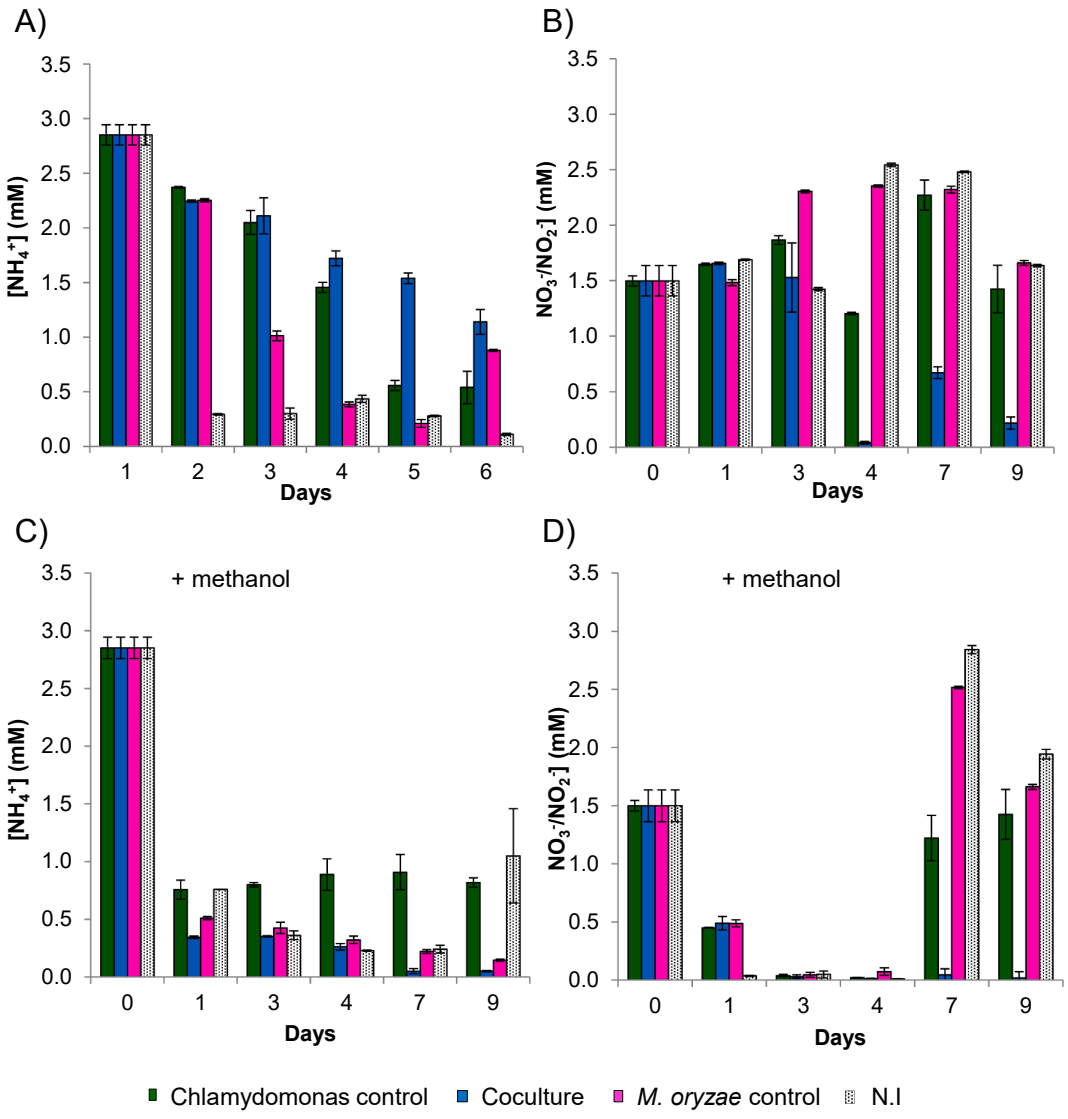

Fig. 3. Ammonium and nitrate/nitrite removal in WW inoculated with the *Chlamydomonas-M. oryzae* consortium. The Chlamydomonas-*M. oryzae* consortium and the respective monocultures were incubated in urban WWs (A and B) or urban WWs supplemented with methanol (5 mL·L^{-1}) (C and D). WW without any inoculum were also used as control (N.I). Initial chlorophyll was set to 5 μg·mL^{-1}. Cultures were incubated under continual illumination (80 PPFD). Data correspond to the means of at least three biological replicates. The graphical representation of some small standard deviation values may not be visible in the current graphic scales. Initial concentrations of ammonium and nitrate/nitrite found in the WW were of 2,8 mM and 1,5 mM, respectively.

previous studies, the Chlamydomonas-*M. oryzae* consortium showed slightly smaller COD decrease.

However, the N removal efficiency was as the highest of those previously reported. Nonetheless, most of the previous reports used WW or synthetic media whose initial N concentrations (ammonium or total N) were lower (ranging from 5 to 260 mg·L^{-1}) than those used in this study. Considering the N removal efficiency and the initial N concentrations, the N removal performance of the Chlamydomonas-*M. oryzae* consortium is among the highest ever reported. Furthermore, as commented before the removal of N probably occurred through assimilation into biomass rather than through denitrification. This feature is of additional biotechnological interest because it can potentially increase biomass yield and can avoid environmental concerns regarding N_2O emission during WWT (Lu et al., 2014; Rahimi et al., 2020). Nonetheless, the possible effects of the Chlamydomonas-*M. oryzae* consortium on 1) the endogenous microbial population of the WWs, 2) the long-term stability of the consortium and 3) the optimization of the process with respect to factors such as continuous mode operation, light–dark cycles or initial inoculum ratios, should be further analyzed.

3.4. Deconvoluting the mutually beneficial metabolic exchange between Chlamydomonas and M. Oryzae

From the data reported in Figs. 2 and 3 (and in e-supplementary materials) it was evident that the Chlamydomonas-*M. oryzae* consortia performance was greatly impacted by the presence of methanol in the medium. To better understand this consortium cooperation, the growth and the consumption/excretion of key metabolites were analyzed in media with different C sources: acetate as the sole C source (MMA), acetate and methanol (MMA+Me), acetate and ethanol (MMA+Et), and acetate, methanol and ethanol (MMA+OH) (Fig. 4).

All alcohol-containing media led to a slight decrease in the chlorophyll content of the algal monocultures (maximal values between 14 and 16 μg·mL^{-1}) relative to alcohol-free media (18 μg·mL^{-1}) (Fig. 4A), thus indicating that the presence of the alcohols in the media had a slight negative effect on the algal growth. These data conflict with findings from a previous report indicating a growth stimulation effect of methanol (30–100 mM) on Chlamydomonas (Stepanov and Zolotareva, 2015), but are consistent with the generally accepted understanding that

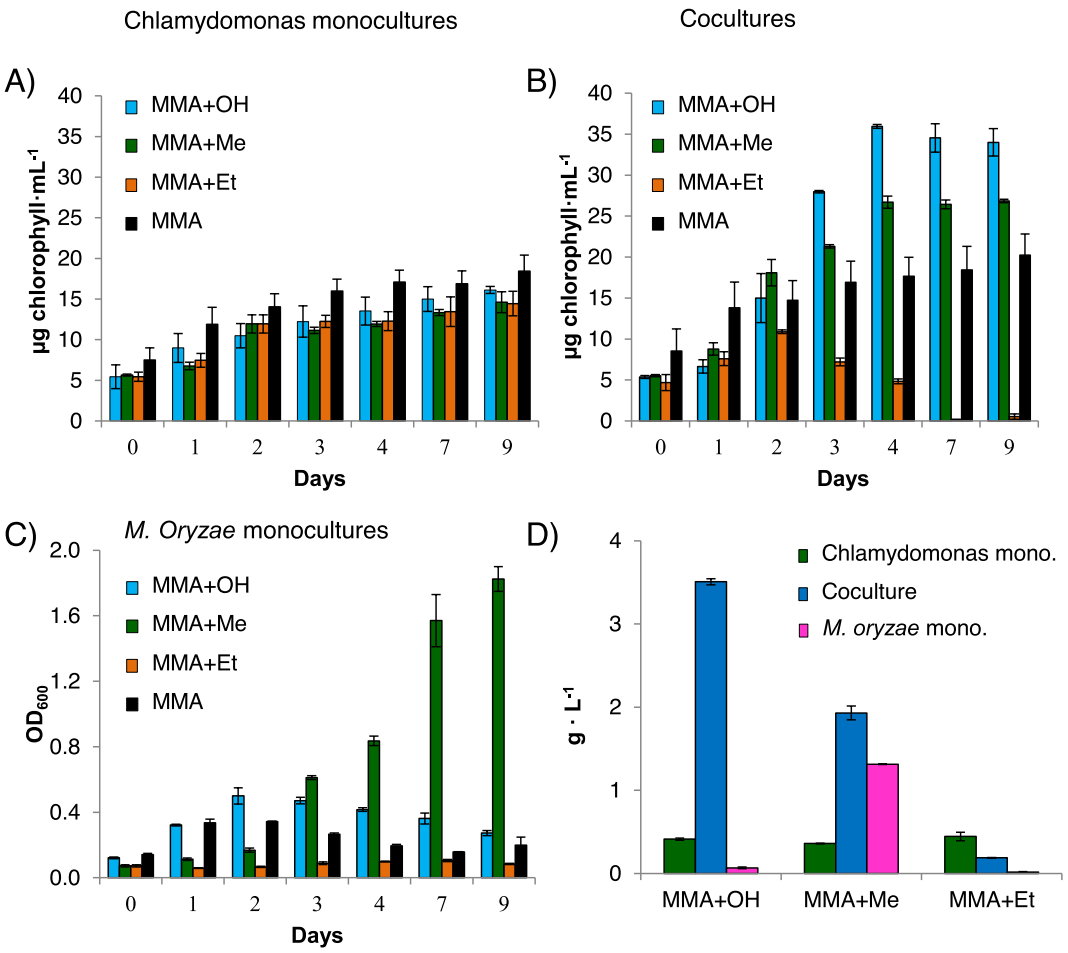

Fig. 4. Growth of the *Chlamydomonas-M. oryzae* consortium in different carbon-containing media. The Chlamydomonas-*M. oryzae* consortium and the respective monocultures were incubated in different carbon-containing media: acetate (MMA); acetate, methanol, and ethanol (MMA+OH); acetate, and methanol (MMA+Me); and acetate and ethanol (MMA+Et). Concentrations were of 5 mM, 120 mM and 85 mM for potassium acetate, methanol, and ethanol, respectively. A and B, total chlorophyll; C, *M. oryzae* monocultures $OD_{600;}$ D, dry weight biomass. Cultures were incubated under a photoperiod of 12:12 h (80 PPFD). Data correspond to the means from at least three biological replicates. The graphical representation of some small standard deviation values may not be visible in the current graphic scales.

acetic acid is the only organic C source that can be efficiently assimilated by this alga (Chaiboonchoe et al., 2014; Harris et al., 2009). On the other hand, the bacterial monocultures showed substantial growth only in MMA + Me (Fig. 4C), thus indicating that the presence of ethanol prevented bacterial growth in MMA+OH medium. In fact, in MMA+Et medium, the bacteria showed no growth at all, thereby suggesting that the initial concentration of ethanol (85 mM) might be toxic to the bacteria when methanol is not present. Finally, bacterial monocultures showed moderate growth when incubated in MMA, thus implying that the bacteria poorly use acetate as the only C source (Fig. 4C).

The presence of alcohols increased the chlorophyll content of the cocultures grown in MMA+Me (maximum of 26.8 μg chl.·mL^{-1}; 183%) but particularly in MMA+OH (maximum of 35.9 μg chl.·mL^{-1}; 210%) media. Of note, in media with no alcohol (MMA medium), the cocultures showed comparable chlorophyll content (maximum of 20.2 μg chl.·mL^{-1}; 109.7%) to the algal monocultures. Finally, in the MMA+Et cocultures, the alga showed poor growth (maximum of 10.8 μg chl.·mL^{-1}) and began to die after 3 days (Fig. 4B), which contrasted with the Chlamydomonas monocultures incubated in MMA+Et where the alga only suffered a slight growth impairment. The dry weight biomass of the consortia incubated in MMA+OH and in MMA+Me media were > 700% and 15% higher, respectively, than the sum of the values for their individual monocultures (Fig. 4D). The small difference between cocultures and monocultures observed in MMA+Me cultures is probably related to the strong ability of *M. oryzae* to grow alone in MMA+Me medium, thus diminishing the relative increase in the biomass of the cocultures.

As expected, in Chlamydomonas monocultures, acetic acid was rapidly consumed, whereas alcohols removal from the media was very slow (101–116 mM and 71–76 mM of remaining methanol and ethanol, respectively, after 9 days) (Fig. 5A, D and G). This decrease in alcohols level might have been due to a simple evaporation phenomenon; thereby, 7–19% and 11–13% of methanol and ethanol, respectively, could be lost over 9 days. On the other hand, *M. oryzae* monocultures consumed the acetic acid from the media when ethanol was not present (MMA and MMA+Me media), being the fastest in the absence of any alcohol (Fig. 5C). Interestingly, in the presence of ethanol, *M. oryzae* monocultures excreted large amounts of acetic acid, being higher in MMA+OH than in MMA+Et (maximum of 17.3 and 11.1 mM, respectively) (Fig. 5C). Furthermore, the *M. oryzae* monocultures completely consumed the initial methanol (125 mM) after 9 days in MMA+Me (Fig. 5F). However, in the presence of ethanol (MMA+OH), the uptake of methanol by the bacterial monocultures was much slower, and 93.2 mM of methanol remained in the medium after 9 days (Fig. 5F). Finally, ethanol uptake in *M. oryzae* monocultures was not very pronounced, showing 66 mM and 49 mM remaining concentrations after 9 days in MMA+OH and MMA+Et cultures, respectively. These values were close to the concentrations obtained in Chlamydomonas monocultures and consequently were probably influenced by evaporation (Fig. 5I). From these observations it can be concluded that in the *M. oryzae*

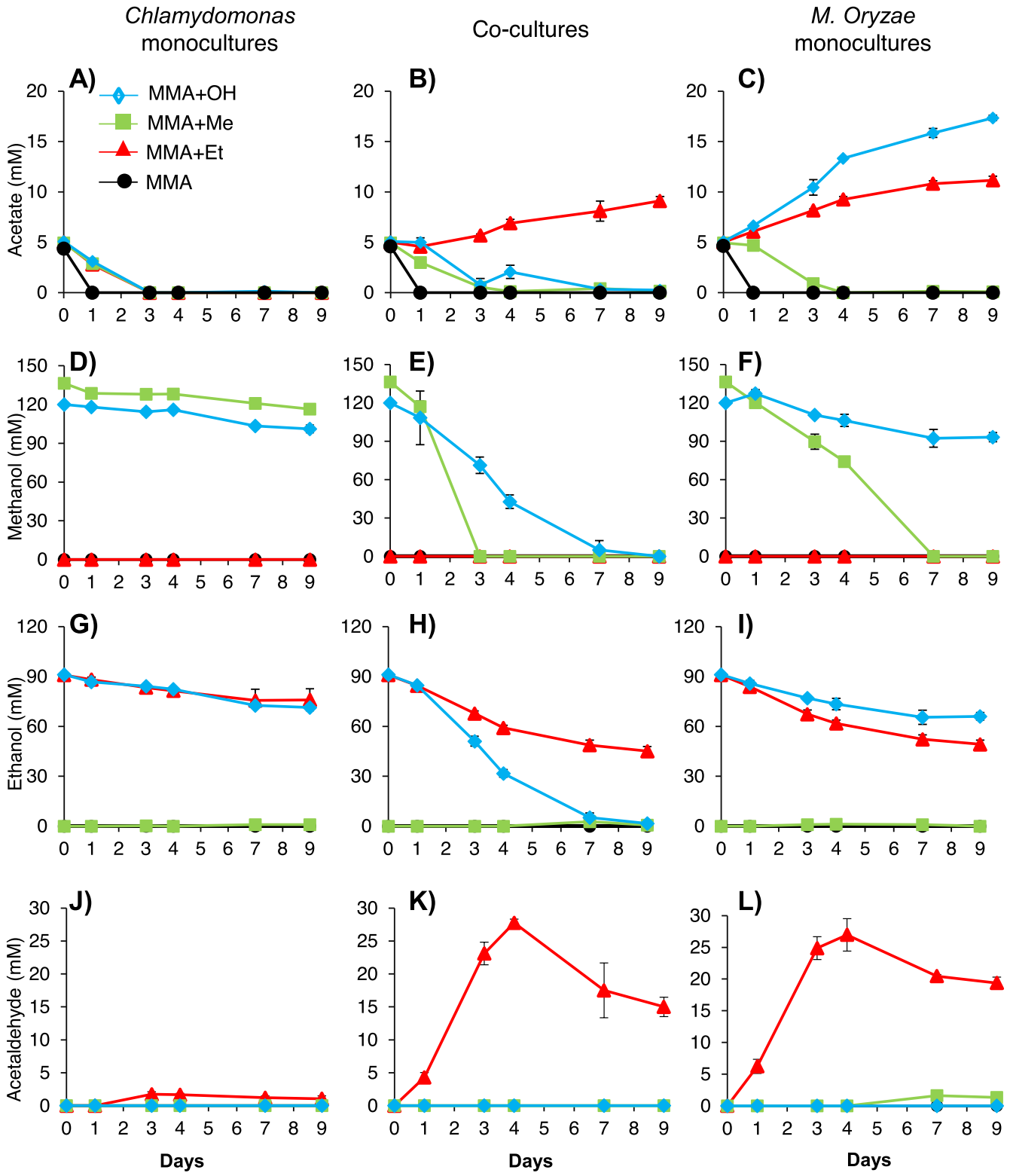

Fig. 5. Acetate, methanol, ethanol, and acetaldehyde concentrations present in the media of *Chlamydomonas-M. oryzae* consortium. The Chlamydomonas-*M. oryzae* consortium and the respective monocultures were incubated with different carbon sources: acetate (MMA); acetate, methanol, and ethanol (MMA+OH); acetate, and methanol (MMA+Me); and acetate and ethanol (MMA+Et). Initial concentrations were of 5 mM, 120 mM and 85 mM for potassium acetate, methanol, and ethanol, respectively. Cultures were incubated under a photoperiod of 12:12 h (80 PPFD). Data correspond to the means from at least three biological replicates. The graphical representation of some small standard deviation values may not be visible in the current graphic scales.

monocultures, methanol and acetic acid were efficiently consumed in the absence of ethanol; otherwise, methanol was consumed much more slowly, and acetic acid was excreted in large quantities.

In the cocultures, the methanol uptake was much higher than that in the respective monocultures (Fig. 5E). In MMA+OH medium, the methanol was fully consumed after 7 days (25 mM/day), whereas 93.2 mM of methanol remained in the bacterial monocultures after 9 days (Fig. 5E vs 5F). In the MMA+Me medium, the cocultures consumed all methanol after 3 days (41.6 mM/day), whereas the bacterial monocultures required 9 days to fully consume all the methanol (13.8 mM/day) (Fig. 5E vs 5F). This highest consumption of methanol by the consortium revealed a better performance of *M. oryzae* when grown together with the alga in both MMA+OH and MMA+Me media. Similarly, the ethanol consumption was also much higher in cocultures than in the corresponding monocultures, particularly in cocultures incubated in MMA+OH, in which the ethanol was almost entirely consumed after 7 days (Fig. 5H). Of note, neither the Chlamydomonas monocultures nor the *M. oryzae* monocultures efficiently consumed ethanol in any media; however, the MMA+OH consortium showed very noticeable uptake of ethanol. Finally, as in *M. oryzae* monocultures, acetate accumulation was observed in cocultures incubated in MMA+Et medium (maximum of 11.15 mM). However, unlike the *M. oryzae* monocultures, no acetic acid accumulation was observed in the MMA+OH cocultures, rather the acetic acid was completely consumed (Fig. 5B).

M. oryzae CBMB20 has been described to use methanol as the sole C source, whereas ethanol supports "weak" growth, and acetate and glucose do not support any growth at all (Madhaiyan et al., 2007). However, in the present study, *M. oryzae* consumed acetic acid (5 mM) in the absence of ethanol, and acetic acid also supported some growth as the sole C source (Figs. 4C and 5C). Moreover, *M. oryzae* grew on methanol but not on ethanol (85 mM) as the sole C source. Indeed, the presence of ethanol prevented bacterial growth on methanol when both alcohols were present in the medium (Fig. 4C). The data indicated that *M. oryzae* transformed external ethanol into acetic acid, which accumulated in the medium (11.1 and 17.3 mM, in MMA+Et and MMA+OH, respectively). The excessive accumulation of acetic acid in the medium (>5 mM) might possibly have prevented *M. oryzae* growth. Ethanol utilization is likely linked in *M. oryzae* to the alcohol dehydrogenase and aldehyde dehydrogenase activities, which sequentially led to the production of acetaldehyde and acetic acid, respectively. At high ethanol concentrations, its incomplete oxidation might have led to excessive acetaldehyde accumulation, which could be toxic to *M. oryzae*. The detection of high levels of acetaldehyde (>25 mM) in cocultures and *M. oryzae* monocultures incubated in MMA+Et (Fig. 5J-L) is consistent with this hypothesis. Moreover, although Chlamydomonas monocultures experienced only a minor decrease in growth when cultivated in MMA+Et, the alga quickly died when co-cultivated in the same media (Fig. 4). Hence, the accumulation of acetaldehyde due to *M. oryzae* activity (Fig. 5K) may also severely poison the alga. Acetaldehyde accumulation was not detected in the *M. oryzae* monocultures or cocultures incubated in MMA+OH, and accordingly, no toxic effect was observed in this medium. A possible explanation for the acetaldehyde accumulation in the MMA+Et medium but not in the MMA+OH medium may be that the accumulation of acetic acid in the absence of methanol may cause an imbalance between the C1 and C2 assimilatory metabolism. It is known that some facultative methylotrophs such as *Methylobacterium extorquens* can assimilate acetic acid very efficiently via the Ethylmalonyl-CoA (EMC) pathway, but requires a fine tuning of the C1 and C2 assimilatory metabolism involving the Serine, EMC and TriCarboxylic Acid (TCA) cycles, which are tightly interconnected to avoid metabolite imbalances (Schneider et al., 2012). Accumulation of glyoxylate and EMC intermediates can be toxic to *Methylobacterium* sp. (Skovran et al., 2010). However, no information is available regarding how *M. oryzae* addresses these C fluxes when growing on acetic acid. As described previously (Madhaiyan et al., 2007) and supported by the present data, *M. oryzae* does not appear to grow efficiently on acetic acid as the sole C source (Fig. 4C), and some metabolic constraints must hinder this metabolite utilization. The enzymatic activities of the alcohol dehydrogenase and aldehyde dehydrogenase require NAD^+ to oxidize ethanol to acetic acid and generate NADH. However, in methylotrophs, the main sinks of NADH are the EMC and Serine cycles (Skovran et al., 2010; Šmejkalová et al., 2010), which may be impaired in the absence of methanol. This might cause an excessive accumulation of NADH and a shortage of NAD^+, which prevented the full oxidation of ethanol and promoted the accumulation of acetaldehyde. In contrast, the presence of methanol in the MMA+OH medium might allowed for the correct functioning of the Serine cycle, which can regenerate NAD^+. In turn, this results in an even greater accumulation of acetic acid than that in MMA+Et medium (Fig. 5C) but in a less toxic environment, owing to the absence of acetaldehyde accumulation (and possibly also glyoxylate and EMC intermediates). In contrast, in the MMA+OH cocultures, the acetic acid excreted by the bacteria was likely consumed by the algae, thereby eliminated the negative effect of acetic acid on the bacterial growth, benefited the algal heterotrophic growth and explained the high consumption rates of alcohols observed in the consortium (Fig. 1 and Fig. 5).

The great improvement in the algal growth in cocultures incubated in MMA+OH was probably due to the CO_2 and acetate excreted by *M. oryzae*, the latter of which is the preferred C source of Chlamydomonas. In the MMA+Me cocultures, the algal growth also benefited from bacterial CO_2, although to a lesser extent than in the MMA+OH medium, probably because of the absence of excreted acetic acid (Fig. 4B and Fig. 5B and C). Moreover, although *M. oryzae* grew alone very efficiently in MMA+Me medium (Fig. 4C), the methanol uptake in MMA+Me cocultures was higher than that in bacterial monocultures (Fig. 5D and F), thereby also indicating better performance of the bacteria in the cocultures. Because methanol is highly abundant in MMA+Me medium and can be efficiently taken up by the bacteria, the exchange in reduced C sources between both microorganisms was unlikely to be beneficial for the bacteria. Instead, O_2 or organic N (e.g. amino acids) provided by the alga may potentially explain the better performance of the bacteria in MMA+Me cocultures. Nonetheless, the precise metabolic relationships benefiting the bacterial growth need to be defined.

Overall, all these data showed a clear metabolic cooperation between Chlamydomonas and *M. oryzae*, when grown together in the presence of methanol and ethanol (MMA+OH), that mutually benefited both organisms. In contrast, the respective monocultures showed little efficient taking up both type of alcohols (Fig. 5). This kind of metabolic complementation is somehow similar to that reported by Calatrava et al. (2018), wherein Chlamydomonas and *Methylobacterium aquaticum* survived in media with peptides/amino acid as the sole C and N sources only when cocultured together. These findings are two examples of how Chlamydomonas-*Methylobacterium* spp. consortia can collaborate 1) to grow on nutrient sources that would otherwise be unsuitable for monocultures and/or 2) to eliminate metabolic intermediaries that are toxic to one organism.

3.5. Hydrogen production by the Chlamydomonas-M. oryzae consortium

To further elucidate other possible biotechnological applications of the consortium, its ability to produce H_2 was tested. The premise for this application was based on the ability of M. *oryzae* to release acetic acid into the media when incubated in the presence of ethanol, and the ability of Chlamydomonas to link acetic acid consumption to H_2 production (Fakhimi et al., 2019b, 2019a; Jurado-Oller et al., 2015).

Cultures were incubated in MMA+OH, MMA+Me and MMA+Et media in sealed bioreactors without purging of the headspaces (Fig. 6). No H_2 production was observed in Chlamydomonas monocultures and *M. oryzae* monocultures. These data revealed that all MMA-derived media, which contained only 5 mM of acetate, were not ideal for H_2 production in Chlamydomonas monocultures under 80 PPFD. Similar results have been previously obtained (Fakhimi et al., 2019b). However,

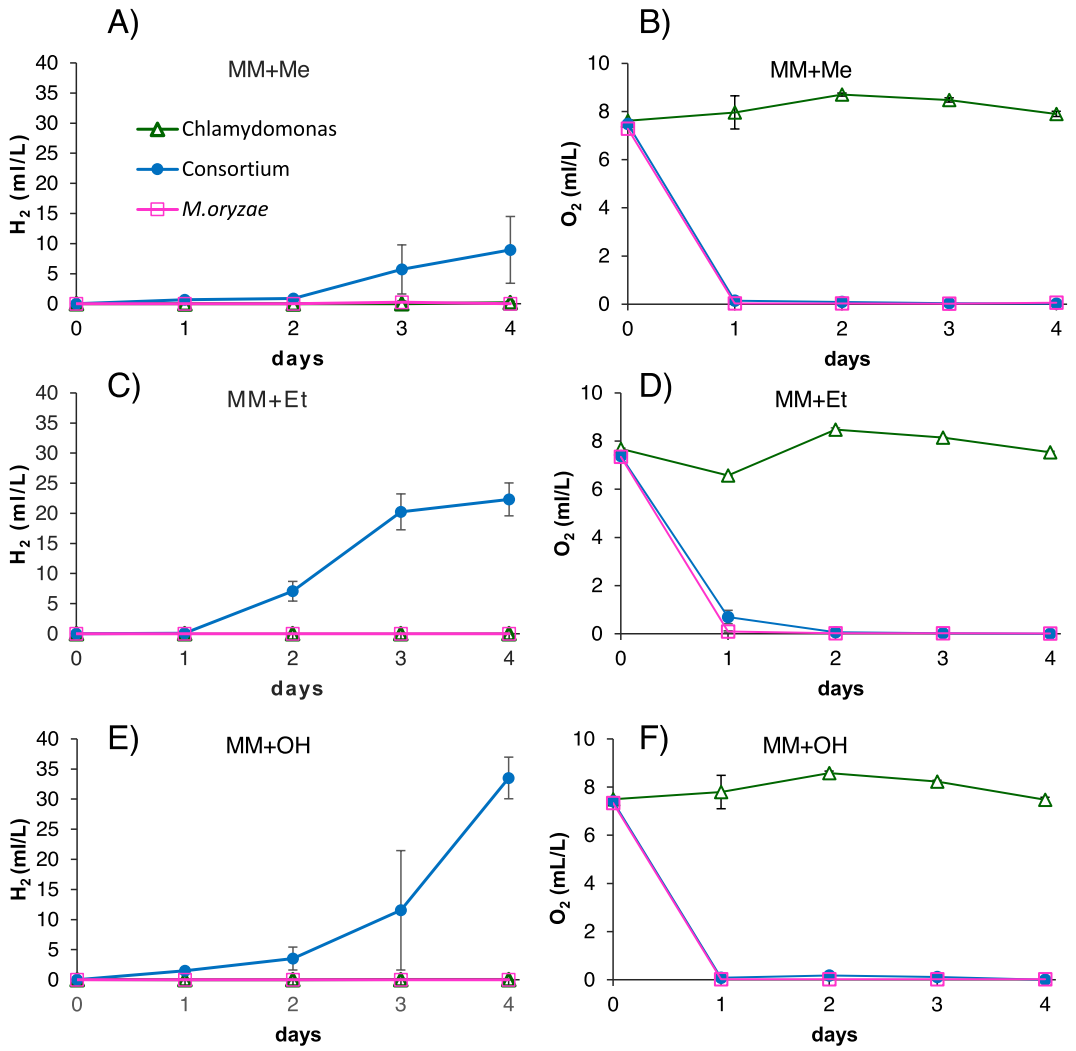

Fig. 6. H_2 production in Chlamydomonas-*M. oryzae* cocultures and monocultures. Chlamydomonas and *M. oryzae* monocultures and cococultures were incubated in MMA+OH, MMA+Me and MMA+Et at 80 PPDF. H_2 (A, C and E) and O_2 (B, D, and F) levels are plotted. Data correspond to the means from at least three biological replicates. The graphical representation of some small standard deviation values may not be visible in the current graphic scales.

H_2 production was observed in cocultures incubated in MMA+OH and MMA+Et (33.5 mL·L^{-1} and 22.3 mL·L^{-1}of cumulative H_2 after 4 days, respectively). Finally, the H_2 production in cocultures incubated in MMA+Me was much lower (8.9 mL·L^{-1}).

The higher H_2 production observed in MMA+OH and MMA + Et media was probably attributable to the ability of *M. oryzae* to excrete acetic acid in these media (Fig. 5C), thus facilitating H_2 production by the alga. Accordingly, in the cocultures incubated in MMA+Me medium, in which no acetic acid was excreted, the H_2 production was low. The lower H_2 production observed in the MMA+Et cocultures relative to the MMA+OH cocultures was probably due to the low cell viability of these cocultures (Fig. 4B). The positive effect of acetic acid on H_2 production in Chlamydomonas has been suggested to be associated with its assimilative metabolism under hypoxia (González-Ballester et al., 2017; Jurado-Oller et al., 2015), rather than only to its contribution to deplete the O_2 levels from the media. Under this condition, the PSII-independent H_2 production pathway may use the reductive power generated during acetic acid assimilation. The H_2 production observed in MMA+OH, MMA+Me, and MMA+Et media represent a good scenario for the confirmation of the assimilative acetate metabolism hypothesis and rejection of a simple O_2 depletion effect, because the three types of cocultures showed comparable levels of hypoxia (Fig. 6B, D, F) but differing potential to accumulate acetic acid in the medium (Fig. 5C). Because of the absence of acetic acid excretion in the cocultures incubated in MMA+Me medium, the H_2 production observed in this medium can be linked to the PSII-dependent pathway and/or the PSII-independent pathway using alternatives sources of reductive power (e.g., from starch degradation). Nonetheless, it can be estimated that in the cocultures incubated in MMA+OH, around 75% (35.5 vs 8.9 mL·L^{-1}) of the total H_2 production could be linked to the PSII-independent pathway and acetic acid assimilation, which is in accordance with previous reports (González-Ballester et al., 2017).

4. Conclusion

A novel Chlamydomonas-*M. oryzae* consortium was identified with high potential for N removal, biomass generation and H_2 production when incubated with methanol and ethanol. Viability of the consortium was extended using nitrate as sole N source rather than ammonium. A metabolic cooperation relying on the exchange of acetate from the bacterium to the alga permitted the consortium growth on ethanol-containing media, which is an unsuitable C source for both organisms.

Further research is needed to optimize the consortiuṁs performance and growth in WW and to delve into its metabolic relationships.

CRediT authorship contribution statement

María Jesus Torres: Investigation, Writing – original draft. **David González-Ballester:** Supervision, Conceptualization, Funding acquisition, Writing – original draft. **Aitor Gómez-Osuna:** Investigation. **Aurora Galván:** Resources, Writing – review & editing. **Emilio Fernandez:** Resources, Writing – review & editing. **Alexandra Dubini:** Supervision, Conceptualization, Funding acquisition, Writing – original draft.

Declaration of Competing Interest

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

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Data availability

All data are available at 10.5281/zenodo.5860577.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.127088.

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