Microalgae based wastewater treatment coupled to the production of high value agricultural products: Current needs and challenges

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Abstract

One of the main social and economic challenges of the 21st century will be to overcome the worlds' water deficit expected by the end of this decade. Microalgae based wastewater treatment has been suggested as a strategy to recover nutrients from wastewater while simultaneously producing clean water. Consortia of microalgae and bacteria are responsible for recovering nutrients from wastewater. A better understanding of how environmental and operational conditions affect the composition of the microalgae-bacteria consortia would allow to maximise nutrient recoveries and biomass productivities. Most of the studies reported to date showed promising results, although up-scaling of these processes to reactors larger than 100 m^2 is needed to better predict their industrial relevance. The main advantage of microalgae based wastewater treatment is that valuable biomass with unlimited applications is produced as a co-product. The aim of the current paper was to review microalgae based wastewater treatment processes focusing on strategies that allow increasing both biomass productivities and nutrient recoveries. Moreover, the benefits of microalgae based agricultural products were also discussed.

Keywords: bioremediation, cyanobacteria, nutrients, water, biostimulants, biofertilisers.

1. Introduction

You never miss the water till the well runs dry. It's an old saying that it is now more relevant than ever: approximately 60% of the world population is expected to suffer a water shortage by 2025 (Schewe et al., 2014). One of the main social and economic challenges of the 21st century will be to overcome the worlds' water deficit expected by the end of this decade. Main causes for this deficit include an increased demand for water, contamination of water resources, and lack of technologies to reclaim used water. The main goal of wastewater treatment processes is to allow the reutilisation or discharge of effluents back into the environment without causing a significant damage. However, conventional nutrient removal methods have restrictions concerning their high-energy requirements or environmental impact (greenhouse gas emissions) and are facing challenges to meet strict nutrient discharge standards (Muga and Mihelcic, 2008).

The search for an alternative economic, sustainable, and effective strategy to process wastewater led to an increased interest in microalgae-based wastewater treatment processes. The use of microalgae is considered as one of the most promising strategies to process wastewater (Acién Fernández et al., 2018; Cai et al., 2013; Lage et al., 2018; Vo et al., 2020) and is already being implemented at commercial scale (e.g. Chiclana and Mérida in Spain or Christchurch in New Zealand). In the current review, the term microalgae will be used indistinctively to refer to prokaryotic cyanobacteria and eukaryotic microalgae. Cyanobacteria are a bacterial phylum capable of performing photosynthesis and for this reason these microorganisms are generally included into the term "microalgae". Recovering nutrients from wastewater using microalgae involves the symbiotic association of microalgae with aerobic and anaerobic microorganisms. It is not possible to produce 100% pure microalgal cultures

at large scale, especially when using open reactors and wastewater. The composition of the microalgae-bacteria consortia depends on several factors including the quality of the water used, environmental conditions and operational conditions (Collao et al., 2021). For example, a low light availability (e.g. high culture depths) (Sánchez-Zurano et al., 2021a) and an increase in temperature (González-Camejo et al., 2017) promote the growth of nitrifying bacteria. If operational conditions are managed properly, over 95% of the produced biomass will be microalgae (Herrera et al., 2020).

The association of microalgae and other microorganisms is generally beneficial for microalgal growth. Yeast such as *Rhodotorula glutinis* promoted biomass and lipid productivity in culture of *Arthrospira platensis* (Xue et al., 2010). Certain bacterial groups can synthesise micronutrients, siderophores, growth stimulants, and antibiotics that promote growth and protect algae from pathogenic microorganisms (Lian et al., 2018). Some of the most common symbiosis factors produced by bacteria to promote microalgal growth are vitamin B_{12}, nitrogen, dimethylsulfoniopropionate, roseobacticides, virbioferrin, and acyl-homoserine lactone, among others (Fuentes et al., 2016; Yao et al., 2019). Bacteria and yeast not only promote growth but also the composition of the produced biomass (Fuentes et al., 2016; Yao et al., 2019). Bacteria present in microalgal cultures such as *Flavobacterium, Terrimonas,* and *Sphingobacterium* produce extracellular metabolites that facilitate harvesting by increasing the floc size and promoting the sedimentation of microalgae (Lee et al., 2013). One of the main advantages of using microalgae-bacteria consortia is their dual role: they not only remove contaminants/nutrients but also produce valuable biomass that can be further used for a wide variety of applications. For example, microalgae produced using cattle dairy wastewater has been used for fertilising pasture and led to higher concentrations of minerals including phosphorus, calcium,

magnesium, and manganese and a higher dry matter content (Lorentz et al., 2020).
Biostimulants are among the most common microalgae derived products currently
available in the market. Biostimulants are considered environmentally friendly and
cost-effective products when compared to their synthetic counterparts. These
products are highly demanded, especially in the organic cropping system (Colla and
Rouphael, 2020; Ronga et al., 2019). Their potential utilisation to obtain increased
yields or improved fruit quality has been demonstrated in several recent reports
(Coppens et al., 2016; Dineshkumar et al., 2020b; Garcia-Gonzalez and Sommerfeld,
2016; Navarro-López et al., 2020; Uysal et al., 2015).

The aim of the current paper was to review recent findings on wastewater treatment
using microalgae-bacteria consortia and to summarise and discuss the potential
utilisation of microalgal biomass as feedstock for the production of biostimulants.
Moreover, this review also discusses the main advantages and limitations of current
microalgae production systems focusing on those processes that are coupled to
wastewater treatment and production agricultural products.

2. Culturing and processing of microalgal biomass

Microalgae biotechnology is a relatively new research area. As highlighted in the
previous section, microalgae have potential applications in both wastewater treatment
and agriculture. Microalgae are unicellular photosynthetic microorganisms with a
simple reproductive and cell growth system, which allows a fast proliferation and long-
term survival in harsh environments (Chiaiese et al., 2018). Although microalgae grow
naturally in lakes, rivers, or oceans, these ecosystems allow very low biomass
concentrations for large-scale harvesting. Thus, to obtain higher concentrations,

microalgae need to be produced in photobioreactors that have evolved from initial open ponds to a wide variety of modern designs. The aim of the current section is to describe and summarise the production and processing of microalgae, focusing on those methods relevant to wastewater treatment and production of agricultural products.

2.1 Culturing of microalgae

Several photobioreactor designs are currently being used for microalgae production, being strains of the genus *Arthrospira, Isochrysis, Nannochloropsis, Tetraselmis, Chlorella, Haematococcus,* and *Dunaliella* the most widely produced. The selection of a certain reactor will depend on the end application of the biomass and the produced strain. For example, open ponds such as those shown in Figure 1 are the preferred option when producing microalgae using wastewater while more complex closed tubular reactors are more suitable when the biomass is used for food, cosmetic, or pharmaceutical applications. Closed systems have the advantage of providing a controlled environment that can be manipulated according to the microalgal requirement. A higher process control allows achieving higher volumetric productivities. For example, the productivity of the microalga *Nannochloropsis gaditana* using closed tubular photobioreactors was 0.6 $g \cdot L^{-1} \cdot day^{-1}$ (San Pedro et al., 2015), while the maximum productivity that could be achieved in an open raceway was 0.2 $g \cdot L^{-1} \cdot day^{-1}$ (San Pedro et al., 2014). Closed photobioreactors can be controlled to satisfy specific biological and physiological demands of microalgae and allow the production of monocultures that cannot be produced in open systems. Still contaminations represent a challenge, even for closed systems. A disadvantage of closed photobioreactors is their high cost investment that can be around 0.6-1.2 $M€ \cdot ha^{-1}$ and leads to higher biomass production costs, in the range 20-30$€ \cdot kg^{-1}$. For

this reason, closed systems are used for the production of high-value strains such as *Haematococcus pluvialis* (Villaró et al., 2021), which is used as a source of astaxanthin, a potent antioxidant carotenoid used in the food and cosmetic industries that can reach a market price of 2,500€·kg⁻¹ (Ledda et al., 2016).

Because of their complexity and higher production costs, closed bioreactors are not recommended for wastewater treatment processes. In turn, open systems are much simpler to operate and their fixed and operational costs much lower – cost investment for these systems range from 0.13 to 0.37 M€·ha⁻¹ (Chisti, 2013; Norsker et al., 2011). After decades of research, biomass production costs using open systems have been reduced to 5.0-10.0 €·kg⁻¹. This value could be further reduced to under 1.0 €·kg⁻¹ if the process is scaled-up sufficiently and biomass production is coupled to wastewater treatment and CO_2 capture from flue gases (Acién et al., 2012). One of the main disadvantages of open systems is their lower biomass productivity when compared to closed photobioreactors. The maximal biomass concentration reached in open systems is lower, around 0.5-0.6 g·L⁻¹, mainly because of the self-shading or shadow effect of microalgae. Raceway reactors can be operated at depths of 0.10-0.15 m (Acién Fernández et al., 2013). However, higher depths of up to 1 m have also been evaluated as a strategy to avoid freezing and minimise heat loss during cold time (Sawant et al., 2018) and lower depths (0.05 m) have been used to increase light availability (Sánchez-Zurano et al., 2021b). Light availability is the most important factor in the growth and productivity of photosynthetic microorganisms and current raceway designs do not allow to optimise light utilisation (Barceló-Villalobos et al., 2019). This drawback has been improved by reducing the culture depth. Thin-layer reactors which use water depths ranging from 0.006 to 0.040 m are highly productive

and allow productivities comparable to those of closed systems (Masojídek et al., 2011; Morales-Amaral et al., 2015; Morillas-España et al., 2021a, 2020).

Artificial illumination is economically prohibitive for low value applications. Therefore, wastewater treatment processes based on microalgae are carried out outdoors using the sun as the source for light. These processes are highly influenced by environmental factors namely temperature and solar radiation. For example, the areal productivity of an optimised large-scale raceway reactor used to produce *Scenedesmus almeriensis* reached 20-25 $g \cdot m^2 \cdot day^{-1}$ (0.14-0.17 $g \cdot L^{-1} \cdot day^{-1}$) in summer and maximum productivities in winter and autumn were close to 15 $g \cdot m^2 \cdot day^{-1}$ or 0.10 $g \cdot L^{-1} \cdot day^{-1}$ (Morillas-España et al., 2020). As open systems are exposed to the environment, other microorganisms can lead to contamination of the culture. Only a limited number of strains that are robust enough, fast-growing, and tolerant to extreme conditions can be produced in open reactors and only those that produce a valuable compound render the process economically viable. This include *Arthrospira platensis* and *Chlorella vulgaris*, mainly used as food, and *Dunaliella salina* used as a source of β-carotene (Lafarga, 2020). The maintenance of monocultures (or cultures high a very high content of one strain) at large-scale remains challenging (Lage et al., 2018). In this sense, several research groups are currently identifying extremophile strains that show potential for being produced outdoors and accumulate valuable biomolecules, generally carotenoids (Lafarga et al., 2021). Indeed, the above-mentioned strains *A. platensis* and *D. salina* are extremophiles as they can be produced at pH values in the range 9-10 and salt concentrations up to 300 $g \cdot L^{-1}$. When coupled to wastewater treatment, contamination of the culture is not a major problem. Microalgae-based wastewater treatment is carried out by consortia of microalgae and bacteria (and other microorganisms such as yeasts and moulds) naturally present in

the wastewater and in the environment. A large biodiversity is generally preferred in wastewater treatment processes. In a recent study, metabarcoding of 16S and 18S genes revealed the dynamics of eukaryotic and prokaryotic communities in a *Scenedesmus dimorphus*-based wastewater treatment process, and identified other microalgae (*Chlorella, Pseudocharaciopsis, Characium*, and *Oocystis*) that co-dominated the system as well as bacteria (*Proteobacteria, Firmicutes, Bacteroidetes*, and *Actinobacteria* were the most abundant phyla), and fungi (Ferro et al., 2020). Similar results were observed in cultures of *Anabaena* sp. and *Dolichospermum* sp. produced using waste streams and raceway reactors. In this case, although the consortia contained a large number of different microorganisms including *Chlorella* strains, both *Anabaena* sp. and *Dolichospermum* sp. managed to be the predominant microorganisms (Morillas-España et al., 2021c).

Overall, the selection of the type of photobioreactor used for microalgal growth will depend on the intended use and desired quality of the biomass. When microalgae are used for wastewater treatment and as a source of bioproducts for agriculture, they are produced using open photobioreactors. Main reasons are the lower production costs and the ease of construction, operation, and scale-up of the facilities. Moreover, the surface-to-volume ration of raceway reactors is higher than that of closed systems or thin-layer cascade designs, allowing the processing larger volumes of water per surface unit.

2.2 Processing of microalgal biomass

Prior to commercialisation of the biomass, microalgae need to be harvested. Harvesting and dewatering contribute 3-15% to algae biomass production costs (Fasaei et al., 2018) and refer to a number of processes where a diluted microalgae

suspension is concentrated into a thick paste. Different methods are available for concentrating microalgal biomass and the main advantages and limitations of current harvesting strategies have been revised previously (Barros et al., 2015; Chen et al., 2011; Kadir et al., 2018; Singh and Patidar, 2018). Ideally, the harvesting step should be effective for a large number of microalgal strains and should allow high biomass concentrations at low (moderate) costs of operation, energy, and maintenance (Danquah et al., 2009). Separating algae from water remains a major hurdle to industrial-scale processing and a universal harvesting method does not exist yet. The method used will depend on the final use of the biomass but also on fundamental properties of the microalgae such as strain, density, particle shape, or particle size (Kadir et al., 2018).

For many applications, microalgal harvesting comprises two steps, pre-concentration or thickening and dewatering. However, sometimes a single step is used depending on the desired water content of the suspension (indeed, the most common current strategy at large scale is centrifugation). Flocculation, flotation, or filtration have been used for pre-concentration, followed by centrifugation or filtration applied subsequently as the main dewatering methods to concentrate microalgal cells into pastes. Pre-concentration using flocculation results in the lowest energy requirements but because of the need for chemicals and loss of flocculants, these systems end at the same cost level as mechanical harvesting systems (Fasaei et al., 2018). Bioflocculation is being evaluated as an inexpensive technology for harvesting microalgae and main findings have been summarised in several review papers (Alam et al., 2016; Nazari et al., 2020; Ummalyma et al., 2017). Bioflocculation is a flocculation process of microalgal cells assisted with microorganisms. The aggregation of bacteria and microalga creates large flocs that settle down by gravity without need for adding chemical aids or

modifying the pH of the culture. For example, the diatom *Skeletonema* was used to form flocs of *Nannochloropsis* (Salim et al., 2011) and *Citrobacter freundii* and *Mucor circinelloides* improved the flocculation of *Chlorella* (Jiang et al., 2021). In addition, the bioflocculant poly ɣ-glutamic acid produced by *Bacillus licheniformis* CGMCC 2876 was effectively used to concentrate the microalga *Desmodesmus* sp. F51 allowing a flocculation efficiency higher than 99% and a harvesting efficiency of 95% (Ndikubwimana et al., 2014). This strategy can also be used as a method to improve nitrogen and phosphorus removal from wastewaters as the microalgal-bacterial flocs tend to adsorb suspended compounds (Ummalyma et al., 2017). Filtration techniques are currently being investigated and membrane bioreactors are widely used for municipal and industrial wastewater treatment. The use of membranes to maximise nutrient recoveries from wastewater is also common (Morillas-España et al., 2021d) One of the main limitations of membrane technologies is that microalgae lead to fouling/clogging and reduced flux and therefore to increased operational costs (Singh and Patidar, 2018). However, a correct design of the process and the use of anti-fouling strategies such as intermittent permeation, membrane backwashing, air backwashing, or air-induced cross flow can minimise this issue. Flocculation and membrane filtration are limited in terms of maximal concentration and are generally followed by a centrifugation step, which has potential to achieve high biomass concentrations. However, we would like to highlight what discussed above: a universal harvesting method does not exist and the selected method should be optimised for each process independently.

Once the biomass is concentrated, it needs to be further processed rapidly to avoid spoilage, especially in hot climates (Lafarga, 2020). When used as a source of bioactive compounds for agriculture as a biofertiliser or biostimulant, microalgal

biomass is commercialised as a liquid suspension. For this reason, drying processes will not be discussed in the current paper. Main drying strategies which include freeze-drying, rotary drying, spray drying, solar drying, and incinerator drying and have been revised previously (Chen et al., 2015; Show et al., 2015).

One of the main challenges of producing valuable bioactive compounds using microalgae is that these are (most of the times) produced and accumulated inside microalgal cells. Because microalgal cell walls are rigid and protective, a disruption step is generally required to allow the extraction of valuable biomolecules. In this sense, several strategies have been studied including enzymatic or chemical hydrolysis, bead milling, high pressure homogenisation, sonication, microwaves, pulsed electric fields, high-voltage electrostatic fields, and high-voltage electrical discharges (Lafarga, 2020). At large scale, the most relevant strategies are high-pressure homogenisation, bead milling and, to a lower extent, sonication. High-pressure homogenisation is a mechanical process during which the solution containing the biomass is forced by high pressure (50-300 MPa) through a micrometric disruption chamber. This increases the velocity and subjects the cells to intense fluid-mechanical stresses that disrupt cell walls and membranes (Carullo et al., 2018). Sonication consists on the use of ultrasonic waves, at frequencies beyond 18 kHz, to generate bubbles that collapse and generate spots of extremely high temperature and pressure that induce cell wall disruption (Nicolau-Lapeña et al., 2019). High-energy demands are a major bottleneck for downstream processing of microalgae. Energy consumption of high-pressure homogenisation is lower than that of sonication and is therefore, together with bead milling, the preferred strategy for large-scale processing of microalgae. Pulsed electric fields are being industrially used in the food industry (Lafarga et al., 2018) and could be used to disrupt the cell wall of microalgae at

industrial level. Although this strategy has not been implemented at large scale (up to the best of the authors' knowledge) several laboratory scale trials have been conducted and results are promising (Carullo et al., 2018; Käferböck et al., 2020). Finally, as mentioned before, microalgae based agricultural products are generally commercialised as a liquid suspension (rich in nutrients). This means that a thermal treatment, generally a pasteurisation step is used to increase shelf life and avoid spoilage of the product. Other technologies used to extend the shelf life of food such as high pressure processing or ohmic heating could be implemented to extend the shelf life of agricultural products, although these have not yet been evaluated (ohmic heating was assessed as a cell wall disruption step (Yodsuwan et al., 2018) but not to increase the shelf life of microalgae products).

3. Microalgae and wastewater treatment

Unfortunately, most wastewater produced globally is discharged into the environment without treatment – over 80% of sewage water is discarded untreated (Khan et al., 2019). Wastewater treatment can include physical, chemical, and biological strategies and it allows releasing the water, once treated, into the environment. One of the main advantages of using microalgae for wastewater treatment, besides high efficiency and safety, is that the generated biomass can be further used for numerous applications. These include the production of biofuels (Malla et al., 2015), animal feed (Zhou et al., 2012), or biofertilisers and biostimulants for agriculture. Another important advantage is that microalgae wastewater treatment can be economically viable and sustainable. Indeed, a techno-economic analysis revealed that (under the optimum scenario) the overall cost of processing wastewater using microalgae could be 0.15 $\cdot m^{-3}$ - which is a 30% lower than for activated sludge and was calculated without considering the revenues obtained from the commercialisation of the end agricultural products (Acién

et al., 2017). Moreover, the process can also show a positive energy balance if coupled with biomethane production, rendering the process economically viable and sustainable (Acién et al., 2017).

3.1. Microalgae-bacteria interactions

The development of processes to remove associated bacteria from microalgal cultures and maintain bacteria-free cultures in large-scale production has been attempted (Wang et al., 2016). However, it is deemed impractical and unsustainable because in most cases, bacteria are introduced into microalgae cultivation systems through algae stocks used as starter cultures, which are often not axenic (Gouveia et al., 2019). Moreover, bacteria can access microalgal cultures via multiple operation processes as the culture media used during dilution or as airborne invaders in open systems. Bacteria and microalgae cannot be understood properly if they are evaluated individually. Both appear together in nature and establish many types of interactions, since mutualism or symbiotic relationship until commensalism or parasitism (Fuentes et al., 2016). Microalgae-bacteria interactions are well known for a long time and were already described by Oswald in 1953 (Oswald, et al., 1953). These interactions occur in the area surrounding microalgal cells, where metabolites are exchanged between microalgae and bacteria (Amin et al., 2012) and are species specific as the microenvironment of each microalga is different.

Currently, it is accepted that the interactions between microalgae and bacteria have potential to improve microalgal biomass production(Fuentes et al., 2016). Nutrient exchange plays a major role. Under illumination, microalgae perform photosynthesis, consuming carbon dioxide and producing oxygen. This oxygen is essential for the degradation of organic matter present in wastewater by heterotrophic bacteria. Simultaneously, during bacterial oxidation of organic matter, carbon dioxide is

produced and is available for microalgae to produce photosynthesis (Quijano et al., 2017). Nitrifying bacteria or nitrifiers also are present in wastewater and also have a symbiotic relationship with microalgae. Indeed, these microorganisms transforms ammonium into nitrate using the oxygen produced by microalgae (Vargas et al., 2016). Vitamins, macronutrients, and plat hormones excreted by bacteria promote microalgal growth. A survey of 326 algal species conducted in 2005 revealed that 171 require exogenous vitamin B_{12}, and that the (unexpected) source of this vitamin were bacteria (Croft et al., 2005). Since then, several studies demonstrated the exchange of vitamin B_{12} from bacteria to microalgae, for example, from *Mesorhizobium* sp. to the B_{12}-dependent microalga *Chlamydomonas nivalis* (Kazamia et al., 2012) or from *Sinorhizobium meliloti* to *Chlamydomonas reinhardtii* (Xie et al., 2013).

The association of microalgae and bacteria during wastewater treatment has advantages as well in terms of nutrient recoveries. For instance, a combination of *C. vulgaris* and a microalgae growth-promoting bacterium as *Azospirillum brasilense*, allowed an increased removal of nutrients (nitrogen and phosphorus) than microalgae alone (De-Bashan et al., 2004). One of the reasons for this effect is that bacteria can increase nutrient availability. For example, in most cases, wastewaters contain organic phosphorus that is not available (or with low availability) for uptake by microalgae. However, bacteria produce enzymes to mineralize the organic phosphorus making it bioavailable for microalgae (Solovchenko et al., 2016). Further studies that allow to understand better the interaction between microalgae and bacteria are needed. This might be the key to maximize productivity, nutrient recoveries and environmental and economic impact (Acién et al., 2016).

3.2. Biomass productivities and nutrient recoveries

Table 1 lists some of the most recent findings on wastewater treatment using microalgae and pilot- or large-scale photobioreactors. The selection of a robust and highly-productive strain capable to grow under a wide range of environmental conditions is of key importance. The most common genus used for wastewater treatment were *Scenedesmus* and *Chlorella* (Table 1). These demonstrated a high tolerance to adverse environmental conditions (high temperatures and solar radiation) and resistance to high $N-NH_4^+$ concentrations. $N-NH_4^+$ inhibits algal growth after a certain strain-dependent threshold (and is naturally present at high concentrations in some types of wastewater). Other microalgae such as *Arthrospira platensins* (Wuang et al., 2016; Zhou et al., 2017), *Tetraselmis* sp. (Andreotti et al., 2020; Michels et al., 2014), or *Haematococcus pluvialis* (Haque et al., 2017; Pan et al., 2021) have been studied as candidates to recover nutrients from wastewater at laboratory-scale using different photobioreactor designs.

The up-scaling of these processes using low cost photobioreactors that allow the processing of large volumes of wastewater is necessary to assess their commercial potential. The most commonly used reactors in the literature are raceways, which was expected because of the above-described advantages of raceways for low-value applications. However, other common designs such as bubble columns and tubular reactors or more innovative reactors have been described. Further efforts are needed to further up-scale these processes as, although some studies were conducted in relatively large reactors, their scale is not yet representative of industrial processes (reports using photobioreactors larger than 100 m^2 are not available).

In terms of nitrogen and phosphorus removal capacities, all the studies demonstrated that microalgae are able to recover large percentages of the total nitrogen and phosphorus present in the wastewater. However, the percentage of nitrogen or

phosphorus recovered will depend largely on the initial concentration in the wastewater. It is more accurate to express removal capacity of a process as grams of nitrogen or phosphorus that can be recovered per square meter and day. Nitrogen and phosphorus removal rates of up to 4286 and 227 $mg \cdot m^{-2} \cdot day^{-1}$ respectively have been reported in pilot-scale raceway reactors (80 m^2, 11.8 m^3) operated using primary wastewater (Morillas-España et al., 2021b). In that study, the authors concluded that approximately 15-30% of the nitrogen removed from the wastewater was stripped into the atmosphere (Morillas-España et al., 2021b). Little is known about the effect of environmental and operational conditions on the composition of the microalgae-bacteria consortia. In addition, the effect of the composition of the consortia on the efficiency of the process and on the overall biostimulant activity of the biomass is not known. A recent study concluded that both, the dilution rate and the depth of the culture can affect significantly the microbial populations of the culture (Sánchez-Zurano et al., 2021a). In that study, the authors observed that the abundance of nitrifiers increased with culture depth and this could lead to the accumulation of nitrate in the system. In a different study, a significant variation in the composition of the consortia during the year was reported, and this partially contributed (together with environmental fluctuations throughout the year) to the observed differences in the nitrogen and phosphorus removal efficiencies (Sánchez Zurano et al., 2020). Similar results were observed in another report, where a strong influence of temperature, solar radiation, and nutrient content on bacterial communities was observed (Collao et al., 2021). In that study, the authors suggested that the excretion of microalgal substances to the medium could modulate bacterial communities and therefore, the performance of the system and the overall quality of the produced biomass. Only a limited number of studies conducted mass balances or reported phenomena that take place during

wastewater treatment (nitrification, stripping, or precipitation of phosphorus, for example). This should be also considered in further studies as, for example, stripping could represent a large percentage of the total nitrogen "consumption".

4. Microalgal agricultural products: A biorefinery approach

Microalgal biomass produced using wastewater can be used for different applications. For example, because of their high content in protein and in valuable bioactive molecules, several animal feeds (especially aquafeeds) enriched using microalgal biomass have been developed and demonstrated antioxidant, antimicrobial, and disease-preventing effects (Dineshbabu et al., 2019). High production costs of microalgal biomass when compared to conventional feed ingredients (soybean, fish oil, etc.) has been suggested as the main challenge that needs to be addressed before fully exploiting microalgae in the animal feed industry (Dineshbabu et al., 2019). In this sense, the use of wastewater as a source of nutrients could contribute to reducing production costs. A recent study suggested that microalgae production costs could be lower than 1 €·kg^{-1} if produced using wastewater and flue gases (Garrido-Cardenas et al., 2018). The utilisation of microalgal biomass, or the "residues" left after the extraction of valuable microalgal products, for producing biogas by anaerobic digestion could allow to increase the economic viability of using microalgae in the feed industry (Uggetti et al., 2014).

4.1. Biofertilisers and biostimulants

Low production yields are mainly caused due to insufficient nutrient availability, which is a major agronomic problem in some parts of the world (while excess nutrients represent a problem in some others). Chemical fertilisers and manure have been key

to agricultural intensification but they also lead to a widespread nutrient pollution and the degradation of lakes, rivers, and coastal ocean while the release of nitrous oxide from fertilised fields contributes to climate change (Foley et al., 2011). This, together with an improved knowledge about the relationship between plants and soil microorganisms occurring in the rhizosphere have led to and increasing development and utilisation of microbial-based fertilisers or biofertilisers worldwide (Malusá and Vassilev, 2014).

Biostimulants offer a novel approach for the regulation or modification of physiological processes in plants. Not only to stimulate growth but also to mitigate stress-induced limitations and finally increase yields (Yakhin et al., 2017) – Figure 3. Both biofertilisers and biostimulants are considered environmentally friendly and cost-effective products and are highly demanded, especially in the organic cropping system (Ronga et al., 2019). Plant biostimulants can be divided into 7 major groups: (i) humic and fulvic acids, (ii) protein hydrolysates and other N-containing compounds, (iii) algal extracts and botanicals, (iv) chitosan and other biopolymers, (vii) inorganic compounds, (vi) beneficial fungi, and (vii) beneficial bacteria. Their nature is very diverse as well as their physiological functions. To date, several physiological functions have been demonstrated and these include the protection of photosynthetic compounds against photo damage or the initiation of lateral roots – Table 2. Microalgal extracts demonstrated gibberellin-like, auxin-like, and cytokinin-like effects in previous reports (Navarro-López et al., 2020). Gibberellins are plant hormones that promote plant growth in a variety of developmental contexts. Mutants defective in gibberellins show reduced elongation of roots, stems, and floral organs (Rizza et al., 2017). Auxins (i.e. indole-3-acetic acid, indole-3-butyric acid, phenylacetic acid) and cytokinins (*trans*-zeatin, kinetin, *N-N'*-diphenylurea) were identified in several microalgal species from

Chlorella, *Scenedemus*, and *Acutodesmus* genus (Piotrowska-Niczyporuk et al., 2020). Auxins have the ability to induce growth responses in plants and have implications in most of the quantitative changes that occur during a plants' life cycle (Keswani et al., 2020). Cytokinins participate in the regulation of plant growth, physiological activities, and yield and play a key role in response to abiotic stress (Li et al., 2021).

Microalgal biomass has potential to prevent nutrient loss by gradually releasing nitrogen, phosphorus, or potassium into the environment. Different microalgae-based biofertilisers or biostimulants are currently commercially available and these include AlgaFert® and AlgaFert Eco® commercialised by the Spanish company Biorizon Biotech SL (Almería, Spain). Almost one century ago, Alfred Redfield concluded that plankton have an average atomic C:N:P stoichiometry of 106:16:1 (Redfield, 1934). In the case of freshwater microalgae, the Redfield ratio is not a rule with N:P molar ratios ranging within 8:1 and 45:1 (Whitton et al., 2016). The C:N:P of microalgae reflects their macromolecular composition: protein is the major reservoir of cellular nitrogen, phospholipids and nucleic acids are the major reservoirs of phosphorus, and protein, lipid, and carbohydrate content determine cellular carbon (Finkel et al., 2016). Significant phylogenetic differences in macromolecular composition of microalgae have been identified after conducting a hierarchical Bayesian analysis using a large number of data compiled from the literature. For example, while cyanobacteria have an averaged protein content of 42.2%, the protein content of other phyla such as Chlorophyta or Bacillariophyta is around 32.8 and 29.2% respectively (Finkel et al., 2016). However, the composition of microalgal biomass also depends on other factors which include culture media composition and environmental factors like temperature or solar radiation (Lafarga, 2020). Thus, the chemical characteristics of microalgae-

based biofertilisers or biostimulants will be highly influenced by all these factors. For example, a recent study demonstrated that the molar N:P composition of the microalgae *C. vulgaris, Stigeoclnium* sp., *S. obliquus,* and *C. sorokiniana* ranged within 7.8 to 20.3 when produced in a growth medium with an N:P ratio of 6:1 and that this difference was reduced to a ratio between 11.3 and 16.3 when the microalgae were transferred to a growth medium with an N:P ratio of 2:1 (Whitton et al., 2016). As an example, the commercial microalgal fertilisers AlgaFert® (Biorizon Biotech SL, Spain) and Spirnature (Agrinature Producciones Agrícolas SL, Spain), both based on Spirulina, have an NPK ratio of 1-7-3.

As highlighted in previous sections, one of the advantages of microalgae is that this valuable resource can be produced in wastewater obtaining a dual role: (i) phycoremediation of wastewater and (ii) biomass rich in macro- and micro-nutrients essential for an optimal crop growth and development – Figure 2. All that glitters is not gold, and microalgae-based agricultural products have some drawbacks. For example, microalgae have the ability to accumulate heavy metals, and for this reason, accurate chemical analysis should be performed to certify safe agricultural microalgae-derived products (Ronga et al., 2019). Moreover, the effect of wastewater on the composition of the biomass, and therefore on the quality and bioactivity of the end product, has been generally overlooked. Wastewaters generally have a low content of phosphorus, and it is known that phosphorus limitation (generally) promotes lipid production and accumulation and a substitution of phospholipids with glycolipids and/or betaine lipids (Huang et al., 2019). Phosphorus limitation also led to an increased content of carotenoids, ascorbic acid, and tocopherols (Gauthier et al., 2020). The exposure of microalgae to heavy metals that can be present in wastewater can also promote the synthesis of valuable compounds such as ascorbate peroxidase,

catalase, superoxide dismutase, or ascorbate among other compounds (Gauthier et al., 2020).

Microalgae have also been suggested as biological biocides to manage pests and diseases (Renuka et al., 2016). Ongoing studies and developments on this field will certainly result in the appearance of commercial microalgae-derived biocides in the future.

4.2 Application of microalgae in soil

Several reports demonstrated the effectiveness of microalgae-based biofertilisers to improve crop yields. Indeed, rice cultivation inoculated with the microalgae *Chlorella vulgaris* or *Arthrospira platensis* led to 7-21% higher rice yields (Dineshkumar et al., 2018). Results were consisted with those reported in a different study, where microalgae and cyanobacteria inoculation enhanced nutrient uptake and rice growth in China – microalgae inoculation also led to reduced arsenic translocation from roots to grains in arsenic-contaminated paddy soils (Wang et al., 2018). The potential utilisation of dried biomass and extracts from *Acutodesmus dimorphus* as biofertiliser in Roma tomato plants was also investigated (Garcia-Gonzalez and Sommerfeld, 2016). The authors of that study reported that *A. dimorphus*, applied 22 days prior to seedling transplant, led to increased plant growth and higher number of branches and flowers. Similar results were observed in a recent study where eighteen liquid extracts obtained from microalgae and cyanobacteria improved root and shoot length of tomato plants by 112 and 53%, respectively (Mutale-joan et al., 2020) – Figure 4. The authors of that study identified the production of a vast array of metabolites induced by microalgal extracts which led to accumulation of palmitic acid and stearic acids in the plants as well as pyridine-3-carboxamide, an active form of vitamin B3. In a different

study, the potential of *Chlorella vulgaris* or *Arthrospira platensis* alone or mixed with cow manure on growth parameters, biochemical composition, and nutritional properties of onions was evaluated (Dineshkumar et al., 2020b). Higher yields and quality were observed when manure was combined with *A. platensis* followed by manure combined with *C. vulgaris.* Both microalgae, when used alone, led to higher yields and quality than manure alone. Not only microalgal biomass but also microalgal co-products from other industrial processes can be used to improve plant yields. For example, Solé-Bundó et al., (2017) suggested that the digestate from microalgae anaerobic digestion and co-digestion with primary sludge could be a promising solution towards both wastewater treatment and, because of its high organic matter and macronutrients, agriculture.

Utilisation of microalgae as a biofertiliser could promote not only yields but also quality. For example, comparable growths after cultivating tomatoes using microalgae-based and commercial organic fertilisers were reported (Coppens et al., 2016). Wwhen cultivated using microalgae-based fertilisers, fruit quality was improved by increasing sugar and carotenoid content. In that study, microalgal biomass was produced using waste streams from aquaculture, demonstrating the previously mentioned dual wastewater treatment/biofertiliser production potential of microalgae. Moreover, microalgae utilisation in agriculture can be beneficial not only to the plant or the fruit but also to soil. Indeed, previous reports assessing the effect of microbial fertilisers from microalgae at four different concentrations (0.0-1.5 dose) on maize and wheat, concluded that when administered at a dose of 1.0, microalgae led to a higher amount of organic matter in soil and its water holding capacity was improved (Uysal et al., 2015).

4.3 Foliar application

The number of studies evaluating the potential utilisation of microalgae and microalgae-derived compounds for foliar application is limited. This strategy is relatively novel and is one of the most innovative agricultural practices as it is environmentally safe and promotes agricultural sustainability (Ronga et al., 2019). Microalgae-derived extracts, even at low concentrations, can induce an array of physiological plant responses. Foliar application of *A. dimorphus* at a concentration of 3.75 g·mL^{-1} led to increased plant height and greater number of flowers and branches per plant in tomato plants (Garcia-Gonzalez and Sommerfeld, 2016). Moreover, application of extracts of *C. vulgaris* at different concentrations to green gram (*Vigna mungo* L.) led not only higher yields but also improved soil physical and chemical parameters (Dineshkumar et al., 2020a). Foliar application was performed ten days before blooming, after berry sitting and 21 days later. Similar results were obtained recently by the same research group on black gram (*Vigna mungo* L.) (Dineshkumar et al., 2020c).

Some microalgal strains such as *A. platensis* and *A. maxima* (*Spirulina*) are naturally rich in protein (Lafarga et al., 2020). These strains contain high contents of amino acids which are well known biostimulants. Protein hydrolysates are among the active ingredients of plant biostimulants (Calvo et al., 2014; Romero García et al., 2012). In this sense, the effect of foliar spraying with enzymatic hydrolysates of *Scenedesmus* sp. and *A. platensis* (10 g·L^{-1}) on *Petunia × hybrida* plant development and lead nutrient status was studied (Plaza et al., 2018). Authors reported that application of *Scenedesmus* sp. five times (days 0, 14, 25, 35, and 42 after transplanting) at a concentration of 10 g·L^{-1} accelerated plant development and fastened flowering. In turn, *A. platensis* at the same studied dose and application dates led to enhanced root dry matter, water content, and number of flowers per plant (Plaza et al., 2018). Foliar

applications of algal extracts seems to be more effective if applied in the morning, when the leaf stomata are open (Battacharyya et al., 2015), although further studies are needed to confirm this hypothesis. In a different study, foliar application of enzymatic hydrolysates of *A. platensis* promoted growth of seedlings in organically grown lettuce – hydrolysates obtained after 4 h of hydrolysis were the most active promoting growth and increasing spermine content (Mógor et al., 2018). Results were in line with those reported in a different study, where seeds treated with *A. dimorphus* culture and with the *A. dimorphus* extracts at concentrations higher than 0.75 g·mL^{-1} accelerated seed germination by two days (Garcia-Gonzalez and Sommerfeld, 2016).

Results reported so far suggest that microalgae are excellent biofertilisers and/or biostimulants with numerous reports demonstrating their potential to improve plant growth, fruit yields, and/or number of flowers per plant, among other benefits. The use of microalgae and microalgal extracts in agriculture is a reality and the number of novel products launched into the market is increasing every year. Overall, the most commonly utilised microalgae are *Spirulina* and *Chlorella*. However, there are thousands of microalgae strains, with varied biochemical compositions, currently available in culture collections around the world. This huge variability suggests a bright future for microalgae-derived bioproducts in agriculture, although further research is needed to identify the most effective microalgae and extract for a given application.

Conclusions

One of the main advantages of using microalgae-bacteria consortia in wastewater treatment processes is their dual role: they not only remove nutrients and contaminants but also produce valuable biomass, which can be used for a wide variety

of applications. The most common used photobioreactor designs for wastewater treatment are raceways. Not only because of their lower construction and operation costs, but also because their low surface-to-volume ration allows to process large amounts of wastewater per surface area. So far, most of the published studies were conducted at laboratory-scale. Further studies using large (over 100 m^2) reactors are needed to better predict the potential of microalgae for the treatment of wastewater. The most common strains studied to date are *Scenedesmus* and *Chlorella*. Microalgae-derived agricultural products showed promising results and potential for being used as a sustainable and environmentally friendly strategy. Indeed, there are several commercial microalgae-based products currently available. Their production could be coupled to wastewater treatment. Moreover, biochemical fractionation of microalgal biomass and extracts derived thereof and agronomic tests of their purified compounds are needed as a useful step for in-depth study of the action mechanisms of microalgae. Overall, the benefits of a microalgal biorefinery approach to treat wastewater and produce valuable products for agriculture including biofertilisers and biostimulants go well beyond environmental health, with implications on human health, energy, food safety, and mitigation of climate change.

Acknowledgements

CRediT author statement

A. Morillas-España: Writing-Original Draft; **T. Lafarga:** Writing – Original Draft, Conceptualisation, Supervision; **A. Sánchez-Zurano:** Writing-Original Draft; **F.G. Acién-Fernández:** Supervision, Funding Acquisition; **C.V. González-López:** Supervision, Funding Acquisition.

Table 1. Outdoor demonstrations of microalgae based wastewater treatment processes

Photobioreactor	Microalgal strain(s)	Culture medium	Operation mode	Biomass concentration/productivity	Nutrient removal	Reference
Raceway (8.3 m², 850 L)	*Scenedesmus* sp.	Secundary urban wastewater	Semi-continuous mode (0.1-0.3 day⁻¹)	Biomass productivity ranged from 4 g·m⁻²·day⁻¹ in winter to 17 g·m⁻²·day⁻¹ in summer.	Average COD, TN, TP and *E. coli* removal efficiencies of 84, 79, 57, and 93%	(Posadas et al., 2015)
Raceway (1.5 m², 470 L)	Dominated by *Stigeoclonium* sp., diatoms, *Chlorella* sp., and *Monoraphidium* during warm seasons and *Chlorella*, diatoms, and *Stigeoclonium* sp, in cold seasons	Urban wastewater	Semi-continuous mode	Biomass productivity ranged from 6-8 g·m⁻²·day⁻¹ in winter to 13-24 g·m⁻²·day⁻¹ in summer.	COD removal rates of 29-58 g·m⁻²·day⁻¹. The removal of microcontaminants was season-dependent.	(Matamoros et al., 2015)
Tubular reactor (890 L)	*Chlorella pyrenoidosa*	Digested wheat starch processing wastewater (filtered)	Batch mode	Biomass productivity ranged from 0.11 g·L⁻¹·day⁻¹ in winter to 0.63 g·L⁻¹·day⁻¹ in summer.	Average COD, TN, and TP removal efficiencies of 66, 83, and 97% respectively	(Tan et al., 2014)
Raceway (7.2 m², 800 L) and tubular reactor (340 L)	*Nannochloropsis gaditana*	Centrate wastewater	Semi-continuous mode (0.2-0.3 day⁻¹)	Maximum biomass productivity values around 25 and 10 g·m⁻²·day⁻¹ for tubular and raceway reactors respectively	TN and TP removal rates of 20-30 and 1-3 mg·L⁻¹·day⁻¹ (tubular reactor) and 20-30 and 0.5-1.5 mg·L⁻¹·day⁻¹	(Ledda et al., 2015)

				(raceway) respectively.	
Thin-layer cascade reactor (32 m², 1600 L)	*Scenedesmus* sp.	Primary urban wastewater	Semi continuous mode (0.3 day⁻¹)	Maximum biomass productivity values varied between 28 and 47 g·m⁻²·day⁻¹ in winter and summer respectively	$N-NH_4^+$, $N-NO_3^-$, and $P-PO_4^{3-}$ removal rates varied within 15-30, 0-2, and 2-6 mg·L⁻¹·day⁻¹ respectively	(Sánchez Zurano et al., 2020)
Semi-open bioreactor (1500 L)	*Chlorella* sp.	Centrate wastewater	Semi-continuous mode	Biomass productivity ranged from 17.7-34.6 g·m⁻²·day⁻¹	Average COD, TN, and TP removal efficiencies of 70, 61, and 61% respectively	(Min et al., 2011)
Raceway (1.9 m², 533 L) and tubular reactor (380 L)	*Scenedesmus obliquus*	Secundary urban wastewater	Semi-continuous mode (HRT 2-5 days)	Maximum biomass productivity was 8.3 and 21.7 g·m⁻²·day⁻¹ for the raceway and tubular reactor, respectively	Average TN and TP removal efficiency of 89.6 and 86.7% (tubular reactor) and 65.1 and 58.8 (raceway) respectively.	(Arbib et al., 2013)
Bubble column (33 L, 4 columns)	*Chlorella zofingiensis*	Artificial wastewater	Batch mode	Biomass productivity of 0.06 g·L⁻¹·day⁻¹	Maximum TN and TP removal efficiencies of 73.5 and 100% respectively	(Zhu et al., 2014)

Rotating modified raceway (4080 L)	Dominated by *Diatoma, Pediastrum,* and *Chlorella*	Tertiary urban wastewater	Fed-batch mode	Biomass productivity of 31 $g \cdot m^{-2} \cdot day^{-1}$	Average TP and TN removal rates of 2.1 and 14.1 $g \cdot m^{-2} \cdot day^{-1}$ respectively.	(Christenson and Sims, 2012)
Photo-membrane bioreactor (1300 L)	*Scenedesmus* sp.	Industrial wastewater (electric factory)	Continuous mode	Biomass concentrations ranged from 0.14 to 0.22 $g \cdot L^{-1}$	Average TP and TN removal efficiencies of 100 and 46% respectively. Phosphorus precipitation was observed.	(Zhen-Feng et al., 2011)
Twin-layer bioreactor with immobilised microalgae (6 m^2, 55 L)	*Halochlorella rubescens*	Primary and secondary urban wastewater	Batch mode	Average biomass productivity of 6.3 $g \cdot m^{-2} \cdot day^{-1}$	Average TN and TP removal efficiencies in the range 70-99%	(Shi et al., 2014)
Modular offshore photobioreactor (83.6 m^2, 21000 L)	*Scenedesmus dimorphus* (shifted to *Chlorella, Cryptomonas* and *Scenedesmus* after 12 months)	Urban wastewater	Semi-continuous mode	Ranged from 3.5 to 22.7 $g \cdot m^{-2} \cdot day^{-1}$	Maximum TN, TP, and BOD removal efficiencies of 75, 93, and 92% respectively.	(Novoveská et al., 2016)
Thin-layer reactors (63	*Scenedesmus almeriensis*	Primary urban	Semi-continu	Average annual productivity of 24.8 $g \cdot m^{-2} \cdot day^{-1}$ with a	TN and TP removal rates depended on	(Morillas-España

m^2, 2400 L; 126 m^2, 3600 L)		wastewater	ous mode	maximum of 32.8 $g·m^{-2}·day^{-1}$ in summer	the season and varied within 695-2383 and 70-118 $mg·m^{-2}·day^{-1}$ respectively	et al., 2021a)
Raceway reactors (80 m^2, 11800 L)	*Scenedesmus almeriensis*	Primary urban wastewater	Semi-continuous mode	Biomass productivities ranging within 20-28 $g·m^{-2}·day^{-1}$ when operating at dilution rates of 0.3-0.5 day^{-1}	TN and TP removal rates higher than 90% and COD removal of 70% approximately	(Sánchez-Zurano et al., 2021a)
Raceway reactors (80 m^2, 11800 L)	*Scenedesmus almeriensis*	Primary urban wastewater	Semi-continuous mode	Maximum biomass productivity of 25.1 $g·m^{-2}·day^{-1}$	Maximum TN and TP removal rates of 4286 and 227 $mg·m^{-2}·day^{-1}$ respectively	(Morillas-España et al., 2021b)

Abbreviations: COD, chemical oxygen demand; TN, total nitrogen; TP, total phosphorus; HRT, hydraulic retention time; BOD, biological oxygen demand.

Table 2. Effect of biostimulants on crop production. Table modified from (du Jardin, 2015) **with permission from Elsevier.**

	Humic acids	Algal extracts	Protein hydrolysates	Glycine betaine	Plant growth-promoting Rhizobacteria
Cellular mechanism (i.e. interaction with cellular components and processes)	Activate plasma membrane proton-pumping ATPases, promote cell wall loosening and cell elongation in roots	Stimulate expression of genes encoding transporters of micronutrients (i.e. Cu, Fe, Zn)	Stimulate phenylalanine ammonia-lyase enzyme and gene expression and production of flavonoids under stress	Protection of photosystem II against photo damage – likely via activation of scavengers of reactive oxygen	Release of auxins and activation of auxin-signalling pathways involved in root morphogenesis

Physiological function (i.e. action on whole plant processes)	Increase linear growth of roots and root biomass	Increased tissue concentrations and root to shoot transport of micronutrients	Protection against UV and oxidative damage	Maintenance of leaf photosynthetic activity under salt stress	Increased lateral root density and surface of root hairs
Agricultural function (i.e. output traits relevant for crop performance)	Increased root foraging capacity and enhanced nutrient use efficiency	Improved mineral composition of plant tissues	Increased crop tolerance to abiotic stress (i.e. salt)	Increased crop tolerance to abiotic stress (i.e. salt)	Increased root foraging capacity and enhanced nutrient use efficiency
Economic and environmental benefits (i.e. changes in yield, quality, ecosystem services)	Higher crop yields, saving of fertilisers and reduced losses to the environment	Enhanced nutritional value and biofortification of plant tissues	Higher crop yields	Higher crop yields	Higher crop yield, savings of fertilisers and reduced losses to the environment

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Figure 1. Open raceway bioreactors located at the University of Almería, Spain.

Figure 2. Dual role of microalgae-based wastewater treatment: Wastewater bioremediation and valuable biomass production

Figure 3. Microalgae-based biostimulants. Biostimulants can not only modify physiological responses but also maximise crop productivities and promote root development, enhancing nutrient uptake and optimise (minimise) fertiliser consumption and use efficiency.

Figure 4. Effect of microalgal extracts on (A) root length and (B) root dry weight of 40 days old tomato plants. Data represent means ± standard errors of five biological replicates. Different letters indicate significant differences (p<0.05). Figure reprinted from (Mutale-joan et al., 2020) **with permission from Nature Research.**

Figure 1

Figure 2

WASTEWATER

SOLAR RADIATION

MICROALGAE

O_2

BACTERIA

COD

CO_2

N, P, CO_2

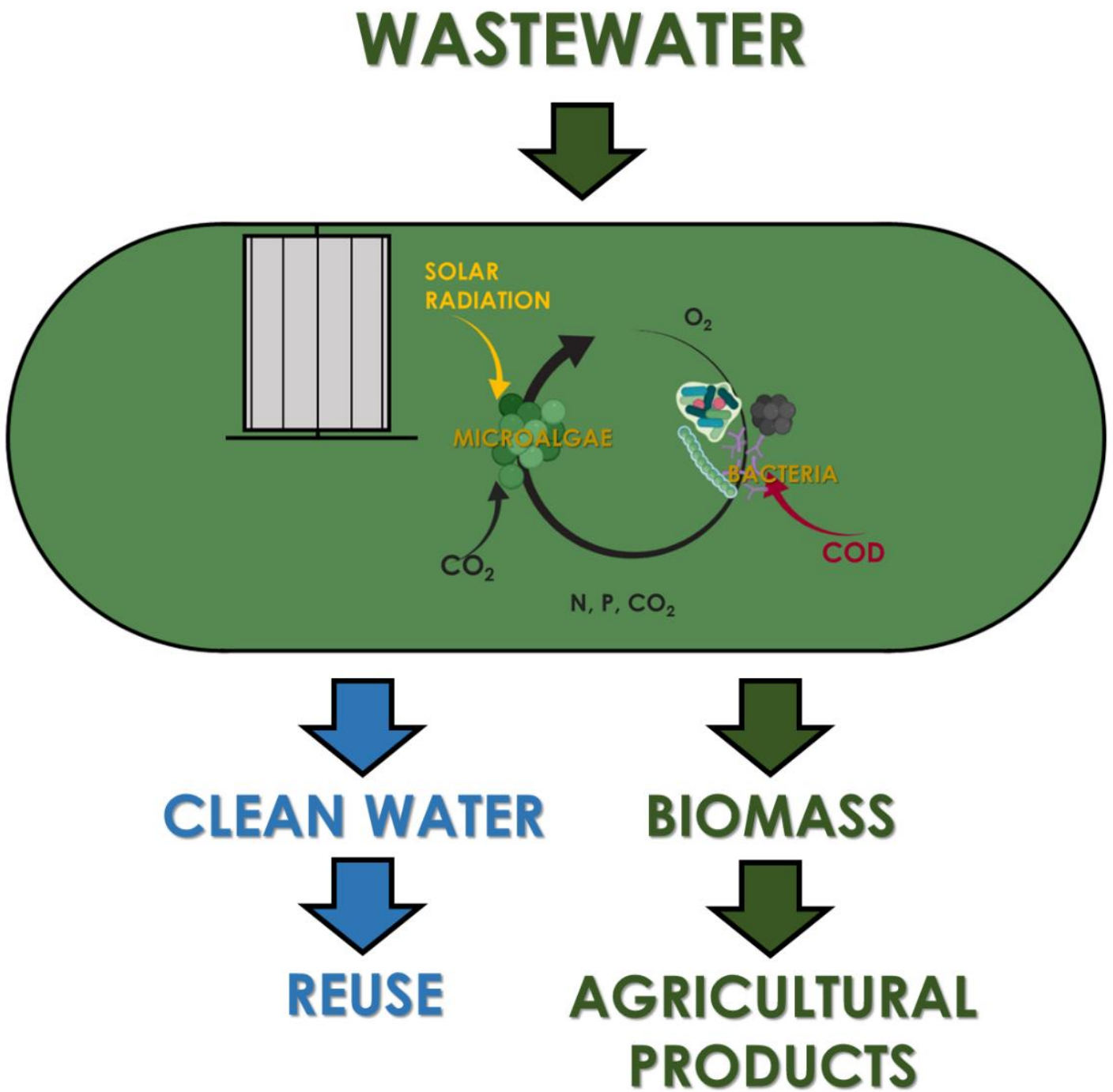CLEAN WATER

BIOMASS

REUSE

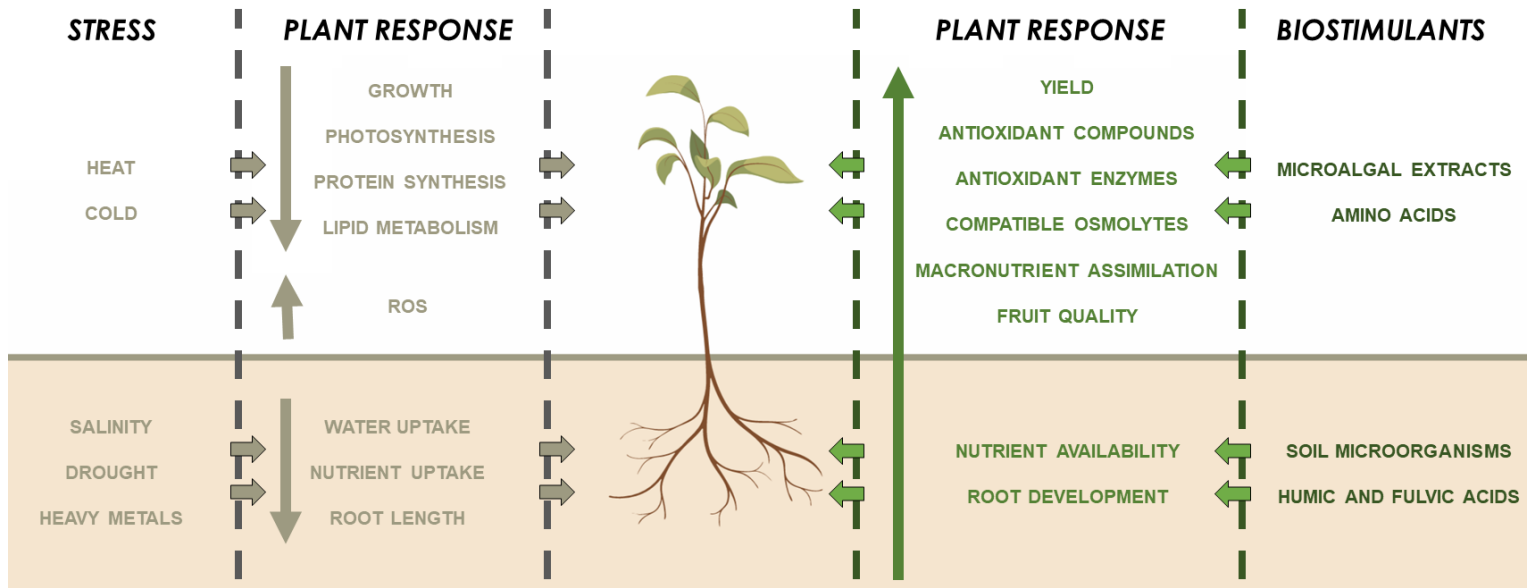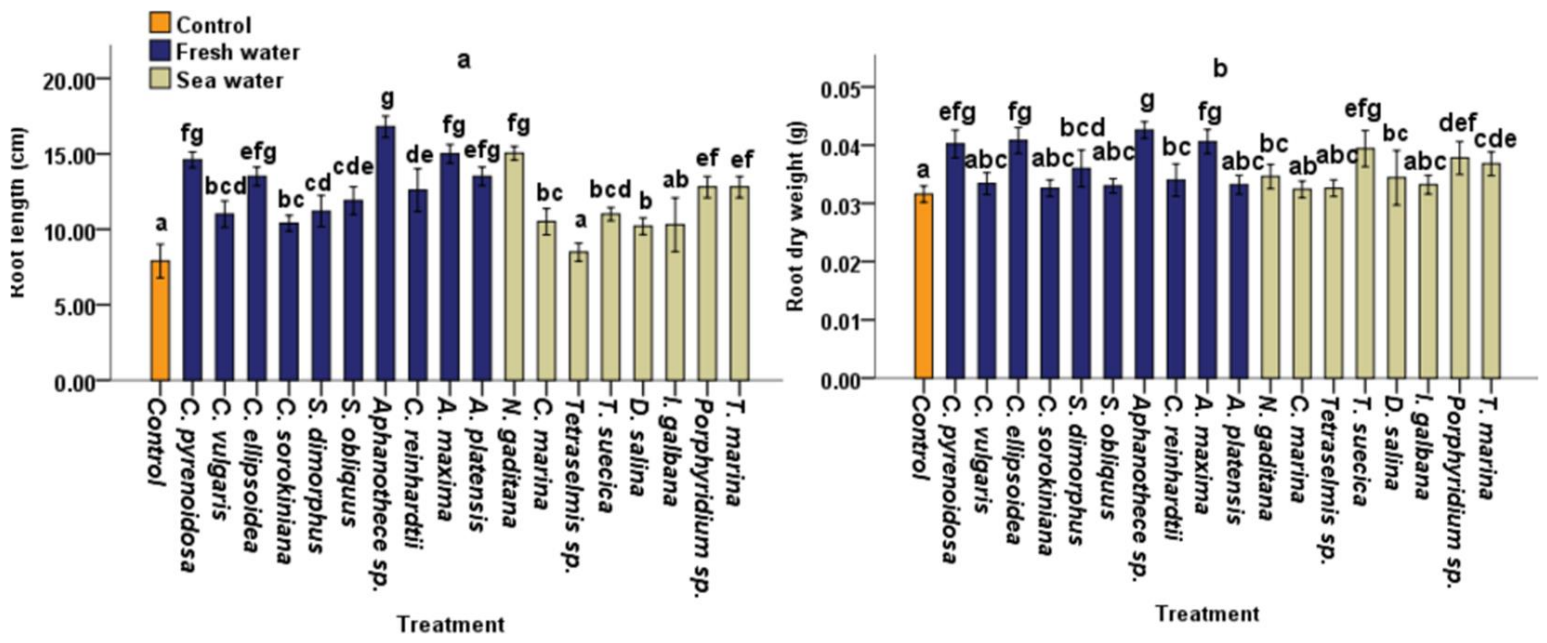AGRICULTURAL PRODUCTS
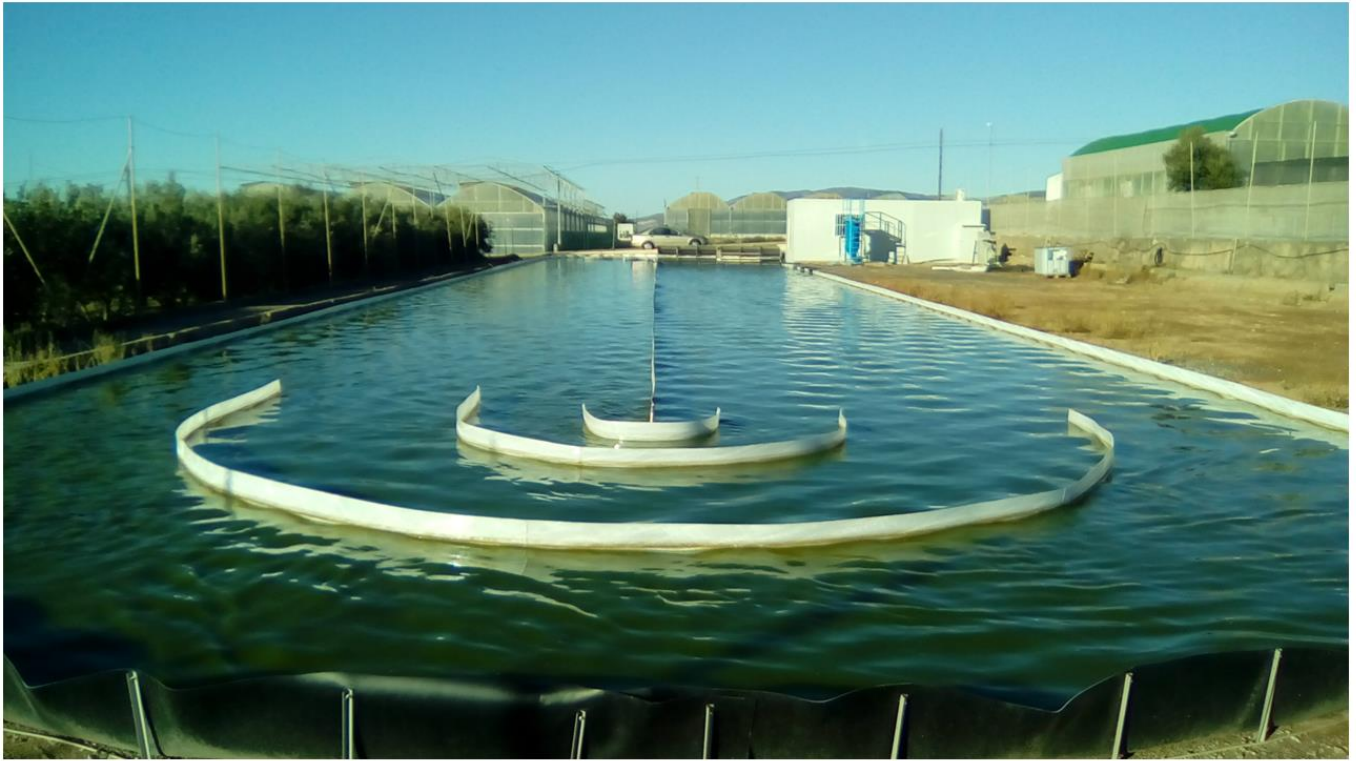
Figure 3

Figure 4

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