

Activity assessment of microalgal-bacterial consortia based on respirometric tests

S. Rossi, M. Bellucci, F. Marazzi, V. Mezzanotte and E. Ficara

ABSTRACT

Respirometric techniques are useful tools to evaluate bacterial activities in activated sludge processes due to their fast execution and the possibility to obtain several kinetic parameters from a single test. Using such techniques in microalgae-bacteria consortia treating wastewater could allow a better understanding of mutual interactions between the microbial populations as a function of environmental parameters. This work aims at developing and testing a novel experimental respirometric protocol to determine oxygen uptake rates and oxygen production rates by a microalgae-bacteria consortium. The defined protocol is characterized by alternating light/dark regimes and by dosing substrates/inhibitors to selectively activate/inactivate microalgal and bacterial metabolisms. The protocol was then applied on microalgal and bacterial consortia, which were grown on the liquid fraction of black water from biogas plants fed on agricultural and municipal waste sludge. Results elucidate the presence and activity of microalgae and nitrifying bacteria in the tested systems, suggesting that the respirometric tests could be included into monitoring procedures of photobioreactors/algal ponds.

Key words | activity assessment, microalgae-bacteria consortium, nitrification inhibition, oxygen mass transfer, respirometry, wastewater treatment

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INTRODUCTION

Biological wastewater treatment with microalgae has gained an increasing interest in the last decades, thanks to the capacity of microalgae to uptake nutrients (ammonia, nitrate and phosphate), to produce bio-fuels and to fix inorganic carbon (Chisti 2007). One of the major advantages of using microalgal-based wastewater treatment is that microalgae produce oxygen with their photosynthetic activity, and the produced oxygen is used by autotrophic bacteria to oxidize ammonia nitrogen. Indeed, the synergism between nitrifying bacteria and microalgae was reported by several authors (Godos *et al.* 2009; González-Fernández *et al.* 2011). The use of microalgal-bacterial (MB) consortia could decrease the operational costs connected to aeration in aerobic wastewater treatment.

Respirometric and titrimetric techniques are useful tools to determine the optimal growth conditions for bacteria in activated sludge systems and have been applied to various biological systems (Spanjers & Vanrolleghem 1995; Munz *et al.* 2009). By means of these techniques, the oxygen consumption of bacterial suspensions can be quantified, from which bacterial activity is then computed. In addition,

respirometry can be successfully applied to characterize influent wastewaters, to identify potential inhibitory effects and to evaluate kinetic and stoichiometric parameters (Vanrolleghem 2002). The knowledge of these parameters is essential for the calibration and validation of mathematical models, describing the behaviour of biological processes, such as the activated sludge models (Henze *et al.* 2000).

The application of respirometric tests to algal-bacterial suspensions could represent a powerful alternative to determine the activity and the suspended solids apportioning among the mass of relevant microbial components. This information allows for a better knowledge and understanding of wastewater-treating photobioreactors/ponds. Nonetheless, to date, the application of respirometric/titrimetric techniques for the characterization of MB suspensions is scarce. Some authors used respirometry for evaluating the effect of different environmental factors, such as light regimes and intensity (Brindley *et al.* 2010), pH, bicarbonates or nutrient sources (Decostere *et al.* 2013, 2016; Tang *et al.* 2014). Respiratory activity tests were also performed to assess the photosynthetic activity or the dark respiration of microalgal cultures (Kliphuis

et al. 2011; Ruiz-Martinez et al. 2016). However, a lack of uniformity in units and procedures makes the comparison of data and protocols quite difficult. Previous works generally refer to different test conditions or experimental devices; significant discordance can be particularly found regarding: light availability (light intensity and light/dark phase durations), biomass characteristics (microalgae strains and initial biomass concentrations), environmental conditions (nutrient and inorganic carbon sources and availability, pH and temperature range) and respirometric devices (volume and geometry of bioreactors). Moreover, except for a few cases (Vargas et al. 2016), respirometric procedures are generally applied to pure microalgal cultures, in the absence of bacteria, which makes those procedures not directly applicable to MB reactors. This work aims at developing a respirometric device and a standardized procedure to study MB suspensions, by evaluating both microalgal and nitrifying activity during the test. To the extent of the authors' knowledge, this combined approach has never been applied before to MB consortia. Specifically, the respirometric protocol was tested on MB consortia grown on agro-zootechnical and municipal black waters.

MATERIALS AND METHODS

Microalgae cultivation

MB suspensions were collected from three microalgal culturing systems (a laboratory-scale system, a pilot-scale bubble-column, a pilot-scale raceway), briefly described below.

The laboratory-scale cultivation system was composed of four glass cylinders (hereafter referred to as 'Lab-C'), with a working volume of 2 L each. Lab-cylinders were operated semi-continuously, with an average hydraulic retention time (HRT) of 20 d and were fed on the liquid fraction of anaerobically digested agro-wastewaters (hereafter referred to as 'black water', BW). During Lab-C cultivation, BW was diluted twice with tap water, due to high nutrient contents. Every cylinder was equipped with an aeration system for mixing and CO₂-supply. Lab-cylinders were exposed to artificial fluorescent lights (OSRAM Fluora, 2 × 30 W), providing a photosynthetically active radiation (PAR) of 68 ± 17 mE m⁻² s⁻¹ with a 12 h/12 h light/dark cycle. The average room temperature was about 24 °C. The MB culture was dominated by *Chlorella* and *Scenedesmus* spp., as confirmed by optical microscopy observations.

The pilot-scale bubble-column (hereafter referred to as 'Pilot-C') operated outdoors and was characterized by a

working volume of 75 L and a diameter of 29 cm. The reactor was fed on the same BW described above, without applying dilutions. Feeding was continuous with an HRT of 20 d and CO₂ was supplied to the culture by means of an air compressor, bubbling air at the bottom of the column. Air bubbling also provided adequate mixing to the suspension. The MB culture was dominated by *Chlorella* and *Scenedesmus* spp.

The pilot-scale raceway was an open pond reactor (hereafter referred to as 'Pilot-RW'), operating in outdoor conditions. The system was characterized by a working volume of about 1 m³, a surface of 5.8 m² and a water depth of 18 cm. Mixing was provided by a paddle-wheel, and a pH-controlled injection of off-gas from the combined heat and power unit was used to transfer CO₂ to the culture. No temperature regulation was implemented. The reactor was fed first in batch and then continuously, with an HRT of 10 d. The feed was the BW of the anaerobically digested waste sludge of a municipal wastewater treatment plant. MB samples observed under the microscope contained *Chlorella*, *Chlamydomonas* and *Scenedesmus* spp., but the dominant algal species in the culture were *Chlorella*.

The average composition of influent wastewaters is reported in Table 1.

Data in Table 1 indicate that all cultivation systems were fed with quite concentrated wastewater, especially in ammonia nitrogen and organic matter (although the latter is expected to consist of recalcitrant organics that the anaerobic process could not convert into biogas). For this reason, bacteria were also expected to develop together with

Table 1 | Composition of the black waters used as influent to the cultivation systems (values are expressed as mean ± standard deviation, N is the number of measurements, Pilot-C is the pilot-scale bubble-column bioreactor, Lab-C is the laboratory-scale cultivation system, Pilot-RW is the pilot-scale raceway reactor)

Parameter		Cultivation system		
		Pilot-C	Lab-C	Pilot-RW
NH ₄ ⁺	mg N L ⁻¹	251 ± 86	134 ± 47	244 ± 79
NO ₃ ⁻	mg N L ⁻¹	8.6 ± 2.9	4.3 ± 1.1	0.2 ± 0.3
PO ₄ ³⁻	mg P L ⁻¹	19.8 ± 8.2	9.1 ± 4.4	5.7 ± 0.8
Soluble chemical oxygen demand	mg O ₂ L ⁻¹	362 ± 153	271 ± 216	122 ± 54
Turbidity	FAU	137 ± 176	76 ± 91	68 ± 48
TSS	mg TSS L ⁻¹	126 ± 119	54 ± 62	80 ± 40
OD ₆₈₀	-	0.16 ± 0.15	0.09 ± 0.06	0.07 ± 0.14
pH	-	7.9 ± 0.2	7.8 ± 0.5	8.1 ± 0.3
Conductivity	mS cm ⁻¹	3.2 ± 0.3	2.7 ± 0.7	1.5 ± 0.3
N	-	36	12	31

microalgae. Indeed, nitrifying consortia easily developed in the bioreactors, as confirmed by high concentrations of oxidized N-compounds (NO_3^- and NO_2^-) measured in the algal suspensions (data not shown). The presence of high concentrations of heterotrophic bacteria (HB) was not expected, since the organic load in BW is generally constituted by recalcitrant or scarcely biodegradable substances, as reported by Akhiar *et al.* (2017). High turbidities, associated with the presence of suspended solids, were sporadically measured in the BW, due to malfunctioning in the dewatering step obtained by centrifuging.

Respirometric equipment and procedure

A simple *respirometer* was set up (Figure 1) to perform respirometric assays, consisting of: a bioreactor (250 mL transparent glass flask) which was covered in aluminium foils during dark phases; a dissolved oxygen (DO) probe (Hach-Lange, LDO101) connected to a multi-meter device (Hach-Lange, HQ40D) recording both DO and temperature data (2 data min^{-1}); an aeration system including an air pump (KW, Mouse 2-Beta, 110 NL h^{-1}) and a porous diffuser; a lighting system made of fluorescent lights (OSRAM Fluora, $2 \times 30 \text{ W}$); and a magnetic mixing system (VELP Scientifica, Microstirrer), operated at a fixed revolution speed of 150 rpm to minimize the oxygen mass transfer.

During the respirometric test, the following solutions were used:

- Substrates solution (SS): 9.5 g $\text{NH}_4\text{Cl L}^{-1}$, corresponding to 3.2 g $\text{NH}_4^+\text{-N L}^{-1}$; 12.3 g $\text{NaNO}_2 \text{ L}^{-1}$ corresponding to 8.2 g $\text{NO}_2^-\text{-N L}^{-1}$, which was dosed in order to achieve an ammonium and nitrate concentration of 10 mg N L^{-1} .

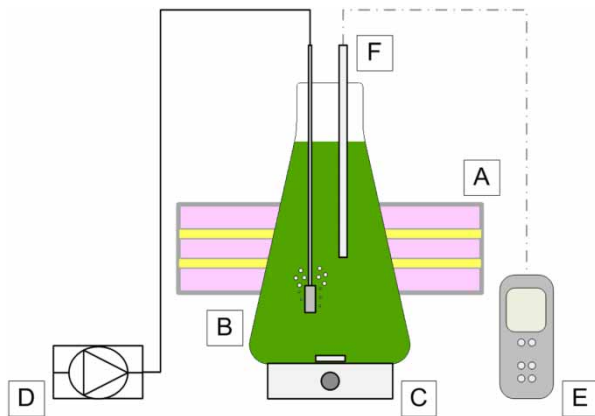


Figure 1 | Lay-out of the respirometer (A: lights, B: 250 mL glass flask, C: magnetic mixer, D: air pump, E: multi-meter and data logger, F: DO probe).

- Inhibitors solutions (IS): allylthiourea (ATU) solution (IS_1 : 2.5 g ATU L^{-1}), chlorate solution (IS_2 : 60 g $\text{KClO}_3 \text{ L}^{-1}$), which were dosed in order to achieve ATU concentration of 10 mg L^{-1} and a chlorate concentration of 1.2 g L^{-1} , known to cause the complete inhibition of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), respectively (Hynes & Knowles 1985; Ginestet *et al.* 1998). Inhibitors of AOB and NOB were dosed simultaneously because preliminary analysis demonstrated that reliable results on the sole NOB activity were difficult to be obtained, probably due to both their limited occurrence in the MB suspensions (as suggested by frequent NO_2^- accumulation) and their limited specific oxygen request compared to AOB.

The sum of injected volumes (10.5 mL) represents an increase lower than 5% in the final respirometer volume. Therefore, dilution correction factors were not considered during calculations, since the volume increase was considered negligible.

To perform respirometric tests, the following *protocol* was set up. First, an aliquot (250 mL) of MB suspension was sampled from the cultivation system. The optical density at the wavelength of 680 nm (OD_{680}) was measured before each test and adjusted to a fixed interval (from 0.4 to 0.6) to obtain comparable light penetration. Therefore, when necessary, a mineral medium was used to dilute the original suspension. The composition of the mineral medium was defined in order to mimic the ionic composition of the original MB samples, thus avoiding the exposure of MB consortia to osmotic shocks. The diluted MB suspension was transferred into the respirometer bioreactor.

Then DO data collection was started. Along the test duration, environmental conditions (light conditions and the presence/absence of specific substrates and/or inhibitors) were modified, identifying different phases (L_i are light phases, D_i are dark phases).

Phase durations were set to 15 min for light phases and 10 min for dark phases, according to Brindley *et al.* (2010). The duration of the first light phase was set to 30 min to let the consortium acclimate to respirometer conditions. Hereafter each phase is described, including environmental conditions and expected biological reactions affecting the DO concentration:

- L_1 : Light on, addition of SS. Oxygen production is expected due to active microalgal photosynthesis; a contribution to oxygen consumption is also expected due to the aerobic activity of nitrifying bacteria (hereafter referred to as 'NIT', including both AOB and NOB), of

HB and of microalgae (MA) respiration; therefore, the net oxygen production rate of this phase (OPR_{L1}) is made of the following contributions:

$$OPR_{L1} = OPR_{MA,P} + OPR_{MA,R} + OPR_{HB} + OPR_{NIT}$$

where $OPR_{MA,P}$ is the photosynthetic MA oxygen production, $OPR_{MA,R}$ is the respiration by microalgae, OPR_{HB} is the oxygen production by heterotrophs (negative), OPR_{NIT} is the oxygen production by nitrifiers (negative).

- D_1 : Light off. Oxygen consumption by MA respiration, NIT activity and HB activity; therefore:

$$OPR_{D1} = OPR_{MA,R} + OPR_{HB} + OPR_{NIT}$$

- D_2 : Light off, addition of IS_1 and IS_2 . NIT are inhibited and the oxygen consumption is only attributed to MA respiration and HB activity; therefore:

$$OPR_{D2} = OPR_{MA,R} + OPR_{HB}$$

- L_2 : Light on. Oxygen production due to MA photosynthetic activity is expected and simultaneous oxygen consumption by MA respiration and HB activity; therefore:

$$OPR_{L1} = OPR_{MA,P} + OPR_{MA,R} + OPR_{HB}$$

- D_3 : Same conditions as D_2 . Oxygen consumption by MA respiration and by HB activity; therefore:

$$OPR_{D3} = OPR_{MA,R} + OPR_{HB}$$

The typical output of a respirometric test and its phases are reported in Figure 2, in which the DO profile and the calculated OPR for each phase are plotted against time. Under the graph, a simplified representation of the activity/inactivity of the main microbial populations is reported.

Then the test was ended and DO data were retrieved and used to compute the OPR (positive/negative when a net production/consumption was observed) in each phase, according to the following *data processing* procedure. Experimental DO data were fitted by a model considering the coexistence of oxygen mass transfer, due to mixing, and the biological DO consumption/production rates, as follows:

$$\frac{dDO}{dt} = k_L a^* (DO_{SAT} - DO) + OPR \quad (1)$$

where: dDO/dt is the variation of DO over time [$mg\ O_2\ L^{-1}\ h^{-1}$], $k_L a$ is the global oxygen mass transfer coefficient [h^{-1}], DO_{SAT} is the oxygen saturation concentration at the actual temperature [$mg\ O_2\ L^{-1}$], DO is the actual DO concentration in the bioreactor [$mg\ O_2\ L^{-1}$], OPR is a constant oxygen production rate [$mg\ O_2\ L^{-1}\ h^{-1}$]. The minimum mean square error criterion was used to assess the optimal fit. The $k_L a$ coefficient was evaluated during abiotic tests in clean water (data not shown), implementing the log-deficit procedure as described in ASCE (1993). A final value of $0.9\ h^{-1}$ was calculated and used for OPR determinations.

OPR values of each phase (OPR_{L1} , OPR_{L2} for the two light phases; OPR_{D1} , OPR_{D2} , OPR_{D3} for the three dark phases) were determined, which depend on the microbial

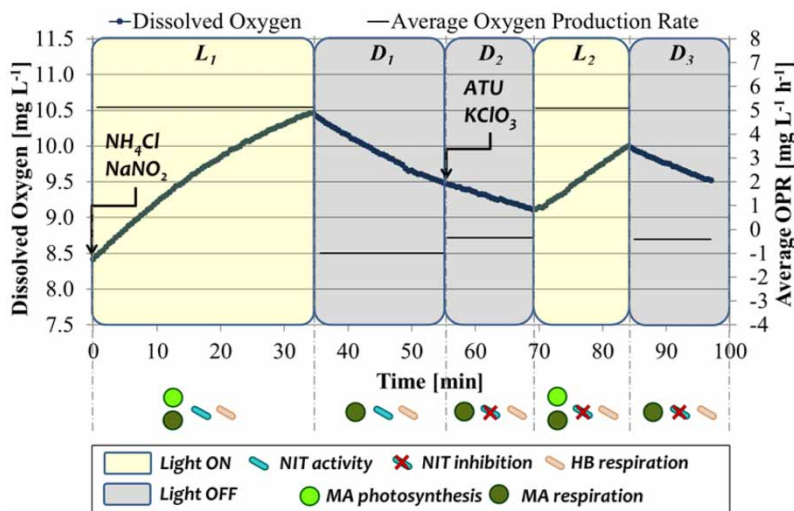


Figure 2 | Typical result of a respirometric test. Phases are reported together with the addition of substrates or inhibitors and the active or inactive populations.

oxygen uptake/consumption as follows:

$$\text{OPR}_{\text{MA,P}_1} = \text{OPR}_{\text{L}_1} - \text{OPR}_{\text{D}_1} \quad (2)$$

$$\text{OPR}_{\text{MA,P}_2} = \text{OPR}_{\text{L}_2} - \text{OPR}_{\text{D}_2} \quad (3)$$

$$\text{OPR}_{\text{MA,P}_3} = \text{OPR}_{\text{L}_2} - \text{OPR}_{\text{D}_3} \quad (4)$$

$$\text{OPR}_{\text{NIT}_1} = \text{OPR}_{\text{D}_1} - \text{OPR}_{\text{D}_2} \quad (5)$$

$$\text{OPR}_{\text{NIT}_2} = \text{OPR}_{\text{L}_1} - \text{OPR}_{\text{L}_2} \quad (6)$$

All the OPR values are expressed as $\text{mg O}_2 \text{ L}^{-1} \text{ h}^{-1}$. From Equations (2)–(6), various estimates were obtained for both microalgal photosynthetic oxygen production ($\text{OPR}_{\text{MA,P}_1}$, $\text{OPR}_{\text{MA,P}_2}$; $\text{OPR}_{\text{MA,P}_3}$) and the nitrifiers' oxygen consumption ($\text{OPR}_{\text{NIT}_1}$, $\text{OPR}_{\text{NIT}_2}$). Mean values were then calculated ($\text{OPR}_{\text{MA,P,mean}}$, $\text{OPR}_{\text{NIT,mean}}$). $\text{OPR}_{\text{MA,P}_1}$ and the mean value of $\text{OPR}_{\text{MA,P}_2}$ and $\text{OPR}_{\text{MA,P}_3}$ were compared by means of heteroscedastic T-tests (computed using data analysis toolbox in Microsoft Office Excel 2016) to exclude the possibility of short-term inhibition effects of ATU and chlorate on MA. The dark oxygen consumption including MA and HB respiration was also obtained, although it gives an aggregated information (OPR_{R^*}). OPR values were corrected by using a temperature-dependent function to compare results obtained under laboratory conditions with results calculated for the pilot-scale bioreactor. OPRs obtained for NIT were corrected with an Arrhenius-type function, as described in the activated sludge models (Henze *et al.* 2000). The OPR for MA was corrected using the temperature correction suggested in Solimeno (2017).

Finally, specific OPRs (sOPRs), expressed as $\text{mg O}_2 \text{ g TSS}^{-1} \text{ h}^{-1}$, could be calculated by dividing the OPR values by the concentration of total suspended solids (TSS) in the suspension:

$$\text{sOPR}(t) = \frac{\text{OPR}(t)}{\text{TSS}(t)} \quad (7)$$

Experimental plan

Preliminary tests were conducted on samples taken from Lab-C and Pilot-C over three consecutive days to assess the repeatability of the described methodology. Pilot-C samples were transported from the pilot plant in white plastic bottles and tested within 2–3 hours, while Lab-C samples were tested immediately after sampling.

After preliminary tests, the described procedure was used to monitor the Pilot-RW system during a 3-month continuous operation (June–August). A respirometric test was

performed every second week (seven tests) to follow the activity of the MB consortium and to evaluate the robustness of the method, by comparing the respirometric results to the parameters (nitrification rates and microalgal growth rates) calculated for the pilot plant from conventional monitoring analyses (nitrogen forms, OD_{680} and TSS trends, data not shown).

Analytical methods

Soluble chemical oxygen demand, ammonia, nitrate- and nitrite-nitrogen and phosphate-phosphorus were determined using spectrophotometric test kits (Hach-Lange, LCK 314, LCK 303, LCK 339, LCK 342 and LCK 348, respectively) on filtered samples ($0.45 \mu\text{m}$). TSS were determined according to standard methods (APHA 2005). Optical density at 680 nm and turbidity were measured in 1 cm and 5 cm cuvettes respectively, by a spectrophotometer (Hach-Lange, DR 3900). pH and DO were measured by a portable multi-meter (Hach-Lange, HQ40D). Microalgae were counted using an optical microscope (Optika, B 350) at $40\times$ magnification.

Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) is a molecular technique that uses fluorescently labelled oligonucleotide probes, which target the 16S rRNA, to detect specific organisms directly into their environmental matrix (Nielsen *et al.* 2009). In this study, it was used to confirm the presence of AOB in MB suspensions. FISH was carried out on samples collected and stored in ethanol (sample/ethanol ratio 1:1 [v/v]) at 20°C prior to fixation, which was performed using 4% paraformaldehyde fixative solution as described by Amann *et al.* (1990) and Bellucci & Curtis (2011). Hybridization was performed with a mix of Cy3 labelled probes and competitors targeting AOB (Nso 1225, NEU, CTE, 6a192, c6a192), according to a protocol described in Bellucci & Curtis (2011). Hybridized MB samples were then visualized under a fluorescent microscope (Zeiss, Axioskop HBO 50) and recorded.

RESULTS AND DISCUSSION

Preliminary tests

Test conditions and main results obtained for Lab-C and for Pilot-C are reported in Table 2. Samples were collected from

Table 2 | sOPR results obtained in preliminary tests on samples collected in Lab-C and Pilot-C (tests were performed in triplicate, results are expressed as mean \pm standard deviation; PAR is the photosynthetically active radiation, sOPR is the specific oxygen production rate). Italic results represent the main output of the respirometric tests

sOPR [mg O ₂ g TSS ⁻¹ h ⁻¹]	Lab-C	Pilot-C
	OD ₆₈₀ = 0.43 \pm 0.02 PAR = 59.2 \pm 3.0 μ E m ⁻² s ⁻¹	OD ₆₈₀ = 0.58 \pm 0.01 PAR = 40.8 \pm 6.7 μ E m ⁻² s ⁻¹
sOPR _{NIT_1}	-4.8 \pm 4.1	-7.2 \pm 8.8
sOPR _{NIT_2}	-6.0 \pm 2.2	-7.8 \pm 5.9
sOPR _{NIT_mean}	-5.4 \pm 2.9	-7.5 \pm 7.0
sOPR _{MA,P_1}	11.7 \pm 3.0	14.1 \pm 2.7
sOPR _{MA,P_2}	12.9 \pm 2.1	14.7 \pm 5.3
sOPR _{MA,P_3}	12.4 \pm 2.2	10.0 \pm 0.6
sOPR _{MA,P_mean}	12.3 \pm 1.9	12.9 \pm 2.0
sOPR _{R*}	-4.3 \pm 0.7	-4.1 \pm 0.9

each plant within 3 days to minimize variations in the MB community and used to perform respirometric tests.

The nitrifying activity could be calculated by both Equation (2) and (3). The results of the two systems are comparable and show that NIT activity is present at a similar level, though slightly higher in the pilot column (7.5 mg O₂ g TSS⁻¹ h⁻¹) than in laboratory reactors (5.4 mg O₂ g TSS⁻¹ h⁻¹). Nonetheless, by taking into account the quite high standard deviation, these two values cannot be considered statistically different. This was verified by applying a heteroscedastic T-test including both sOUR_{NIT_1} and sOUR_{NIT_2} (6 data per set: sOUR: specific oxygen uptake rate). A *p*-value of 0.16 was obtained, suggesting that the difference in the NIT activity in the two culturing systems was not significant at a significance level of 0.05. As stated before, the presence of NIT in the MB community of both Lab-C and Pilot-C culturing systems was confirmed by the presence of oxidized nitrogen forms (both as nitrite and nitrate) in the algal suspension. In contrast, higher variability was found for results from the Pilot-C, probably associated with possible slight alterations (considering the short time interval) in the outdoor conditions, which may have affected the microbial consortium.

The gross photosynthetic activity was very similar for the Lab-C samples (12.3 mg O₂ g TSS⁻¹ h⁻¹) and the Pilot-C (12.9 mg O₂ g TSS⁻¹ h⁻¹), corresponding to 5.1 and 6.4 mg O₂ L⁻¹ h⁻¹ respectively, which is comparable to the results obtained by Decostere et al. (2013) and Decostere et al. (2016) in which the gross OPR was found to be lower than 10.4 mg O₂ L⁻¹ h⁻¹. By comparing OPR_{MA,P_1} to the mean value of OPR_{MA,P_2} and OPR_{MA,P_3}, the potential inhibition of ATU and chlorate to microalgae photosynthetic activity could be

assessed. No significant difference was found by applying a paired T-test (*p*-level = 0.39); therefore no short-term inhibition could be demonstrated. Therefore, nitrification inhibitors (allylthiourea and potassium chlorate) used during the test were proven to not affect the microalgal photosynthetic activity.

Results about algal respiration during the dark phases (OPR_{R*}) were very similar in the two sets of experiments (4.3 and 4.1 mg O₂ g TSS⁻¹ h⁻¹ for Lab-C and Pilot-C, respectively). Microalgal dark respiration rates were consistent with those indicated by Ruiz-Martinez et al. (2016) (0.9–5.1 mg O₂ g TSS⁻¹ h⁻¹). The variability was generally lower than that recorded for bacterial activity. This may be due to the stronger effect of photosynthesis compared to nitrification on DO data, which makes the photosynthetic activity easier to discriminate from the oxygen mass transfer (see Equation (1)). Reducing the relevance of the oxygen mass transfer compared to the biological oxygen production/consumption is therefore a desirable improvement.

As previously mentioned, the endogenous respiration of heterotrophic biomass cannot be computed, and it is eventually embedded in the measurement of the OPR_{R*}.

Overall, the similar feed and HRT applied to the two culturing systems promoted the development of similar MB communities in terms of sOPR_{NIT}, sOPR_{MA}, and sOPR_{R*}.

Monitoring tests

In this second experimental campaign, the respirometric protocol was applied to monitor the Pilot-RW. A selection of operational data for the Pilot-RW is reported in Table 3. As suggested by Marazzi et al. (2017), average values of the 4 days preceding the day of test were considered for irradiance and temperature. Relevant variations of OD₆₈₀ were observed, as a consequence of the changing environmental conditions. Similarly, a quite high variability was observed

Table 3 | Environmental conditions and operating parameters in the Pilot-RW in relation to the respirometric tests

Test	Date	OD ₆₈₀ [-]	Temperature [°C]	PAR [μ E m ⁻² s ⁻¹]	NH ₄ ⁺ [mg N L ⁻¹]	NO ₂ ⁻ [mg N L ⁻¹]	NO ₃ ⁻ [mg N L ⁻¹]
1	31/05/17	0.62	24.2	1520	33.0	10.9	3.1
2	14/06/17	0.71	25.5	1000	80.9	16.4	2.0
3	28/06/17	1.05	21.9	672	60.9	107	12.0
4	12/07/17	1.37	24.9	1290	43.2	109	7.1
5	26/07/17	1.41	24.1	1320	65.9	145	12.0
6	04/08/17	1.68	30.7	1340	60.9	157	12.4
7	25/08/17	1.79	25.9	1120	40.2	146	13.2

in the oxidized nitrogen forms, though constant ammonium concentrations were observed.

Respirometric tests were performed on the MB suspension after dilution (applied dilution factors: 1–2.9) to a standardized OD_{680} of 0.63 in order to improve repeatability.

The sOPRs detected by respirometric tests are summarized in Figure 3. As for the MA, $sOPR_{MA,P,mean}$ is reported together with the standard deviation for the three measures (i.e. computed from $OPR_{MA,P,1}$, $OPR_{MA,P,2}$, $OPR_{MA,P,3}$). A generally satisfactory reproducibility was obtained, especially for tests 2–6, with an average coefficient of variation of 10.7%. The precision of the method for the mentioned experiments is similar to that calculated by Brindley et al. (2010) for determining microalgal photosynthetic rates, where values of 2.8–7.6% were obtained during repeatability tests. Average standard deviation of 8–10% was observed by Rozzi et al. (2003) while applying respirometric tests on HB in activated sludge samples using three different respirometric protocols. Results are also consistent with the study of Ricco et al. (2004), in which coefficients of variation of 2–10% were determined during toxicity assessments for xenobiotic compounds in a closed respirometer. The specific oxygen production is quite constant during the whole experimental period with a significant decrease in test 7. The test was executed at the end of the summer, when the outdoor irradiance and temperature were lower than in July and August.

The specific oxygen consumption during the dark phase ($sOPR_{R^*}$) varied significantly over time (2–19 $mg\ O_2\ g\ TSS^{-1}\ h^{-1}$). During the first two tests the highest results were recorded, later microalgal dark respiration decreases reaching stability around 2 to 5 $mg\ O_2\ g\ TSS^{-1}\ h^{-1}$. This is consistent with the values obtained by Ruiz-Martinez et al. (2016). The initial higher respiratory activity during test 1 and 2 might be explained with a fast microalgal growth

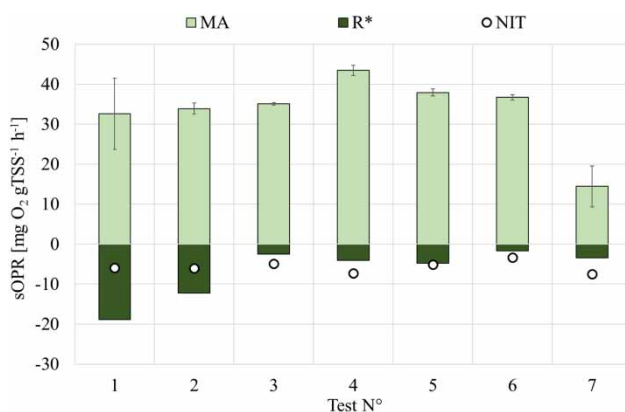


Figure 3 | Average sOPR measured during Pilot-RW monitoring tests.

rate, resulting in an increased energy requirement, as suggested by Kliphuis et al. (2011). It should be considered that algal respiration may also include heterotrophic oxygen uptake, although its contribution to the overall oxygen consumption is not expected to be relevant because of the recalcitrant nature of the organic matter in the BW that was used to feed the raceway.

The OPR of microalgae during light phases was 10–25 $mg\ O_2\ L^{-1}\ h^{-1}$, slightly higher than that obtained by Decostere et al. (2013), probably because of the different operating conditions applied. However, results are consistent with that obtained for *Chlorella vulgaris* by Tang et al. (2014), under similar conditions. The gross specific oxygen production rate ($sOPR_{MA,P,mean}$) faced an initial increase (from about 33 to 44 $mg\ O_2\ g\ TSS^{-1}\ h^{-1}$), then decreased in the last test. The photosynthetic activity of MA detected by the respirometric assay could not be directly linked to any calculated parameter in the open pond. However, comparisons of the obtained data with the OD_{680} variation rate ($rOD_{680} = \Delta OD / \Delta t, h^{-1}$), which is commonly related to the concentration of microalgal biomass (Figure 4), suggest that the respirometric assay is a reliable tool to follow the evolutionary trend of microalgae activity in the photobioreactor. The few discrepancies could be justified by the fact that the microalgae growth rate and OPR values are influenced by other operating and environmental parameters that varied during the course of the experimentation, such as the light/dark daily cycle.

The sOPR for nitrifiers reported in Figure 5 suggests that the activity of nitrifying bacteria was substantially stable in Pilot-RW from test 3 onward. This is in agreement with the stable concentration of NO_x ($NO_2^- + NO_3^-$) measured in the Pilot-RW (see Table 3).

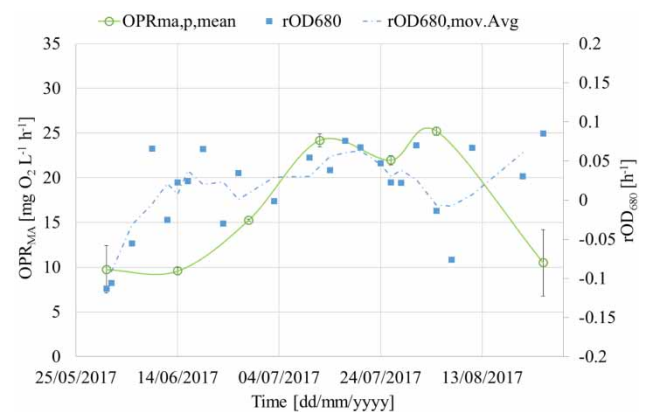


Figure 4 | Calculated microalgal oxygen production rates using the respirometric protocol ($OPR_{MA,P,mean}$) and optical density variation rates (rOD_{680}) from RW monitoring data. Dotted line represents the 3-points moving average on rOD_{680} .

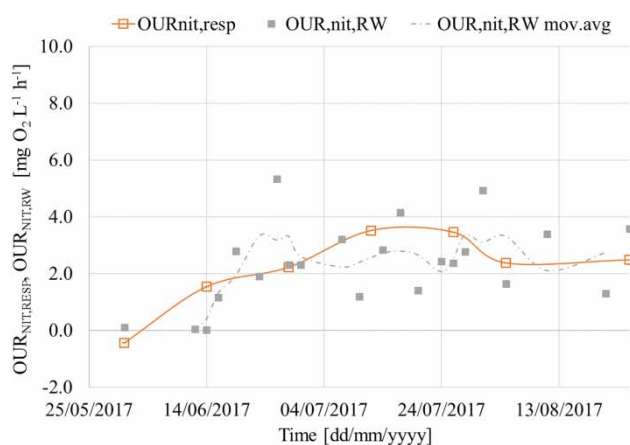


Figure 5 | Comparison between calculated oxygen uptake rate for nitrification from Pilot-RW monitoring data ($OUR_{NIT,RW}$) and its 3-points moving average ($OUR_{NIT,RWmov}$) and nitrifying activity obtained by respirometric tests ($OUR_{NIT,RESP}$).

From the trend of the NO_x concentrations assessed in the Pilot-RW, and assuming a conventional stoichiometric oxygen request for nitrification, the oxygen consumption rate by nitrifiers could be estimated and compared to the oxygen uptake rate evaluated with the respirometric assay. The oxygen uptake rate, OUR, detected in the respirometric assays (Figure 5) seemed to be well correlated to the average level of the oxygen uptake estimated for the Pilot-RW. Only the first OUR value was slightly negative, indicating that the nitrifying activity was scarce and the low oxygen consumption was poorly detected with the respirometric procedure. These results suggest that respirometric tests could provide a rough estimation of the potential nitrifying activity of the consortium.

FISH analyses also confirmed a stable occurrence of AOB in the MB suspensions (Figure 6).

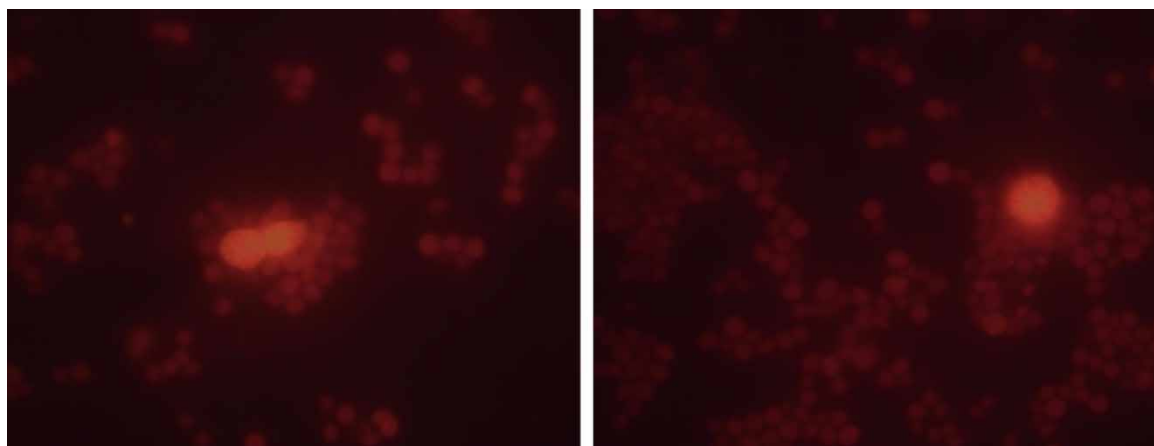


Figure 6 | Microalgae (dark grey; dark red in online version) and AOB (light grey; light red in online version) colonies observed in MB samples collected in Pilot-RW using a fluorescent microscope. Please refer to the online version of this paper to see this figure in colour: <http://dx.doi.org/10.2166/wst.2018.078>.

CONCLUSIONS

This study demonstrated that respirometry is a promising technique to gain insights into the oxygen dynamics in microalgal-bacteria systems. The proposed protocol involved the implementation of changing environmental conditions (in terms of light availability and presence of inhibitors) and successfully distinguished specific oxygen production/consumption rates deriving from the different guilds of microbes involved. Nevertheless, the experimental setup needs to be further improved to be routinely applied. Suggestions are: (i) the reduction in the oxygen mass transfer during the test; (ii) the search for specific inhibitors of algal dark respiration, which would help to discriminate between algal dark respiration and oxygen consumption due to HB in mixed MB consortia. Once optimized, the protocol could become a very simple and effective tool for monitoring outdoor and indoor MB bioreactors treating industrial and municipal wastewaters as well in the study of optimal operational conditions to be applied to these bioreactors.

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