A novel photo-respirometry method to characterize consortia in microalgae-related wastewater treatment processes

Ana Sánchez Zurano, Cintia Gómez Serrano, Jose M. Fernández Sevilla, Acién-Fernández F.G., Molina-Grima E.

Chemical Engineering Department, University of Almeria, Ctra. Sacramento, s/n, 04120 Almería, Spain

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Abstract

In this paper, we present a method for determining the different prevailing metabolisms of microalgae bacteria consortia taking place in microalgae-based wastewater treatment processes. The three main microorganism types have been considered: heterotrophic and nitrifying bacteria, and microalgae. The method has been optimized in terms of the operation strategy, including the starvation period required, and the biomass concentration and irradiance during the measurements. The results show that a starvation period of one to three days can be necessary depending on the type of wastewater being processed - the lower the nutrient concentration, the shorter the starvation time required. The measurements were taken close to 100 %Sat. to avoid limitation or inhibition phenomena. By providing light, the microalgae's oxygen production rate was determined whereas the oxygen consumption rate of the heterotrophic bacteria was quantified by adding sodium acetate; lastly, the oxygen consumption rate of the nitrifying bacteria was measured by adding ammonium chloride. The optimal experimental conditions determined were a biomass concentration of 0.5 g/L and an irradiance of 200 $\mu E/m^2 \cdot s$. The methodology's accuracy was verified, thus confirming it as a valuable tool for rapidly characterizing the consortia in microalgae-based wastewater treatment. The analysis of samples from a range of different reactors/substrates confirmed that the prevailing metabolisms in these consortia are modified mainly as a function of the wastewater characteristics. Whatever the wastewater type, the oxygen production rate is the main metabolism, with heterotrophic activity increasing along with increasing chemical oxygen demand (COD) content in the wastewater. Nitrifying activity was only observed when high ammonium concentrations were provided and microalgae growth was unable to consume it in a short time. The developed method is a powerful tool to adequately manage and operate wastewater treatment processes using microalgae/bacteria consortia; it also provides valuable information for modelling purposes.

1. Introduction

Over recent decades, microalgae have been used to produce high-value compounds such as vitamins, pigments and biologically active compounds for the cosmetic, pharmaceutical, nutraceutical and food industries (Spolaore et al., 2006). Microalgae biomass is also a valuable product for direct human consumption and animal/aquaculture-related applications, mainly for its high nutritional value and the health-improvement compounds it contains such as antioxidants, amongst others (Catarina & Xavier, 2012; Gouveia, Batista, Sousa, Raymundo, & Bandarra, 2008). Furthermore, microalgae have been proposed for different services such as soil remediation, flue-gas cleaning, biogas upgrade and wastewater treatment (Acién, Gómez-Serrano, Morales-Amaral, Fernández-Sevilla, & Molina-Grima, 2016; Acien, González-López, Fernández-Sevilla, & Molina-Grima, 2012). The utilization of microalgae in wastewater treatment processes is a highly relevant application due to the microalgae's capacity to recover nutrients such as nitrate/ammonium, phosphorus and others from the wastewater and to transform it into valuable biomass (Olguín, 2012b). Due to the origin of the produced biomass, it cannot be used for human-related applications but it has great potential for agriculture and aquaculture-related applications (Acién et al., 2016). Consequently, microalgae production using wastewater as the nutrient source is a promising alternative, which allows not only a low biomass production cost but also has less environmental impact compared to cultivation systems that use freshwater and fertilizers (Gómez et al., 2013; Park et al., 2011).

Wastewater treatment is the largest bioprocess carried out worldwide, and microalgae can contribute to improving it. Microalgae-related wastewater treatment is performed by microalgae-bacteria consortia. The schematic functioning of this consortia has been previously described. When illuminated, the microalgae consume inorganic carbon, nitrogen and phosphorus, as well as other compounds, to produce biomass while, at the same time, releasing oxygen from photosynthesis. This activity is beneficial in wastewater treatment processes because the oxygen produced by microalgae can be used by aerobic bacteria to biodegrade pollutants so they are capable of oxidizing organic matter into inorganic compounds mainly containing nitrogen and phosphorus (Muñoz et al., 2009). Moreover, the carbon dioxide produced by bacterial respiration is consumed by the microalgae, completing a photosynthesis-respiration cycle (Zambrano et al., 2016).

However, the reality concerning this consortia is more complex, with different microalgae and bacterial metabolisms active at the same time; the overall proportion of these metabolisms determining the capacity of the biological process to treat the wastewater and produce valuable biomass. Therefore, depending on the microalgae species, the growth rate and the capacity to remove nutrients can vary significantly; it always being determined by the light availability inside the reactor. Regarding the bacteria, the two main types of bacterial populations are: (i) the heterotrophic bacteria capable of degrading the organic matter present in the wastewater, and (ii) the nitrifying bacteria responsible for converting ammonia into its most oxidized form as nitrites and nitrates. The existence of denitrifying bacteria is usually disregarded in these systems.

In microalgae-based wastewater treatment, it is considered that an equilibrium exists between microalgae and bacteria-related processes. However, this is not always true because, depending on the operational conditions, the prevalence of microalgae or bacteria varies greatly (Cabanelas et al., 2013). Accordingly, recent studies have shown that the bacterial contribution to a consortium's performance is lower than that from the microalgae; this is due to the fact that the bacteria's metabolism is faster than the microalgae's so only a low bacterial mass is necessary to degrade organic compounds into inorganic compounds. Moreover, the amount of oxygen produced in this process by the microalgae population is far higher than that required by the low bacterial mass. Consequently, the relationship between microalgae and bacteria in a consortium is determined by the wastewater composition and its feed rate. An increase in either the wastewater's organic matter concentration or the feed rate produces a higher proportion of bacteria within the consortium. For this reason, it is essential to understand and model these phenomena so as to adequately design and operate microalgae-based systems for wastewater treatment (Acién et al., 2016).

Respirometry techniques have been used to distinguish between different biological processes such as heterotrophic substrate removal and nitrification in activated sludge. Traditionally, the use of carbonaceous substrates has allowed heterotrophic biomass quantification (including not only bacteria and their storage materials but also protozoa and other higher organisms) by measuring the oxygen consumption. Likewise, the oxygen consumed by autotrophic bacteria such as nitrifying bacteria has been measured using inorganic compounds (Vanrolleghem, 2002). Furthermore, respirometry techniques based on dissolved oxygen measurements have been developed to study the photosynthesis rates and the respiration rates in different microalgae cultures under the

influence of different environmental factors (Costache et al., 2013; Ippoliti et al., 2016). However, respirometry methods are generally applied to pure microalgal cultures without considering the existence of microalgae-bacteria consortia in wastewater treatment processes. On this issue, some authors have started to develop respirometry methods for studying microalgae-bacteria consortia by evaluating both microalgal and nitrifying activity during the test (Rossi et al., 2018).

This work aims to develop a complete photo-respirometry method to quantify the microalgae-bacteria consortia found in wastewater treatment processes, distinguishing between microalgal, heterotrophic and nitrifying activity using the oxygen production/consumption rates. The method's operational conditions have been optimized to define a standardized protocol for characterizing this type of consortia. Furthermore, the developed method has been used to compare the composition of microalgae-bacteria consortia prevailing in different wastewater treatment processes and in pure microalgae cultures, thus showing the large variability of these types of consortia. The methodology described here is a valuable tool for optimizing any microalgae-based process although especially those related to wastewater treatment, which are expected to expand greatly in the near future.

2. Materials and methods

2.1. Photosynthesis and respiration rate measurements

A hand-made photo-respirometer device was designed and built. The equipment allows us to determine any variation in dissolved oxygen concentration in microalgae culture samples under controlled conditions. It comprises an 80 mL jacketed transparent cylindrical glass flask (connected to a temperature-controlled water reservoir for the device's temperature control), which is magnetically stirred and artificially illuminated using two power-controlled LED lamps placed to the right and left of the glass chamber (Figure 1). The light provided by the lamps can be automatically regulated to obtain the desired irradiance inside the centre of the chamber once the sample is added**.** The device is also equipped with a diffuser through which gases (air, O_2, N_2 and CO_2) can be supplied at a low flow rate to modify the culture's dissolved oxygen or pH. To achieve this, the device is also equipped with sensors for irradiance (QSL-1000, Walz, Germany), temperature (PT-100), pH (Crison 5343, Barcelona, Spain) and dissolved oxygen (Crison 5002, Barcelona, Spain) located inside the flask.

An adequate protocol was developed to determine the microalgae cultures' photosynthesis and respiration rates. The developed methodology allows us to distinguish between the metabolisms of the three main populations: the microalgae, the heterotrophic bacteria, and the nitrifying bacteria. Firstly, samples of the microalgae cultures were taken and subjected to nutrient starvation (continuous light of 200 $\mu Em^{-2} s^{-1}$ and an aeration rate of 0.2 $v \cdot v^{-1} \cdot min^{-1}$) to remove the organic matter and the ammonium present in the medium. It was previously demonstrated that one day of starvation was enough to remove the organic matter and ammonium from the samples used. Subsequently, the samples were placed inside the jacketed flask and the variation in dissolved oxygen over time was measured under different conditions. To determine the microalgae's net photosynthesis rate and the respiration rates of the heterotrophic and nitrifying bacteria, each sample was subjected to four light–dark periods of four minutes, during which the variation in dissolved oxygen over time was measured and registered. These values allow us to calculate the respective metabolic rates. The first minute of exposure was disregarded as it was considered to be adaptation time.

The variation in dissolved oxygen was measured in the 90-130 %Sat range, in which the oxygen mass transfer was confirmed as being negligible whilst the performance of the different microorganisms was optimal. The entire system was computer controlled using DaqFactory software. In the following section, each part of the process is described, including the expected biological reactions affecting the dissolved oxygen concentration:

- Microalgae net photosynthesis rate (MNPR). A culture sample was placed inside the photo-respirometer and then exposed to four light–dark cycles of four minutes each to measure and register the variation in dissolved oxygen under whichever condition. Between the dark and light periods, air was provided to recover the 100 %Sat of the dissolved oxygen. During the light periods, oxygen production is expected as the result of active microalgae photosynthesis whereas during the dark periods, the oxygen is consumed by the endogenous respiration rate. Endogenous respiration is defined as the culture's oxygen consumption rate when subjected to starvation and when substrate from an external source is absent, which is indicative of the active biomass concentration (Vanrolleghem, 2002). The net photosynthesis rate was calculated as the sum of the slope of dissolved oxygen accumulation during light and dark periods.
- Heterotrophic bacteria respiration rate (HBRR). Another culture sample was used for this measurement, to which 0,8 mL of sodium acetate (30 g/L) was added as an organic matter

source. Acetate has been described as a substrate for use in wastewater respirometry tests (Vanrolleghem, 2002). The sample was exposed to four light–dark cycles of four minutes each. Between each light and dark period, air was provided to recover the 100 %Sat of the dissolved oxygen. The oxygen consumption in the dark phase allows us to determine the oxygen consumed by the heterotrophic biomass. The respiration rate of the heterotrophic bacteria was calculated as the slope of dissolved oxygen accumulation minus the dissolved oxygen accumulation during the dark period in the endogenous culture.

- Nitrifying bacteria respiration rate (NBRR). Another sample of culture was used for this measurement by providing 0,8 mL of ammonium chloride (3g/L) as the ammonium source. Different ammonium sources have been used to evaluate nitrifying activity in activated sludge processes and microalgae-bacteria consortia, of which ammonium chloride has been the most extensively utilized (Rossi et al., 2018; Vanrolleghem, 2002). The sample was exposed to four light–dark cycles of four minutes each. In the middle of each light and dark period, air was provided to recover the 100 %Sat of dissolved oxygen. The oxygen consumption in the dark phase allows us to determine the oxygen consumed by nitrifying biomass. The nitrifying bacteria's respiration rate was calculated as the slope of dissolved oxygen accumulation minus the dissolved oxygen accumulation during the dark period in the endogenous culture.

  A simplified scheme of the proposed methodology is shown in Figure 2. One can see that during the dark phase (D1-D4), the dissolved oxygen is consumed by microalgal-bacterial endogenous respiration. During the light phase (L1-L4), the microalgae perform photosynthesis and dissolved oxygen production increases while, simultaneously, it is consumed by the respiration processes. The microalgae net photosynthesis rate (MNPR) is calculated as the difference between the oxygen production rate (OPR) during the light period and the oxygen consumption rate (OCR) during the dark period, divided by the dry weight of total biomass (Cb) (Equation 1).

$$\mathrm{MNPR} = \frac{\mathrm{OPR} - \mathrm{OCR}}{Cb} \qquad \textit{Equation 1}$$

  After adding sodium acetate or ammonium chloride, the same measurements are performed to determine both the heterotrophic and nitrifying metabolisms, always starting with new samples. Thus, the heterotrophic bacteria respiration rate (HBRR) was calculated as the difference between the heterotrophic oxygen consumption (HOCR) rate, after providing acetate, and the oxygen consumption rate (OCR) without adding sodium

acetate, divided by the dry weight of the total biomass (Equation 2). Similarly, the nitrifying bacteria respiration rate (NBRR) was calculated as the difference between the nitrifying oxygen consumption (NOCR) rate after providing ammonium chloride and the oxygen consumption rate (OCR) without adding ammonium chloride, divided by the dry weight of the total biomass (Equation 3).

$$\mathrm{HBRR} = \frac{\mathrm{HOCR} - \mathrm{OCR}}{Cb} \qquad \textit{Equation 2}$$

$$\mathrm{NBRR} = \frac{\mathrm{NOCR} - \mathrm{OCR}}{Cb} \qquad \textit{Equation 3}$$

2.2. Microorganisms and culture conditions

2.2.1. Samples from laboratory culture

The *Scenedesmus almeriensis* strain was used as the control microorganism. Stock cultures were maintained photo-autotrophically in spherical flasks (1.0 L capacity) using Arnon medium (Allen and Arnon, 1955). The culture was continuously bubbled with an air–1 % CO_2 mixture to control the pH at 8.0. The culture temperature was set at 22°C, controlled by regulating the air temperature in the chamber. The culture was artificially illuminated in a 12:12 h L/D cycle using four Philips PL-32W/840/4p white-light lamps, providing an irradiance of 750 μE/m^2 s on the spherical 1.0 L flask surface.

For the experiments, this inoculum was transferred to laboratory-scale photobioreactors and industrial-scale outdoor photobioreactors. Details of the reactors and culture medium used in each one are given below. The average composition of the wastewaters used is reported in Table 1.

2.2.2. Samples from bubble columns fed with crop residue leachate

Experiments were performed in 12 bubble column-type reactors with spherical bases (3 cm in diameter, 45 cm in height and with a 300 mL capacity) filled up to 250 mL with leachate from crop residues diluted in water (10% crop residues, 90% water) and 20% of *Scenedesmus almeriensis* inoculum. Each reactor was aerated at a rate of 0.2 v/v/min, with CO_2 injected on demand (pH=8). The reactors were artificially illuminated using eight 28 W fluorescent tubes (Philips Daylight T5), on a simulated daylight cycle. The maximum irradiance (PAR) inside the columns in the absence of cells was 1,850 μEm^{-2} s^{-1}, measured using an SQS-100 spherical quantum sensor (Walz GmbH, Effeltrich, Germany). The cultures' temperature was kept at 25 °C by controlling the temperature of

the culture chamber in which the reactors were located. The reactors were operated in batch mode for 6 days, after which they were operated in a semi-continuous mode. For this, 20 % of culture volume was harvested every day and replaced with fresh culture medium.

2.2.3. Samples from stirred-tank reactors fed with sewage

Experiments were performed in four 1 L stirred-tank reactors (9 cm in diameter, 30 cm in height and with a 1.5 L capacity) operated in the laboratory but simulating outdoor conditions. These reactors were filled with 1 L of sewage taken directly after primary treatment from the wastewater treatment plant in Roquetas de Mar (Almería) and 20% of *Scenedesmus almeriensis* inoculum. To prevent the adverse effect of excessive dissolved oxygen accumulation, the dissolved oxygen was controlled below 200%Sat by supplying air on demand; CO_2 was also injected on demand to control the pH at 8. The reactors were artificially illuminated using eight 28 W fluorescent tubes (Philips Daylight T5) on a simulated daylight cycle. The maximum irradiance (PAR) inside the reactors in the absence of cells was 1,850 $\mu Em^{-2}\ s^{-1}$, measured using an SQS-100 spherical quantum sensor (Walz GmbH, Effeltrich, Germany). The cultures' temperature was kept at 25 °C by controlling the temperature of the culture chamber in which the reactors were located. The reactors were operated in batch mode for 6 days, after which they were operated in semi-continuous mode. For this, 20 % of culture volume was harvested every day and replaced with fresh culture medium.

2.2.4. Samples from an outdoor raceway reactor fed with sewage

A 32 m^2 (4.4 m^3) open raceway reactor operated at a 0.12 m water depth was used. The reactor is equipped with a 1 m^3 sump where pH is controlled at 8 by the on-demand injection of pure CO_2 at 5 l min^{-1}, or air supplied at 50 l min^{-1} to remove oxygen. In the raceway reactor, the culture is circulated at 0.2 m s^{-1} using a rotating paddlewheel (1 m in width and 0.40 m in height) actuated by an electric motor (Morales-Amaral et al., 2015). A SCADA system monitors and controls the reactor's overall operation, including environmental parameters such as solar radiation and ambient temperature, and culture parameters such as pH (Crison 5333T + MM44), temperature (PT1000) and dissolved oxygen (Crison 9336 + MM44). The experiments were performed in semi-continuous mode, by initially filling the reactor with wastewater inoculated with 10% total volume of *Scenedesmus almeriensis* culture from a 3.0 m^3 tubular photobioreactor, which was

operated in batch mode for one week, after which it was operated in semi-continuous mode at a daily dilution rate of 20%.

2.2.5. Samples from an outdoor thin-layer cascade reactor fed with diluted manure

An open 32 m^2 (1.2 m^3) thin-layer cascade reactor operated at a 0.02 m water depth was used. The reactor is equipped with a 1 m^3 sump where pH is controlled at 8 by the on-demand injection of pure CO_2 at 5 l min^{-1}, or air supplied at 50 l min^{-1} to remove oxygen. In the thin-layer reactor, the culture is circulated at 0.2 m s^{-1} using a pump that pushes up the culture to the first layer, lifting it 0.5 m from the culture level in the sump (Morales-Amaral et al., 2015). A SCADA system monitors and controls the reactor's overall operation, including environmental parameters such as solar radiation and ambient temperature, and culture parameters such as pH (Crison 5333T + MM44), temperature (PT1000) and dissolved oxygen (Crison 9336 + MM44). The experiments were performed in semi-continuous mode by initially filling the reactor with pig manure diluted in water (10% pig manure, 90% water) and inoculated with a 10% total volume of *Scenedesmus almeriensis* culture from a 3.0 m^3 tubular photobioreactor, which was operated in batch mode for one week, after which it was operated in semi-continuous mode at a daily dilution rate of 30%.

2.2.6. Samples from an outdoor tubular photobioreactor fed with fertilizers

A 3.0 m^3 capacity industrial tubular photobioreactor (T-PBR) was used for the *S. almeriensis* culture. The facility consists of ten tubular fence-type photobioreactors built as previously described (Fernández et al., 2014). Each photobioreactor is made of a 400 m-long PMMA tube, 0.09 m in diameter, with a bubble column (3.5 m in height and 0.4 m in diameter) for degassing and heat exchange. The pH, temperature and dissolved oxygen are measured at the end of the loop using Crison probes (Crison Instruments, Spain), connected to an MM44 control-transmitter unit (Crison Instruments, Spain), which in turn is connected to a PC control unit, allowing the facility's complete monitoring and control. Each reactor is bubbled at a constant airflow rate of 200 l·min^{-1} while the pH is controlled by the on-demand injection of pure CO_2 at 3 l min^{-1}. The culture temperature is controlled by passing cooling water at 1500 L h^{-1} (when needed, as determined by the computer control) through an internal heat exchanger located in each photobioreactor's bubble column. The reactor was operated in continuous mode by harvesting 20% of the culture volume daily, which was then replaced by fresh medium.

2.3. Biomass concentration and analytical methods

The microalgae biomass concentration was measured by dry weight. We used 100 mL aliquots of the culture filtered through Macherey-Nagel glass fiber MN 85/90. Then, the filters were dried in an oven at 80ºC for 24 h. Standard official methods approved by the Spanish Ministry of Agriculture were used to analyze the composition of the wastewater samples and the water from the reactors (*Ministerio de Agricultura* 1982). The phosphorus was measured by visible spectrophotometry through the phospho-vanado-molybdate complex. Nitrates were quantified at between 220 and 275 nm using a spectrophotometer. Ammonium was measured using the Nessler reactive method. The Chemical Oxygen Demand (COD) was determined by spectrophotometric measurement using Hach-Lange kits (LCl-400).

2.4. Software

The DaqFactory programme (Azeotech, USA) was used to gather the photosynthesis and respiration rate data. Data analysis was carried out using the Statgraphics Centurion XVI software package, in which non-linear regression was used to fit experimental data to the proposed models, and to determine the characteristic parameter values. These models were used to obtain simulations in Microsoft Excel.

3. Results

3.1. Development of the proposed methodology

To order the photo-respirometry methodology, different tests were performed using microalgae-bacteria cultures obtained from sewage. An example of one of these tests is shown in Figure 3. One can observe that during the light phase, the dissolved oxygen level increased rapidly from 100 %Sat to 110 %Sat.; the OPR being 15.89 $mgO_2/L \cdot h$. During the dark phase, the dissolved oxygen level dropped to 98 %Sat.; the OCR being 1.79 $mgO_2/L \cdot h$. From these values, we calculated that the microalgae net photosynthesis rate (MNPR) was 37.3 $mgO_2/g \cdot h$, a normalized value to the biomass dry weight. Another sample of culture was used to determine the heterotrophic bacteria respiration rate (HBRR), which was calculated by adding sodium acetate to the sample after starvation. In this case, the dissolved oxygen level dropped to 93.83 %Sat.; the HBRR being 11.49 $mgO_2/g \cdot h$. Lastly, the nitrifying activity was determined by adding ammonium chloride as the nitrogen source. In these experiments the dissolved oxygen concentration during the dark phase dropped to 96.87 %Sat., corresponding to an NBRR of 7.2 $mgO_2/g \cdot h$.

Table 2 summarizes the overall values determined and the measurements' standard deviation, confirming the reliability of the process. The results confirm the accuracy of the measurements, with the standard deviation being less than 10% of the values obtained. To achieve this, it was critical to perform the experiments in triplicate and to disregard the initial value – the cells' metabolisms are still adapting so these first measurements are always affected by the conditions the cells were previously kept under.

3.2. Determination of optimum light availability and biomass concentration

One of the main factors influencing microalgae behaviour is light availability, and thus the irradiance to which the cells are exposed in the culture. This is determined by the external irradiance and the biomass concentration as well as the culture system's diameter. To determine the optimal irradiance at which the measurements should be taken, experiments were performed using samples from laboratory stirred-tank reactors fed with sewage. These samples were selected because they are the most relevant in terms of the further application of the methodology proposed. Experiments were carried out at a fixed biomass concentration of 0.5 g/L, the external irradiance was modified to achieve the target irradiance inside the sample, measured with the interior sensor. The results show that, at low irradiance values (50 $\mu E/m^2 \cdot s$), the microalgae photosynthesis rate is also low, increasing with light availability up to values of 500 $\mu E/m^2 \cdot s$, and then remaining constant up to values of 2000 $\mu E/m^2 \cdot s$ (Figure 54). Regarding the heterotrophic and nitrifying bacteria, they did not show any relevantly different behaviour whatever the irradiance values imposed. Microalgae activity, on the other hand, was maximal at values of 500 $\mu E/m^2 \cdot s$ but to avoid saturation during photosynthesis, an irradiance of 200 $\mu E/m^2 \cdot s$ was selected for the measurements.

Regarding the biomass concentration, there is a direct relationship between the production/uptake of oxygen and the relative biomass concentration in the cultures. For this reason, determining the optimal biomass concentration at which the measurements should be performed is essential. This variable greatly impacts the method's accuracy and sensitivity. Consequently, experiments were also performed using microalgae cultures from laboratory stirred-tank reactors fed with sewage as the most representative sample type, with distilled water as the control. Measurements were carried out at different biomass concentrations up to 0.8 g/L, determining the three main metabolisms: (i) photosynthesis by microalgae, (ii) respiration by heterotrophic bacteria and (iii) respiration by nitrifying bacteria (Figure 5). The results show that if low biomass

concentration values were used, even as low as 0.1 g/L, the photosynthesis rate was high enough to provide a significant response, far greater than the measurements' standard deviation. The oxygen production rate's standard deviation, based on the photosynthesis rate, was similar whatever the biomass concentration, so values from 0.1 to 0.8 can be used for the standard method. However, the oxygen consumption rate from the respiration measured was much lower than from photosynthesis; it being negligible at biomass concentrations of 0.1 g/L. Only at biomass concentrations of 0.2 g/L was there a measurable oxygen consumption rate from photosynthesis, but the standard deviation was lower than for measurements with biomass concentrations above 0.5 g/L. This is because the homogeneity of light distribution inside the flask reduces with increasing biomass concentration; thus an optimal standard biomass concentration value of 0.5 g/L was obtained for the method.

3.3. Evaluation of the metabolisms prevailing in different cultures

Once the methodology was defined, it could be used to evaluate the metabolism in different samples from a range of different cultures. Therefore, photo-respirometry measurements were carried out to determine the prevailing microalgae-bacteria consortia metabolisms in samples from different culture media and reactors (both in the laboratory and outdoors) (Table 1). The samples were subjected to an inanition period of one to three days, depending on the content of organic or inorganic matter in the culture - one day for urban wastewater, two days for animal manure and three days for leachate from crop residues. A sample from pure microalgae culture was also measured as the "control" in order to determine the differences between pure cultures and wastewater cultures.

The results show that the oxygen production rate from photosynthesis was higher than the heterotrophic and nitrifying activity in all cases, with maximal values being obtained from pure microalgae cultures (Figure 6); especially in *S. almeriensis* culture from spherical flasks (122.9 mgO_2/gbiomass·h). The microalgae net photosynthetic activity from *S. almeriensis* in bubble columns was comparable to the activity in spherical flasks (114.2 mgO_2/gbiomass·h). The photosynthetic activity of *S. almeriensis* in the tubular photobioreactor was likewise very similar to that in culture produced using animal manure in a thin-layer reactor (86.7 and 86.5 mgO_2/gbiomass·h, respectively). Both values were higher than the oxygen production obtained using leachate from crop residues (53.7 mgO_2/gbiomass·h) and urban wastewater in the raceway reactor (32.9 mgO_2/gbiomass·h). The microalgae activity in cultures obtained using sewage as the

nutrient source was similar to that using the laboratory-scale photobioreactor and the external raceway reactor (36.3 and 32.8 mgO_2/gbiomass·h, respectively).

The heterotrophic bacteria respiration rate was very similar in vegetal compost leachate culture (8.9 mg O_2 /gbiomass.h) and animal manure culture (7.6 mg O_2/gbiomass.h), corresponding to the highest heterotrophic activity values measured. The results from the two systems using primary domestic wastewater were comparable, showing that heterotrophic activity was present at a similar level, though slightly lower in the raceway reactor (2.6 mg O_2/gbiomass.h) than in the laboratory reactors (4 mg O_2/gbiomass.h). The respiration rate using *S. almeriensis* culture in the pilot column system (3.8 mgO_2/gbiomass.h) and in the tubular cultures (3.7 mgO_2/gbiomass.h) was similar to that obtained from wastewater, indicating that organic matter removal from wastewater treatment using microalgae was quite efficient. The heterotrophic activity measured in the laboratory cultures (2.3 mgO_2/gbiomass) was the expected response given the symbiotic interaction between the microorganisms in the microalgae cultures.

With regard to performing the nitrifying activity measurements, after the starvation period, it was necessary to check that the nitrogen remaining in the cultures in the form of ammonium was below 2 $mg \cdot L^{-1}$. The results showed that maximal nitrifying activity was obtained using leachate from crop residues as the culture media (7.8 mgO_2/gbiomass·h). The nitrifying activity measured in *S. almeriensis* laboratory cultures was 5.63 mgO_2/g·h, higher than the nitrifying activity measured when different types of wastewater were used. The nitrifying activity was also measured for *S. almeriensis* in tubular reactors (3.2 mgO_2/gbiomass.h). A similar value was obtained when animal manure was used as the substrate (3 mgO2/gbiomass·h). The results for the two systems using primary domestic wastewater are comparable although they show that nitrifying activity was present at a slightly higher level in the laboratory reactors (2.9 mg O_2 /gbiomass.h) than in the external raceway (0.6 mg O_2/gbiomass.h), with both primary domestic wastewaters containing a low ammonium concentration (70 mg/L) diluted by 20-10%, respectively (Figure 6).

4. Discussion

Bacteria have often been considered a contaminant of microalgae cultures. However, the use of microalgae in multiple biotechnology processes, such as in wastewater treatment, have required an understanding of the mechanisms involved in microalgae-bacteria interaction (Fuentes et al., 2016). Knowledge of the microalgae-bacteria consortia which

appear in the treatment of fouled wastewaters (from urban, industrial, agricultural and animal-use sources) is essential to maximise the benefits of microalgae wastewater treatment, as previously reported (Gómez-Serrano et al., 2015; Muñoz and Guieysse, 2006; Olguín, 2012a). Over recent years, studies have focused on how microalgae, through photosynthesis, can convert CO_2 to biomass and produce O_2 to support bacterial growth in wastewater. In turn, the bacteria decompose the organic matter which supplies the CO_2 for photosynthetic activity. Nevertheless, the behaviour model for microalgae-bacteria consortia faces challenges as well (Wang et al., 2016). To develop a method for studying microalgae-bacteria consortia, some authors have tried to adapt the conventional respirometry techniques used in wastewater. By evaluating both the microalgae and nitrifying activity, it has been possible to develop a photo-respirometry protocol to study microalgae-bacteria suspensions (Rossi et al., 2018; Vargas et al., 2016). However, a photo-respirometry method for studying the three relevant populations (microalgae, nitrifying bacteria and heterotrophic bacteria) which appear in wastewater treatment has not been developed.

The results reported here show that a respirometry method based on oxygen production/consumption is a useful and rapid technique. This led us to study the contribution of each population in the microalgae-bacteria consortium, by distinguishing the oxygen production rate (OPR) from microalgae photosynthetic activity, the oxygen consumption rate (OCR) from endogenous respiration, the microalgae net photosynthesis rate (MNPR) in the microalgae, the heterotrophic bacteria respiration rate (HBRR) in the heterotrophic bacteria, and the nitrifying bacteria respiration rate (NBRR) in the nitrifying bacteria. The results from these preview tests, and their variability, were comparable to preview studies focusing on the activity of microalgal-bacterial wastewater consortia using respirometric tests. The oxygen consumption rate (OCR) results from endogenous respiration (3.78 mgO2/gbiomass.h) were quite similar to the results described by Rossi et al. (2018) of 4.3 and 4.1 mg O2/gTSS.h, and were within the range indicated by Ruiz-Martinez et al. (0.9-5.1 mg O2/gTSS); the MNPR and NBRR values being higher than those previously described (Rossi et al., 2018).

To take the measurements properly, it was necessary to subject the culture samples to starvation in order to add different substrates, thus allowing us to distinguish between the respiration rates of the two types of bacteria studied. Heterotrophic biomass (including not only bacteria but also protozoa and other higher organisms) uses substrate consisting of carbonaceous material; therefore, we checked for the absence of organic matter in the

samples after starvation. Nitrifying bacteria, on the other hand, are autotrophic bacteria which use dissolved carbon dioxide to oxidise ammonia to nitrite and nitrite to nitrate. In turn, starvation is applied to consume the residual ammonium in the culture and use ammonium chloride to distinguish the nitrifying activity. We experimentally determined that one day of starvation is required for primary domestic wastewater, two days for animal manure wastewater and three days for lixiviated compost wastewater. Following starvation, photo-respirometry tests were performed at a fixed irradiance of 200 $\mu E/m^2 \cdot s$. As with the preview studies, our results have verified that under real conditions, the cultures are mainly photo-limited, the average irradiance being from 100 to 300 $\mu E/m^2 \cdot s$ (Acién et al., 1998, 1999; Costache et al., 2013). Moreover, the samples were standardized to 0.5 g/L in order to impose comparable light penetration, avoid shaded areas and better distinguish the three populations.

We found that the microalgae activity was significantly higher than the heterotrophic and nitrifying activity in whichever culture medium used, with the contribution of photosynthetic activity being essential in preserving the consortium. Pure microalgae from the spherical flask showed the maximum rate of photosynthesis activity (122.9 $mgO_2/g \cdot h$), analogous to that obtained when using bubble-column reactors as the microalgae production system (114.2 $mgO_2/g \cdot h$). Microalgae activity was similar in samples from the tubular photobioreactor using pure fertilizers to that in the thin-layer reactor fed with pig manure (86.7 and 86.5 $mgO_2/gbiomas \cdot h$, respectively). These results suggest that using pig manure as the microalgae substrate is an excellent alternative method for treating animal manure and producing microalgae biomass. Although the most common way of reusing pig manure is to spread it on farmland, some authors have described using it to produce microalgae biomass (Bai et al., 2012; Wilson and Houghton, 1974). Not only have we been able to demonstrate that pig manure serves as a good microalgae substrate, but the data also show the high microalgae activity achieved from using agricultural leachate wastes as the substrate, with microalgae activity of 53.7 $mgO_2/gbiomass \cdot h$. In the outdoor raceway reactor using primary domestic wastewater, microalgae activity was lower (32.9 $mgO_2/gbiomass.h$), similar to that achieved using the laboratory-scale photobioreactor. As the preview studies reported, this was possible because the thin-layer reactor was more photosynthetically efficient at producing *Scenedesmus sp.* than the raceway reactor and the closed tubular photobioreactor (Acién et al., 2012; Morales-Amaral et al., 2015).

Heterotrophic activity in the activated sludge treatment process has been studied and described for decades because it is responsible for oxidizing the organic material and is capable of forming flocs, which also facilitate effluent clearing (Gayford and Richards, 1970). Accordingly, it is necessary to determine the heterotrophic population which appears in microalgae-bacteria consortia wastewater treatment. Our results show that maximal heterotrophic activity occurred when agricultural waste leachate and pig manure wastewater were used. These results were in agreement with the chemical oxygen demand (COD) values recorded in animal manure and compost leachate, corresponding to 20.200 mg/L and 33.200 mg/L, respectively. However, heterotrophic activity in primary domestic wastewater was lower, similar to the activity in *S. almeriensis* cultures.

Regarding the nitrifying activity, the presence of nitrifying microorganisms in the wastewaters used in this study (agricultural waste leachate, animal manure wastewater and primary domestic wastewater) was determined by their growth in a selective nitrifying media. Preview studies have detected nitrifying bacteria in different materials, such as horticultural waste and sewage sludge, using the same materials and method described (Vargas-García et al., 2010). Microalgae culture from agricultural waste leachate showed the highest nitrifying bacteria respiration rate (7.2 mgO_2/gbiomass·h). Our results were supported by previous studies on agricultural waste composting, which described the presence of ammonia-oxidizing archaea and bacteria; these transform NH3 to NO3 during nitrification. (Zeng et al., 2011). The nitrifying activity measured in *S. almeriensis* laboratory cultures was 5.6 mgO_2/g.h, higher than the nitrifying activity measured when different types of wastewater were used. Preview studies have described that most microalgae culture collections exist in a non-axenic state because other organisms, such as bacteria and microfungi, are present in the culture due to co-insolation (Amaral et al., 2013). This activity was similar to samples from the tubular photobioreactor system, or from cultures grown using pig wastewater in thin-layer cascades, or samples from laboratory photobioreactors fed with primary domestic wastewater. Nitrification in samples obtained from microalgae cultures grown in animal manure have been reported by some authors; this is because nitrogen in the form of ammonia nitrogen is present at very high concentrations in animal manures such as pig waste (Blouin et al., 1990; Evans et al., 1986), with ammonium comprising up to 70% of the nitrogen present in liquid manure (Baumgarten et al., 1999). The animal manure used in this study contained up to 2.97 g NH4/L and it was diluted to 10% for use in the microalgae cultures. These results suggest that the thin-layer reactor was so efficient

because the nitrifying activity was lower than expected. Regarding the laboratory photobioreactors (2.9 mgO_2/g), nitrifying activity of 5.4 mg O_2 /gTSS.h was previously reported in activated sludge with a similar photobioreactors system.

5. Conclusions

The photo-respirometry method developed allows us to quantify the contribution of each of the three main microorganism types that appear in wastewater treatment: microalgae, heterotrophic bacteria and nitrifying bacteria. The method has been applied to microalgae/bacteria consortia established in different wastewater treatment systems (different reactors, water types and operating conditions). The data confirm that microalgae are the main microorganism contributing to the system behaviour, heterotrophic bacteria maintain a relatively stable contribution whatever the operational conditions whereas the nitrifying bacteria contribution largely depends on the nitrogen load and the microalgae performance. This method is a powerful tool for improving the performance of microalgae-based wastewater treatment processes. However, the method needs to be further improved in certain aspects such as searching for specific algal photosynthetic inhibitors, which would help discriminate between microalgae activity and respiration from nitrifying bacteria.

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Table 1. Composition of the waters used as influent in the cultivation systems

	Cultivation systems			
Parameters	Arnon medium	Primary domestic wastewater	Pig manure wastewater	Agricultural waste leachates
pH	7.86	7.60	7.73	-
Conductivity, mS/cm^{-1}	2.33	1.87	16.79	-
Turbidity, FTU	0.00	16.95	7280	1861.00
SST, g/L	0.61	-	11.70	8.24
N-NH_4, mg/L	0.00	59.64	2975.93	3990.00
N-NO_3, mg/L	139.97	2.96	739.74	186.78
P-PO_4, mg/L	-	-	-	819.00
COD, mg/L	-	500	65800	33205

Table 2. Results obtained in preliminary tests showing the method precision. The net photosynthesis rate variability was lower than the obtained rates for bacteria populations because the photosynthetic capacity was greater than the nitrifying and heterotrophic activity on dissolved oxygen values.

Parameters	**Rates**
OPR, mgO_2/L·h	15.89 ± 1.10
OCR, mg O_2/L·h	-1.89 ± 0.12
MNPR, mgO_2/gbiomass·h	37.28 ± 1.46
HBRR, mg O_2/gbiomass·h	-11.49 ± 1.26
NBRR, mg O_2/gbiomass·h	-7.22 ± 0.56

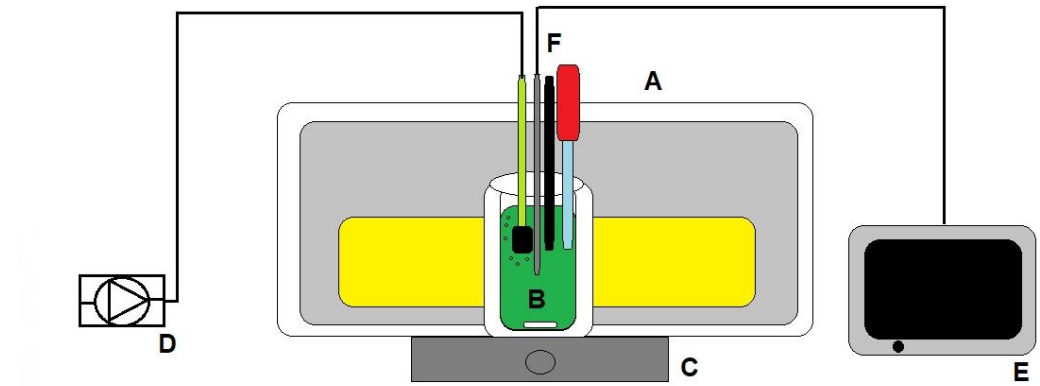

Figure 1. Layout of the respirometer (A: lights, B: 250 mL glass flask, C: magnetic mixer, D: air pump, E: multi-meter and data logger, F: DO probe).

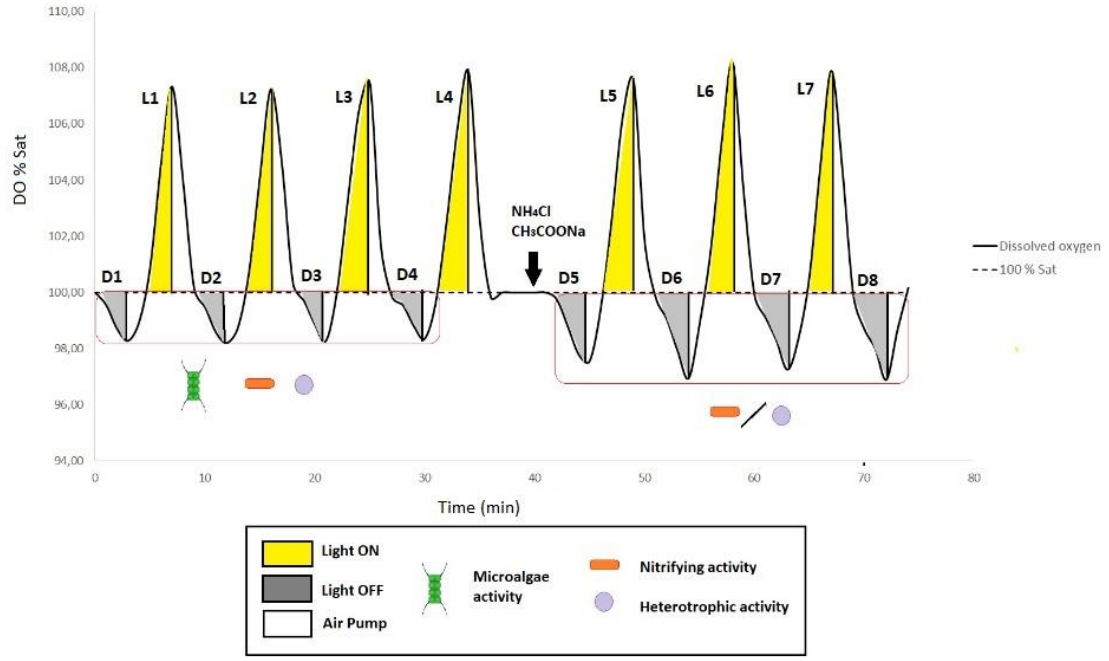

Figure 2. Expected result of a respirometric test to estimate the microalgae net photosynthesis rate (MNPR), the heterotrophic bacteria respiration rate (HBRR) and nitrifying bacteria respiration rate (NBRR). Dark-light periods are reported, showing the variation in dissolved oxygen with time in each of the phases before and after the addition of substrates which activate bacterial populations.

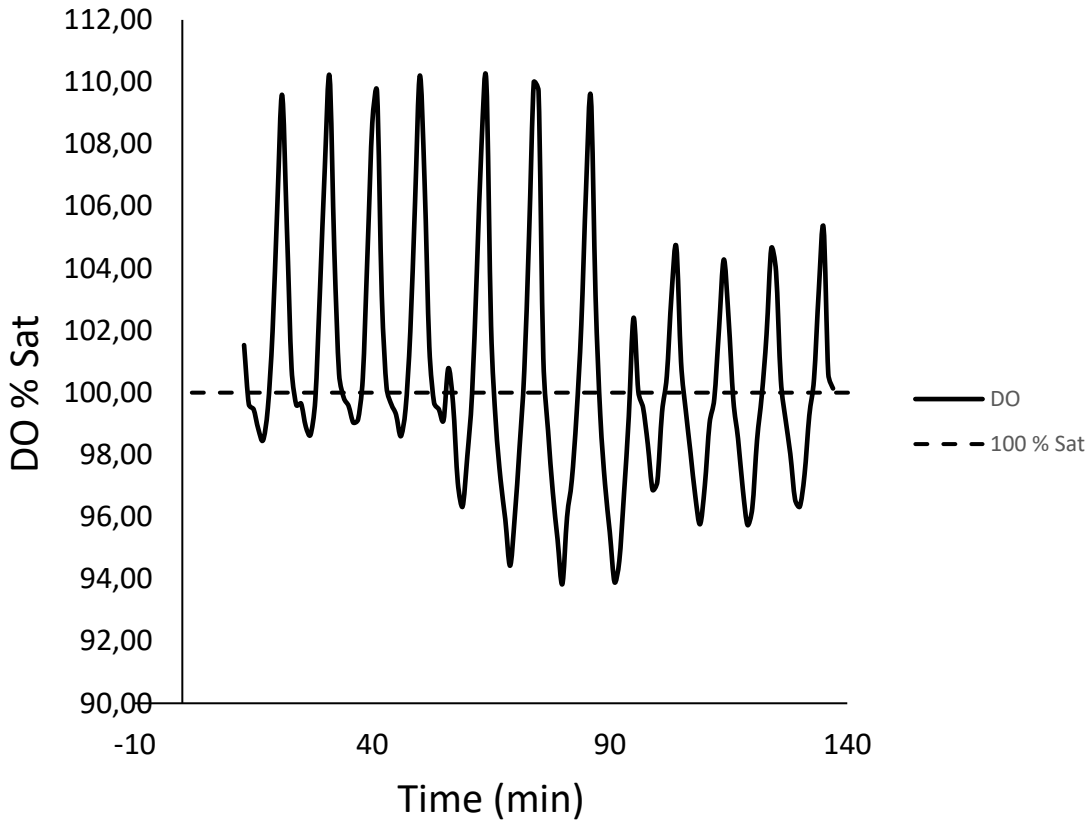

Figure 3. An example of how the dissolved oxygen concentration in the culture modifies in a respirometric test because of the different microalgae and bacteria metabolisms.

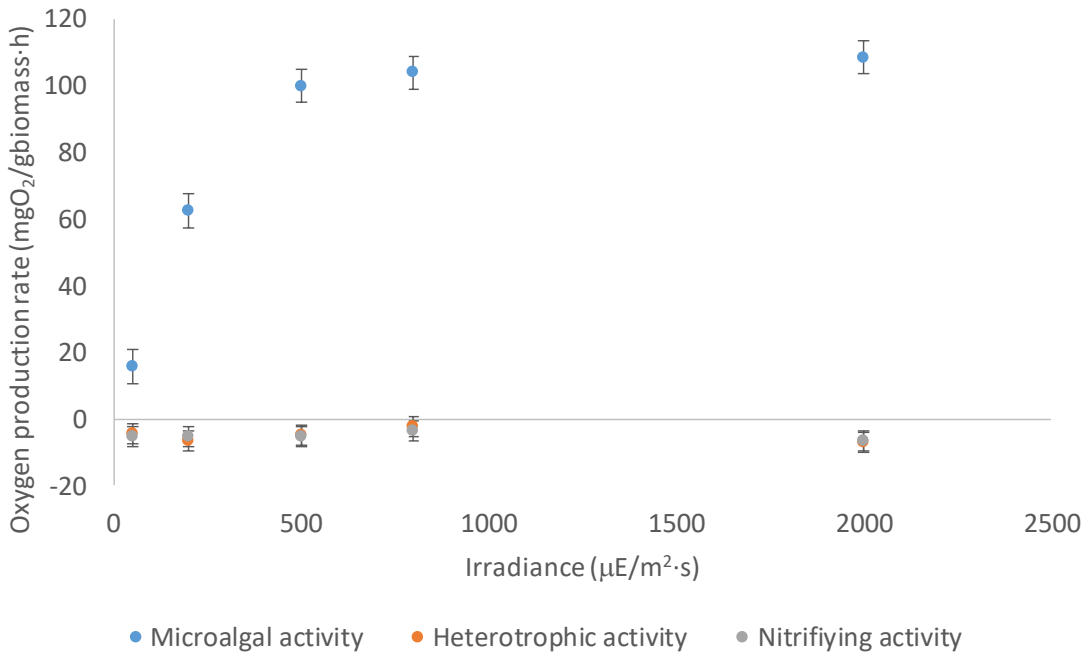

Figure 4. Influence of irradiance on the different net metabolisms considered. Microalgal activity increased with light availability up to values of 500 µE/m^2·s, then remained constant up to values of 2,000 µE/m^2·s.

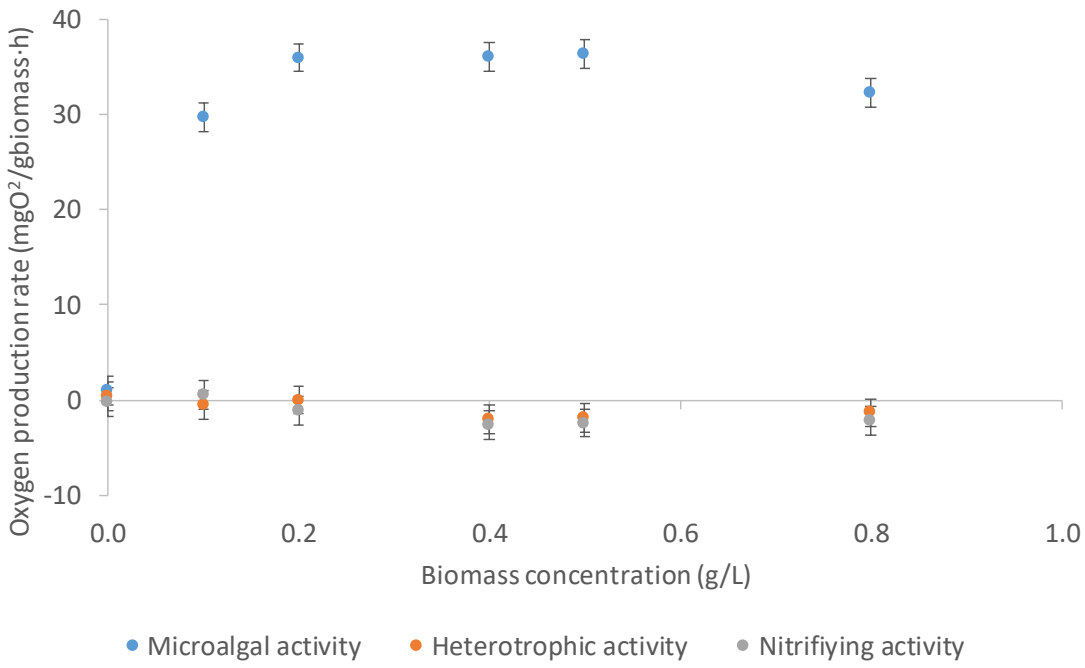

Figure 5. Influence of biomass concentration on the different net metabolisms considered. At low biomass concentration, the variability was higher than the 0.5-0.8 g/L concentration.

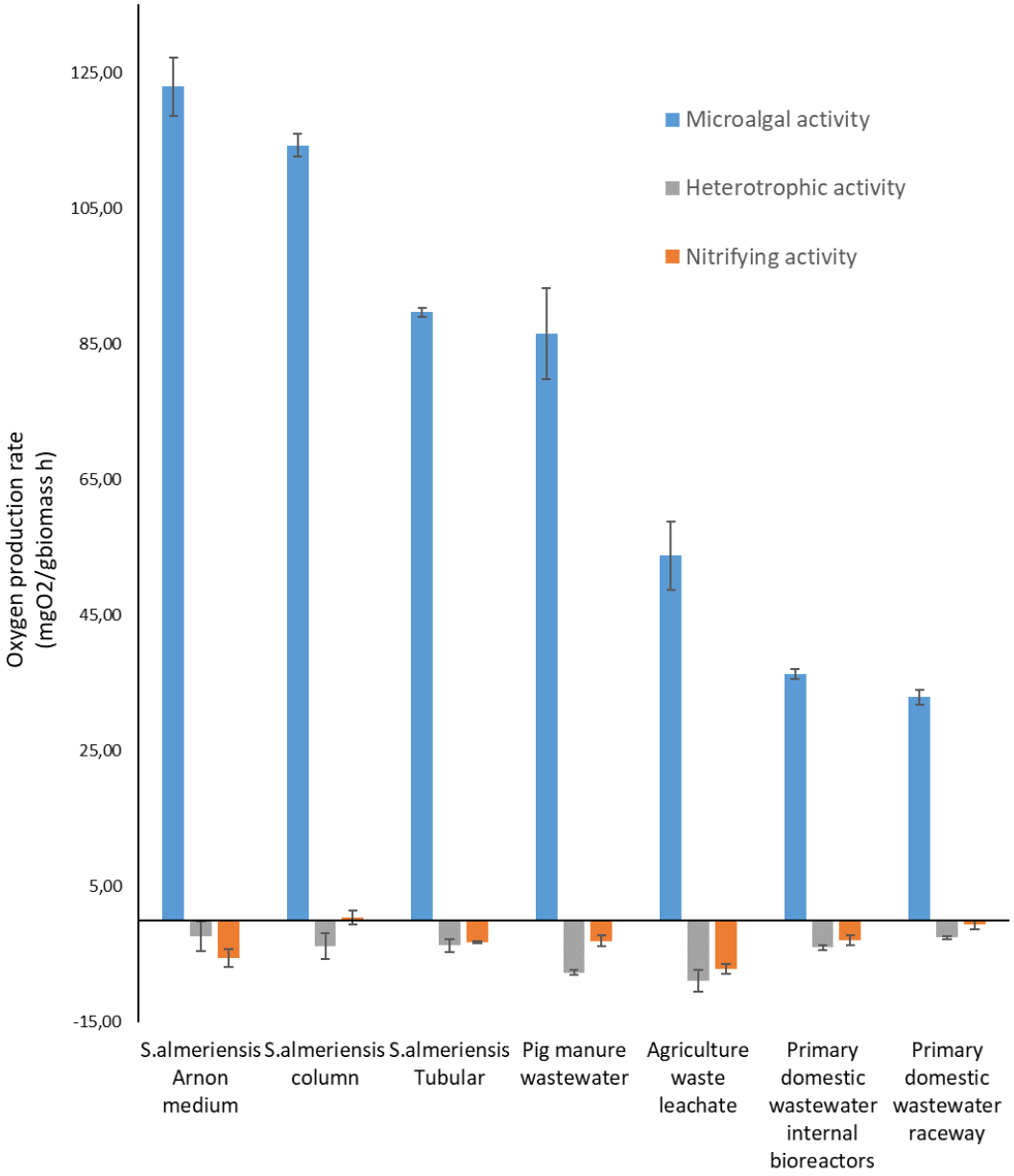

Figure 6.- Distribution of microalgae, nitrifying bacteria and heterotrophic bacteria in pure *S. almeriensis* cultures and in the different wastewaters used. The microalgae activity was greater than the nitrifying and heterotrophic activity in each of the culture mediums used.