Bioresource Technology 352 (2022) 127116

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Respirometric assessment of bacterial kinetics in algae-bacteria and activated sludge processes

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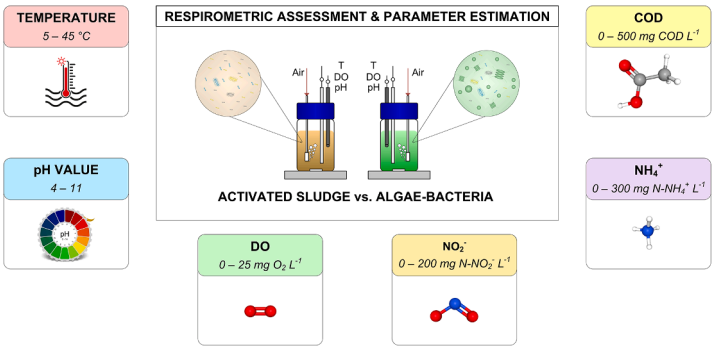
HIGHLIGHTS

- Respirometric procedure to estimate bacterial activities in algae-bacteria systems.
- Comparison of bacterial kinetic parameters in algae-bacteria and activated sludge.
- Strong influence of pH, temperature, oxygen, and substrates on oxygen uptake rates.
- Cardinal models described the effects of different temperature and pH on bacteria.
- Monod/Andrews models described the effects of oxygen and substrate availability.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Wastewater treatment
Microalgae-bacteria consortia
Activated sludge
Respirometry
Mathematical modelling
Kinetics

ABSTRACT

Algae-bacteria (AB) consortia can be exploited for effective wastewater treatment, based on photosynthetic oxygenation to reduce energy requirements for aeration. While algal kinetics have been extensively evaluated, bacterial kinetics in AB systems are still based on parameters taken from the activated sludge models, lacking an experimental validation for AB consortia. A respirometric procedure was therefore proposed, to estimate bacterial kinetics in both activated sludge and AB, under different conditions of temperature, pH, dissolved oxygen, and substrate availability. Bacterial activities were differently influenced by operational/environmental conditions, suggesting that the adoption of typical activated sludge parameters could be inadequate for AB modelling. Indeed, respirometric results show that bacteria in AB consortia were adapted to a wider range of conditions, compared to activated sludge, confirming that a dedicated calibration of bacterial kinetics is essential for effectively modelling AB systems, and respirometry was proven to be a powerful and reliable tool to this purpose.

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https://doi.org/10.1016/j.biortech.2022.127116
Received 25 February 2022; Received in revised form 30 March 2022; Accepted 1 April 2022
Available online 6 April 2022

1. Introduction

Biological secondary treatment is traditionally employed in wastewater treatment plants (WWTPs) to remove the dissolved nutrients (i.e., carbon (C), nitrogen (N) and phosphorous (P) compounds), the most widely applied system being the activated sludge (AS) process (Hreiz et al., 2015; Orhon, 2015). In this bioremediation process, diverse groups of microorganisms are responsible for wastewater treatment, the main actors being heterotrophic bacteria (HB), and autotrophic nitrifying bacteria, i.e., ammonia-oxidizing Bacteria (AOB), and nitrite-oxidizing bacteria (NOB). Despite the high Chemical Oxygen Demand (COD), N and P removal efficiencies obtained by AS processes, high energy demands, and operating costs are caused by aeration, mixing, and reagents required to properly operate the process (Crini and Lichtfouse, 2019). Nutrient losses and greenhouse gas emissions are also reported as major disadvantages of conventional bioremediation systems (Campos et al., 2016; Capodaglio and Olsson, 2019). To overcome these drawbacks, algae-bacteria (AB) consortia has been proposed as a sustainable alternative, as this biotechnology exploits renewable sunlight, consumes atmospheric CO_2, and allows for N and P removal and recovery, while generating valuable bio-products from the algal biomass (Chan et al., 2022; Mantovani et al., 2020; Mennaa et al., 2019; Nguyen et al., 2019).

AB consortia are generally cultivated for wastewater treatment in large-scale outdoor raceway ponds (RWPs), that are exposed to continuous variations in the environmental conditions (mainly: temperature, irradiance, and evaporation rates), driving the algal and bacterial growth kinetics to follow both daily and seasonal patterns. As a result, other key parameters such as pH and dissolved oxygen (DO) noticeably vary during the day, further influencing the water chemistry and growth kinetics (Casagli et al., 2021a; Robles et al., 2020). Moreover, the availability of nutrients in AB wastewater systems is in low amounts, or in large excess, introduce other limitation or inhibition factors for both the algal and bacterial growth (Aparicio et al., 2022; Rossi et al., 2020a; Rossi et al., 2020b; Rossi et al., 2020c). This makes microalgae-bacteria modelling especially challenging, and imposes the calibration of many kinetic parameters, for which few experimental guidelines have been released (Shoener et al., 2019). Within this context, respirometry have been widely applied as a rapid tool for the assessment of kinetic parameters of AS, aimed at the calibration of Activated Sludge Models (ASM) (Henze et al., 2015; Mainardis et al., 2021). On the other hand, photo-respirometry can be successfully applied to assess the activity of AB consortia treating municipal and industrial wastewaters (Flores-Salgado et al., 2021; Rossi et al., 2018; Sánchez-Zurano et al., 2020). Respirometric data have been profitably used to calibrate mathematical models describing AB systems for wastewater treatment (Casagli et al., 2021b; Sánchez-zurano et al., 2021). However, only a few studies proposed specific protocols to assess bacterial activities in AB systems (Flores-Salgado et al., 2021; Sánchez-Zurano et al., 2020), these experiments being generally conducted on the algal biomass alone, which has more inter-species variability than bacteria (Rossi et al., 2020b). In AB modelling, it is generally assumed that the bacterial populations can be modelled by using parameters that are conventionally adopted in ASMs. However, adaptation phenomena in the AB unit can be expected, driven by continuous environmental perturbations and nutrient/substrates competition with algal species (Fallahi et al., 2021; González-Camejo et al., 2020a; González-Camejo et al., 2020b; Ramanan et al., 2016).

In this work, a comprehensive respirometric study of the dependence of bacterial activities on environmental conditions (temperature, pH and DO) and substrates concentrations (COD, N-NH_4^+, and N-NO_2^-) was carried out. The procedure was developed and applied to samples collected from a full scale conventional activated sludge tank (fed on municipal wastewater), and from a pilot-scale algae-bacteria raceway pond (fed on the liquid fraction of anaerobic digestate), both in operation at the same WWTP. The respirometric study allowed to model the behaviour of aerobic bacteria (namely, HB, AOB, and NOB) in the two systems, and to identify the most relevant kinetic parameters describing the effect of the above-mentioned conditions. Parameter identification using experimental data made it possible to compare these effects on each bacterial population, aiming at providing a suitable methodology and an extensive dataset to calibrate existing AB growth models, thus improving their efficiency, and making them effective tools for the improvement of the AB-based process performances. Indeed, by a model-based optimization of crucial parameters, such as temperature, DO, pH and nutrient loads, biomass production and wastewater treatment efficiency can be enhanced.

Table 1
Comparison of the main wastewater characteristics and reactor conditions in the two treatment systems. Results are reported as average ± standard deviation. n. a.: not available.

Parameter	Unit	Activated Sludge (AS)		Algae-Bacteria (AB)	
		Influent	Reactor	Influent	Reactor
N-NH_4^+	mg N·L^{-1}	29.4 ± 10.9	1.0 ± 0.9	219 ± 51	34.5 ± 21.1
N-NO_2^-	mg N·L^{-1}	–	n.a.	0.31 ± 0.08	75.5 ± 50.9
N-NO_3^-	mg N·L^{-1}	–	10.1 ± 3.1	0.32 ± 0.75	58.3 ± 57.2
Total N	mg N·L^{-1}	32.1 ± 10.3	6.4 ± 3.6	220 ± 52	168 ± 71
P-PO_4^{3-}	mg P·L^{-1}	n.a.	0.74 ± 0.30	9.2 ± 2.4	6.3 ± 2.5
Total P	mg P·L^{-1}	4.6 ± 2.2	0.8 ± 0.4	13.1 ± 1.1	12.4 ± 1.5
COD_S	mg COD·L^{-1}	n.a.	n.a.	164 ± 60	205 ± 154
COD_{TOT}	mg COD·L^{-1}	308 ± 128	17.9 ± 8.7	301 ± 40	669 ± 103
$BOD_{TOT,5}$·COD_{TOT}^{-1}	–	0.57 ± 0.09	n.a.	0.30 ± 0.08	0.09 ± 0.01
TSS	g TSS·L^{-1}	0.15 ± 0.08	7.3 ± 1.3	0.21 ± 0.09	0.47 ± 0.24
OD_{680}	–	n.a.	n.a.	0.10 ± 0.06	0.42 ± 0.21
HRT	d	n.a.	0.7 ± 0.1	n.a.	15.0 ± 0.0
Temperature	°C	n.a.	18.3 ± 4.6	n.a.	20.2 ± 6.0
Temperature range	°C	n.a.	13.0–21.9	n.a.	4.7–32.4
pH	–	7.5 ± 0.3	6.9 ± 0.1	8.6 ± 0.2	7.1 ± 0.3
pH range	–	n.a.	6.7–7.1	n.a.	5.9–7.9
DO	mg DO·L^{-1}	n.a.	0.27 ± 0.43	n.a.	8.1 ± 3.1
DO range	mg DO·L^{-1}	n.a.	0.0–3.4	n.a.	0.3–20.8

2. Materials and methods

2.1. Wastewater characteristics, treatment systems and climate

The biomass used for respirometric tests was sampled from two wastewater treatment systems, both located in the WWTP of Bresso-Niguarda (Milan, Italy): (i) a full-scale AS tank receiving pre-treated (screening, sand/grit removal, primary settling) municipal wastewater, and (ii) a pilot-scale AB RWP, treating the liquid fraction of centrifuged digestate originated from the anaerobic digestion of excess activated sludge.

The AS tank (6100 m^3) was operated continuously with an average HRT of 17 h. The tank was located outdoor, and it was subject to weather conditions and low temperatures during winter. However, the large volume and the fact that it was built underground, made it possible to maintain relatively high temperatures throughout the year (13–22 °C). The influent wastewater had average total COD, NH_4^+, and total P concentrations of 307 mg COD·L^{-1}, 22.9 mg N-NH_4^+·L^{-1}, and 4.6 mg

Table 2
Experimental design describing the environmental conditions and nutrient concentrations maintained during respirometric tests.

Parameter	Unit	Target Populations	Range tested
Temperature	°C	HB, AOB, NOB	5–45
pH value	–	HB, AOB, NOB	4–11
Dissolved oxygen	mg DO·L^{-1} (%DO_{SAT})	HB, AOB, NOB	0–25 (0–275%)
COD	mg COD·L^{-1}	HB	0–500
NH_4^+	mg N·L^{-1}	AOB	0–300
NO_2^-	mg N·L^{-1}	NOB	0–200

P·L^{-1}, respectively. Under typical operational conditions, the average pH and DO values in the AS tank were 6.9 pH units and 0.3 mg DO·L^{-1}.

The AB cultivation unit was a 0.87 m^3 RWP, which was operated in continuous mode to maintain an HRT of 6 d. The reactor had a 0.15 m liquid height and a total surface of 5.8 m^2. The RWP was located into a greenhouse, to mitigate the cold winter conditions of Northern Lombardy. More details about the AB unit are available in a previous work (Mantovani et al., 2020). The AB culture was periodically examined for identifying algal species and other microorganisms. During the experimentation, the dominant algae species were *Scenedesmus sp.* and *Chlorella sp.* ($1.6{\cdot}10^6 \pm 1.9{\cdot}10^6$ and $1.1{\cdot}10^6 \pm 1.3{\cdot}10^6$ cells·mL^{-1}, respectively, on average). The temperature range and the maximum solar radiation to which the algal culture was exposed in the RWP were 5–32 °C and 1010 W·m^{-2}, respectively. The pH was maintained within 6–8 by temporized bubbling of pure CO_2 coming from the full-scale biogas upgrading unit. The DO was measured online, reaching minimum and maximum values of 0.3–20.8 mg DO·L^{-1}, respectively. The influent digestate had average concentrations of 220 mg N-NH_4^+·L^{-1}, 177 mg COD·L^{-1}, and 9 mg P-PO_4^{3-}·L^{-1}, respectively. Nitrite was almost absent in both the WW and influent digestate (<0.3 mg N-$NO2^-L^{-1}$), however partial nitrification occurred in the RWP, with NO_2^- accumulation peaks up to 144 mg N-NO_2^-·L^{-1}. The main characteristics of the influent wastewater and treatment systems are reported in Table 1.

2.2. Respirometric device

The respirometer used to evaluate respiration rates was composed of two 500 mL glass bottles filled to 300 mL with biomass suspensions. The device was equipped with probes for temperature, DO and pH. Also, the device was equipped by a control unit collecting and communicating the data every 3 s. Tests were conducted in a thermostatic chamber to maintain the desired temperatures. The pH was controlled at the desired levels by automatic titration of concentrated HCl or NaOH solutions (0.1–0.5 M), while the DO concentration was controlled by on-demand aeration. A more detailed description of the respirometric equipment is available elsewhere (Rossi et al., 2020a,b, 2021).

2.3. Respirometric procedures and experimental design

The experimental procedure was inspired by typical respirometric protocols available for the AS process (Vanrolleghem et al., 1999) and for AB consortia (Rossi et al., 2020a,b; Sánchez-Zurano et al., 2020). For each parameter tested (i.e., temperature, pH, DO, and substrate concentrations), a different respirometric protocol was applied for each bacterial population (AOB, NOB, HB).

First, AB samples were concentrated 10 times by centrifugation at 10,000 g (Filtermaxx VWO, USA) to remove residual culture nutrients and to adjust the biomass concentration, then the samples were immediately resuspended in fresh Bold's Basal Medium (BBM) to avoid stress on the algal populations, which could have resulted in the release of organic matter (González-Camejo et al., 2020b). The composition of the BBM used to resuspend the biomass was previously described (Rossi et al., 2021). AS samples were left under dark conditions for 24 h, bubbling unfiltered ambient air to reach substrate depletion and endogenous conditions.

After pre-treatments, the environmental conditions were modified according to the experimental design (see Table 2), and the tests started. Respirometric protocols were constituted by a series of three re-aeration cycles. The environmental parameter under investigation was later varied, and the re-aeration cycles were further repeated for all parameters' combination. When a certain parameter was varied, all other parameters were kept at reference levels (T = 20 °C, pH = 7.5, DO = 5–6 mg DO·L^{-1}), and the substrate concentrations were maintained to non-limiting concentrations. The control was reactor A (with substrate availability), and the limited reactor was reactor B (with no substrates or with inhibitors). For tests performed on AOB, NH_4^+ (100 mg N·L^{-1}) was added in both respirometric vessels, while reactor B was supplemented with 10 mg·L^{-1} of ATU to stop ammonia oxidation (Rossi et al., 2018). For NOB and HB, reactor A was maintained under endogenous and substrate-limited conditions, while the reactor B was supplemented with the relevant substrate (NO_2^- and COD at concentrations of 25 mg N·L^{-1} and 100 mg COD·L^{-1}, respectively (Sánchez-Zurano et al., 2020). In substrates tests, each substrate was added through successive spikes, aimed at increasing the substrate availability, up to the desired concentrations (see also Section 2.4). The tests were divided into four series, each targeting temperature, pH, dissolved oxygen, and substrate concentrations. The values of the parameters to be tested were chosen to cover their range in outdoor AS and AB systems (see Table 1), as summarised in Table 2.

2.4. Numerical methods

A detailed description of numerical methods to calculate the OURs is reported in previous studies (Rossi et al., 2020a,b, 2021; Sánchez-Zurano et al., 2020). Briefly, a DO mass balance was applied to the respirometric bottles (Eq. (1)), allowing to define relevant rates affecting the dynamic evolution of the DO during each phase, i.e.: the oxygen uptake rate (OUR) and the oxygen transfer rate (OTR).

$$\frac{d\,(DO)}{d\,(t)} = OUR_i + OTR, \quad (i = 1, \ldots, 3) \tag{1}$$

where: OUR_i is the oxygen uptake rate for the considered phase *i* [mg DO·L^{-1}·h^{-1}], and OTR is the oxygen transfer rate for the given respirometer characteristics [mg DO·L^{-1}·h^{-1}].

To describe the oxygen mass transfer, the following equation was used (Eq. (2)):

$$OTR = \theta^{(T-T_{REF})}{\cdot}k_L a_{20}{\cdot}(DO_{SAT}\text{-}DO) \tag{2}$$

where: $\theta = 1.024$ [−] is the temperature correction coefficient according to previous guidelines (ASCE, 1993), T_{REF} = 20 °C is the reference temperature, k_La_{20} = 1.06 [h^{-1}] is the volumetric gas–liquid mass transfer coefficient, estimated through dedicated reaeration tests in clean water at 20 °C (see also supplementary material), DO_{SAT} [mg DO·L^{-1}] is the oxygen saturation at the considered temperature, calculated from the appropriate Henry constant (Rossi et al., 2021).

Given that the DO dynamics were recorded online, and the OTR was calculated by knowing the volumetric mass transfer coefficient k_La, the OUR could be estimated for each phase. Specific oxygen uptake rates ($SOUR_i$, [mg DO·g TSS^{-1}·h^{-1}]) were calculated for each phase, by dividing the obtained OUR_i values by the TSS concentration expressed in [g TSS·L^{-1}] (Eq. (3)).

$$SOUR_i = \frac{OUR_i}{TSS}, \quad (i = 1, \ldots, 3) \tag{3}$$

The bacterial activity of each population ($SOUR_X$ in Eq. (4), where X = HB, AOB, or NOB) was determined by difference among the activity recorded in the control reactor A and the limited reactor B.

$$SOUR_{X,i} = SOUR_{A,i} - SOUR_{B,i} \ (X = HB,\ AOB,\ NOB;\ i = 1, \ldots, 3) \tag{4}$$

Table 3
Results of parameter identification for heterotrophic bacteria (HB), ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB) in activated sludge (AS) and algae-bacteria (AB) consortia. Results are expressed as value (standard error). n.a.: not applicable.

Variable	Fitting Function	Model parameter	Unit	HB		AOB		NOB	
				AS	AB	AS	AB	AS	AB
T	CTMI (Eq. (6))	T_{MIN}	°C	−7.7 (4.3)	−4.3 (4.2)	1.3 (2.5)	0.48 (2.88)	−7.8 (5.4)	−4.3 (5.2)
		T_{OPT}	°C	36.1 (1.1)	35.59 (0.87)	30.19 (0.84)	34.13 (0.80)	36.0 (1.3)	34.4 (1.3)
		T_{MAX}	°C	42.90 (0.14)	40.52 (0.17)	41.78 (0.31)	43.75 (0.26)	43.43 (0.73)	41.85 (0.50)
pH	CPM (Eq. (7))	pH_{MIN}	–	4.19 (0.17)	2.93 (0.50)	4.24 (0.21)	4.43 (0.22)	4.40 (0.10)	4.04 (0.13)
		pH_{OPT}	–	8.02 (0.28)	8.77 (0.35)	8.48 (0.26)	9.45 (0.18)	7.46 (0.23)	7.83 (0.23)
		pH_{MAX}	–	11.06 (0.14)	11.13 (0.11)	10.80 (0.08)	10.82 (0.02)	11.21 (0.17)	11.00 (0.13)
DO	Monod (Eq. (8)), Andrews (Eq. (9))	k_{DO}	mg DO·L^{-1}	1.25 (0.25)	n.a.	n.a.	n.a.	2.12 (0.16)	2.28 (0.41)
		α	L·d·mg DO^{-1}	n.a.	8.75 (3.06)	0.59 (0.15)	1.45 (0.31)	n.a.	n.a.
		DO_{OPT}	mg DO·L^{-1}	n.a.	1.29 (0.21)	6.64 (0.62)	3.27 (0.29)	n.a.	n.a.
COD	Monod (Eq. (8))	k_{COD}	mg COD·L^{-1}	4.39 (0.83)	3.46 (0.61)	n.a.	n.a.	n.a.	n.a.
NH_4^+	Andrews (Eq. (9))	α	L·d·mg N^{-1}	n.a.	n.a.	3.08 (0.60)	2.38 (0.32)	n.a.	n.a.
		NH_{4OPT}	mg N·L^{-1}	n.a.	n.a.	10.55 (3.08)	10. 86 (0.98)	n.a.	n.a.
NO_2^-	Monod (Eq. (8))	k_{NO2}	mg N·L^{-1}	n.a.	n.a.	n.a.	n.a.	0.76 (0.12)	4.58 (0.59)

To compare test results among different conditions, OUR data were finally normalized by the maximum experimental value recorded, i.e., $SOUR_{MAX}$ ($SOUR_{NORM,X,i}$, Eq. (5)).

$$SOUR_{NORM,X,i} = \frac{SOUR_{X,i}}{SOUR_{MAX}} \quad (X = HB, AOB, NOB;\ i = 1, \ldots, 3) \tag{5}$$

To model the respiration dependence from each tested condition, commonly applied models were used (Casagli et al., 2021b; Sánchez-zurano et al., 2021; Solimeno et al., 2019).

The temperature dependence was modelled using the cardinal temperature model with inflection (CTMI), shown in Eq. (6) (Rosso et al., 1995):

$$\frac{SOUR}{SOUR_{MAX}} = \begin{cases} 0, \text{ if } T < T_{MIN} \\ \dfrac{(T\text{-}T_{MAX})\cdot(T\text{-}T_{MIN})^2}{(T_{OPT}\text{-}T_{MIN})\cdot((T_{OPT}\text{-}T_{MIN})\cdot(T\text{-}T_{OPT})\text{-}(T_{OPT}\text{-}T_{MAX})\cdot(T_{OPT}+T_{MIN}\text{-}2\cdot T))}, \text{ if } T_{MIN} < T < T_{MAX} \\ 0, \text{ if } T > T_{MAX} \end{cases} \tag{6}$$

Where: T_{MIN} is the minimum cardinal temperature below which the respiration rate is zero [°C], T_{OPT} is the optimal temperature for which the respiration rate is maximum [°C], T_{MAX} is the maximum cardinal temperature above which the respiration rate is zero [°C].

To evaluate the dependence on the pH value, the cardinal pH model (CPM) in Eq. (7) (Rosso et al., 1995) was fitted to experimental data:

$$\frac{SOUR}{SOUR_{MAX}} = \begin{cases} 0, \text{ if } pH < pH_{MIN} \\ \dfrac{(pH\text{-}pH_{MIN})\cdot(pH\text{-}pH_{MAX})}{(pH\text{-}pH_{MIN})\cdot(pH\text{-}pH_{MAX})\text{-}(pH\text{-}pH_{OPT})^2}, \text{ if } pH_{MIN} < pH < pH_{MAX} \\ 0, \text{ if } pH > pH_{MAX} \end{cases} \tag{7}$$

where: pH_{MIN} is the minimum cardinal pH value below which the respiration rate is zero [-], pH_{OPT} is the optimal pH value for which the respiration rate is maximum [-], pH_{MAX} is the maximum cardinal pH value above which the respiration rate is zero [-].

The dependence on DO and nutrients (NH_4^+, NO_2^-, COD) was expressed as either the Monod function with nutrient limitation in Eq. (8) (Monod, 1942), or Andrews kinetics, if inhibition at high substrate concentrations occurred, as shown in Eq. (9) (Turon et al., 2015).

$$\frac{SOUR}{SOUR_{MAX}} = \frac{S}{S + K_S} \tag{8}$$

where: S represents either the concentration of DO or the relevant substrate (NH_4^+ for AOB, NO_2^- for NOB, and COD for HB) [mg·L^{-1}], K_S is the half-saturation constant for DO or for the substrate [mg·L^{-1}];

$$\frac{SOUR}{SOUR_{MAX}} = \frac{S}{S + \frac{SOUR_{MAX}}{\alpha}\cdot\left(\frac{S}{S_{OPT}}\text{-}1\right)^2} \tag{9}$$

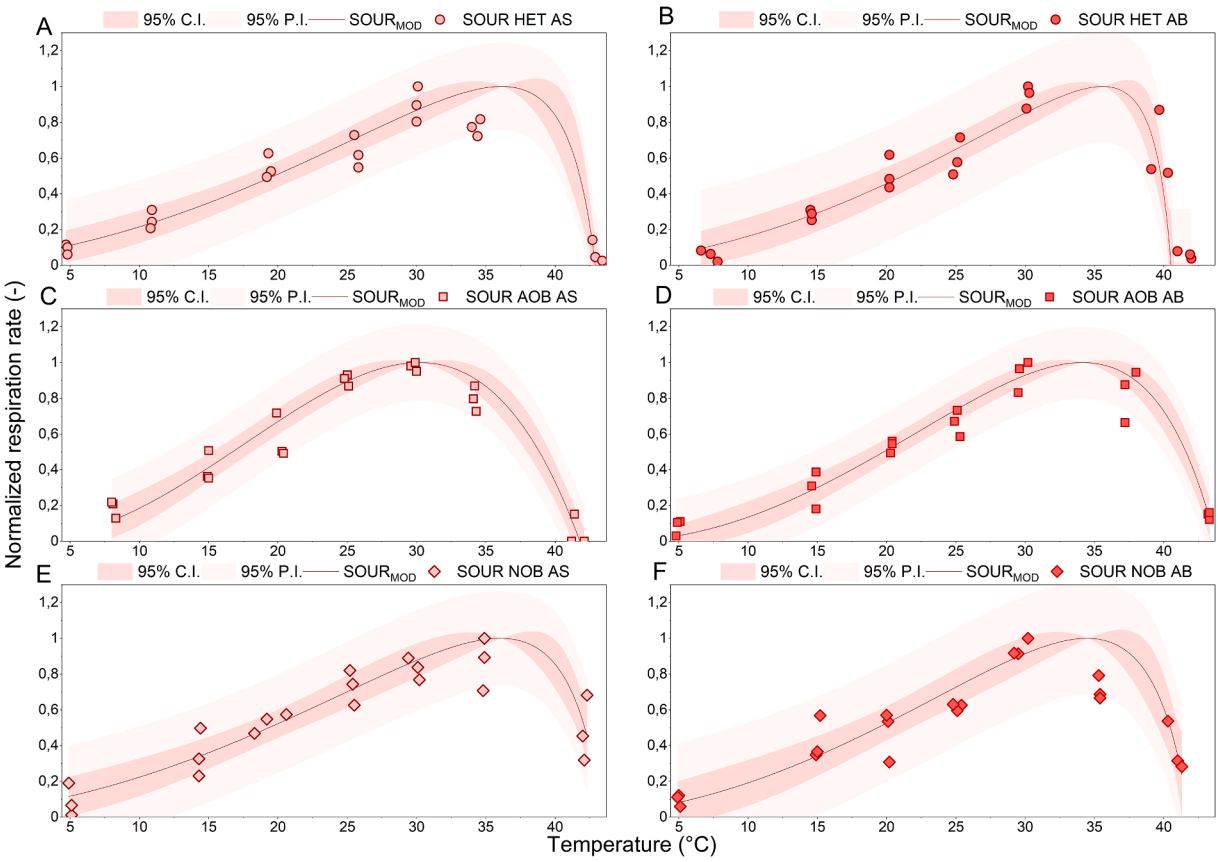

Fig. 1. Effect of temperature on bacterial populations in activated sludge (AS) and algae-bacteria (AB) samples: heterotrophic bacteria in AS (A), heterotrophic bacteria in AB (B), ammonia-oxidizing bacteria in AS (C), ammonia-oxidizing bacteria in AB (D), nitrite-oxidizing bacteria in AS (E), nitrite-oxidizing bacteria in AB (F).

where: α [L·d·mg^{-1}] is the initial slope coefficient; S_{OPT} [mg·L^{-1}] is the optimum concentration of DO or of the considered substrate, for which the respiration rate is maximum.

2.5. Statistical methods and software

Raw data were exported in the software Excel 365 (Microsoft) and organized as input tables for subsequent elaborations. Data were then imported in MATLAB R2021b (The Mathworks) and the SOUR values were estimated from raw data using the *Optimization Toolbox* (function: *lsqcurvefit*). Further elaborations, and data plotting, was performed using OriginPro 2020b (OriginLab Corporation), including SOUR data normalization, nonlinear curve fitting, and model statistics. The reduced chi-squared, residual sum of squares, and the adjusted r-squared were used to express the goodness of fit for selected models. Confidence and prediction intervals were calculated at the significance level of 95% (α = 0.05).

2.6. Analytical methods and reagents used

The TSS concentrations were measured according to Standard Methods (APHA, 2017). Triplicate measurements of the OD_{680} were assessed using a 1-cm path plastic cuvette and read using a spectrophotometer (Hach Company, Model: DR 3900). For AB, the linear regression among the TSS and OD_{680} was used to rapidly estimate the TSS concentration based on faster OD_{680} measurement. All chemicals used to prepare synthetic media (i.e., the synthetic BBM and the concentrated NH_4, NO_2, COD and ATU solutions) were reagent-grade from Sigma-Aldrich.

3. Results and discussion

The results of the fitting procedures for the AS and AB samples (i.e., the estimated model parameters describing the dependence on temperature, pH, DO and substrates) are given in Table 3, respectively. Detailed residual analyses and model statistics, including the reduced Chi-square, residual sum of squares, and adjusted R-square, are reported in see supplementary materials. All models were able to represent the experimental dataset (adjusted R-square values: 0.54–0.94), confirming both the adequacy of the equations used in algae-bacteria models, and the versatility of respirometric tests to determine bacterial kinetics rapidly and reliably.

Results can be discussed by considering that AS and AB were sampled from systems subjected to different conditions, which could have potentially strong impacts on the response of bacterial communities and their kinetic parameters. As the AS and AB conditions significantly varied, bacterial response was expected to reflect certain differences among the two samples. Regarding the distinct conditions experienced by bacteria, the main differences mainly arose from the presence of microalgae, the reactor geometry/scale, and the wastewater characteristics, influencing the environmental conditions of the suspension and the composition and activities of the microbial community. In particular, the scale and geometry of reactors mainly had an impact on the thermal properties and thermal inertia of the biomass suspension. Indeed, the AS had a volume of 6100 m^3, which is several orders of magnitude higher than the scale of the RWP (0.87 m^3). In addition, the AS tank of the Bresso-Niguarda plant was built underground, so that thermal excursions were further buffered, and the AS culture had a more stable temperature throughout the year. On the other hand, the AB pond had higher thermal dispersions, since the pilot plant floor was also in

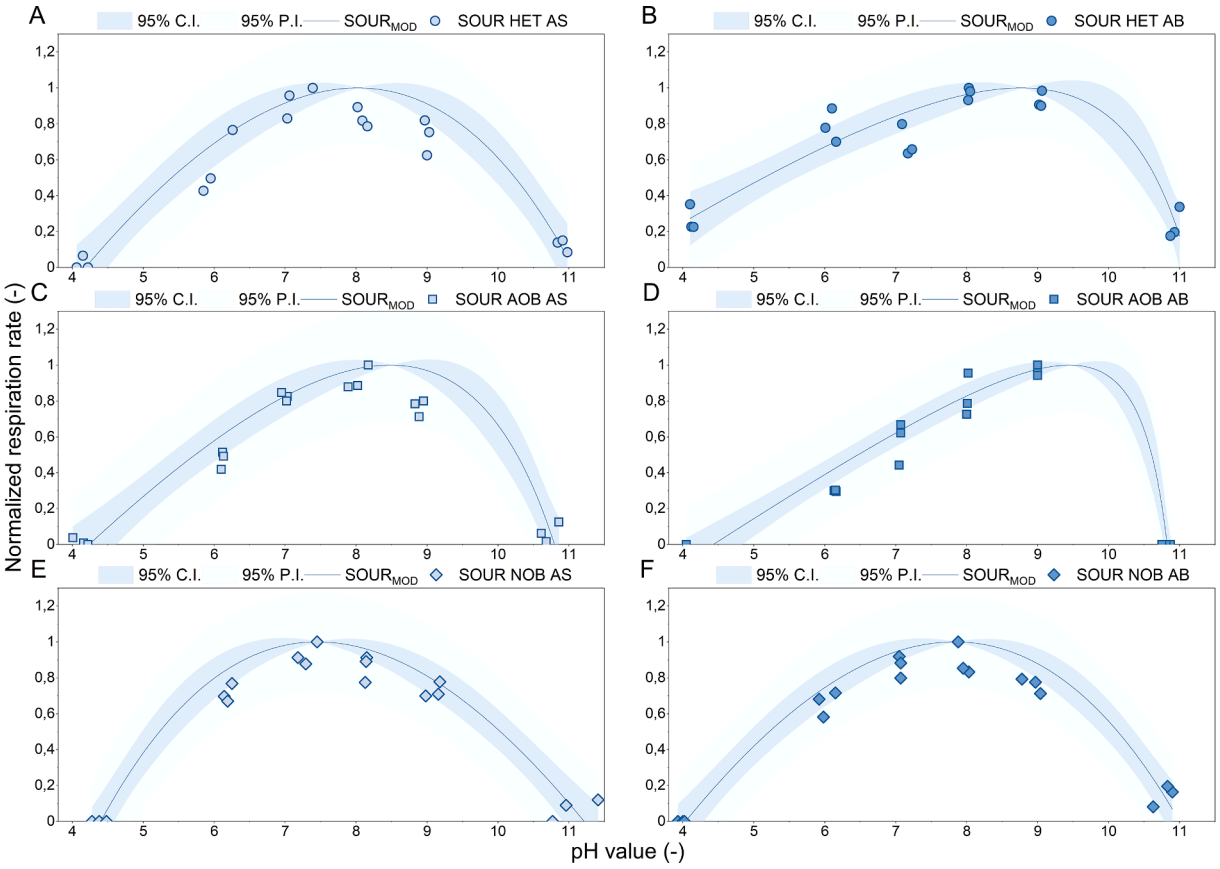

Fig. 2. Effect of pH on bacterial populations in activated sludge (AS) and algae-bacteria (AB) samples: heterotrophic bacteria in AS (A), heterotrophic bacteria in AB (B), ammonia-oxidizing bacteria in AS (C), ammonia-oxidizing bacteria in AB (D), nitrite-oxidizing bacteria in AS (E), nitrite-oxidizing bacteria in AB (F).

exchange with the surrounding air, being placed on a metal structure at approximately 0.8 m above the ground. The geometry of the reactor was further responsible for emphasizing thermal variations, as RWPs are specifically designed to maximize the surface/volume ratio, and to minimize the liquid height, thus maximizing the light penetration for photosynthesis. Indeed, typical liquid heights for RWPs are in the range of 0.1–0.3 m, compared to AS systems in which the tank height can typically reach 3–6 m, depending on the type of installed aeration devices. In addition, the pH and DO were subject to a marked increase in the algal-bacterial suspension, following the photosynthetic activity. Another major difference among the two systems is that the biomass concentration was kept at very high levels in AS (up to 3.7 g TSS·L^{-1}, through settled sludge recirculation). On the contrary, lower TSS concentrations were reached in AB (up to a maximum value of 0.9 g TSS·L^{-1}), as it is common in these systems, to guarantee a sufficient light penetration.

Regarding nutrient sources, as reported in Table 1, the AS received primary effluent, rich in degradable organic matter, and with moderate concentrations of N and P. On the contrary, the AB was fed on nutrient-rich digestate with low concentrations of biodegradable organic matter (Akhiar et al., 2017). This possibly led to the predominance, in the AB system, of autotrophic microalgae and nitrifying bacteria over HB, as testified by the high NH_4^+ removal rates (greater than 84%) and the high NO_X effluent concentrations (up to 245 mg N-NO_2^-+NO_3^-·L^{-1}), coupled with a negligible soluble COD removal efficiency. It should be finally noticed that the bacterial biomass concentrations in AB systems are generally much lower than the algal concentrations, typically showing a ratio of bacterial to algal TSS lower than 1:10 (Casagli et al., 2021a). On the contrary, the biomass in the AS tank is practically constituted only by bacterial biomass, and the concentration of HB in AS is expected to be much higher than AOB and NOB, due to the larger availability of degradable organics and the high growth rates of HB. In the following sections, the results obtained for each parameter are reported and discussed.

3.1. *Effect of temperature*

The effect of temperature on bacterial populations of AS and AB is reported in Fig. 1. As shown, the trend for all populations followed the typical asymmetric curve of algal and bacterial cultures (Rosso et al., 1995), in which the optimal temperature is closer to the maximum than to the minimum temperature. The values of the parameters estimated for the CTMI are reported in Table 3.

Regarding the estimated optimal temperatures, these ranged from 30 to 36 °C, which is common for a wide variety of bacterial strains and species (Rosso et al., 1995). For AS, optimal temperatures were 36.1 °C for HB, 30.2 °C for AOB and 36.0 °C for NOB, while in AB samples, the optimum for growth resulted to be 35.6 °C for HB, 34.1 °C for AOB and 34.5 °C for NOB. Since, to the best knowledge of the authors, no studies are available in which the cardinal temperatures were experimentally determined for AS, it is not possible to directly compare these results with other literature experiences. However, previous studies reported that the optimal temperature for nitrifiers is approximately 30 °C, even though values close to the maximum activity could be observed in the entire range from 15 °C to 35 °C (Shammas, 1986), with a strong decrease in the activity below 15 °C, and almost no activity could be detected at 5 °C. In this work, the optimal temperature for AOB was found to be lower in AS than in AB, while the optimum for NOB was similar in both systems. In most cases, the dependence of AS on temperature is modelled by using Arrhenius-type models, which is only suitable to represent the activity below the optimal temperature, therefore no maximum values are found in the literature.

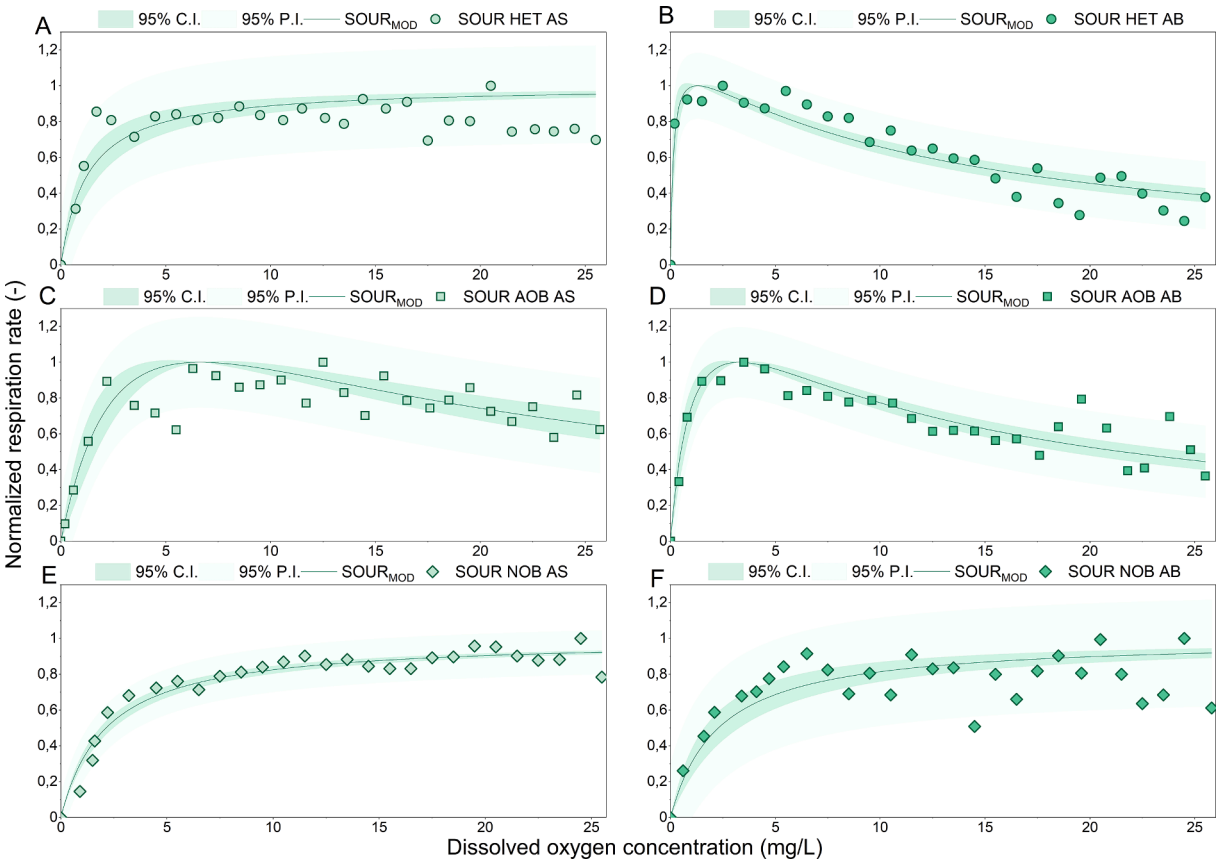

Fig. 3. Effect of dissolved oxygen on bacterial populations in activated sludge (AS) and algae-bacteria (AB) samples: heterotrophic bacteria in AS (A), heterotrophic bacteria in AB (B), ammonia-oxidizing bacteria in AS (C), ammonia-oxidizing bacteria in AB (D), nitrite-oxidizing bacteria in AS (E), nitrite-oxidizing bacteria in AB (F).

Regarding bacterial populations in AB, the temperature dependence was recently modelled based on the CTMI (Sánchez Zurano et al., 2021), obtaining very similar results for HB (T_{OPT} = 36 °C) and for nitrifying bacteria, even if these were assessed as a single bacterial group (T_{OPT} = 33.6 °C). These results were also used as nominal values for the ABACO model (Sánchez-zurano et al., 2021). A further confirmation on the reliability of respirometric estimates comes from the calibrated cardinal temperature models available in recently published AB models. In both the ALBA (Casagli et al., 2021b) and BIO_ALGAE (Solimeno et al., 2019), calibrated values for bacteria are close to the reported estimations.

As previously reported in other studies, bacteria can survive in a wide range of temperatures (Alisawi, 2020; Rosso et al., 1995). This was also confirmed by the experimental data, for which the thermal niche was of 40–50 °C. The minimum tolerable temperature among all bacterial populations spanned from −7.7 °C to 1.3 °C, although with large standard errors, suggesting that the experimental design should be improved to better target this parameter. These results were higher than those reported in a similar AB system (Sánchez Zurano et al., 2021), though in this case the climate conditions at which the AB was operated were much warmer than those described here, possibly suggesting an adaptative behaviour. All the bacterial populations could resist up to more than 40 °C, and maximum tolerable temperatures ranged from 40.5 °C to 43.8 °C, coherently with previous findings (Sánchez Zurano et al., 2021). Maximum temperatures were more precisely identified (i.e., with lower standard errors) compared to minimum temperatures (see Table 3 and supplementary material for more details). The results reported in this section are a great example of how respirometric methods could allow gathering relevant information related to wastewater treatment processes. In particular, the use of the CTMI allows to predict the effect of temperature on bacteria, over a wide range of operational conditions. However, despite the availability of this useful model, the majority of literature works on activated sludge bacteria make use of Arrhenius-type equations, which are only suitable to describe the effect of temperature below the optimum, providing no information about bacterial decay at high temperatures. Furthermore, the existence of slight, yet potentially relevant differences among the kinetic models for bacterial communities in activated sludge and AB were quantified in this work.

3.2. *Effect of pH values*

The effect of different pH values on AS and AB is shown in Fig. 2. The CPM described well the respirometric dataset of all bacterial populations, with satisfactory fits and acceptable adjusted r-square values (0.84–0.90).

The findings indicated that bacteria in both AS and AB systems could resist to wide intervals of pH, still showing some residual activity at pH 4 and 11. Regarding the optimal pH, estimated values varied among the different populations (between 7.5 and 9.5). For AS, optimal pH values were 8.0 for HB, 8.5 for AOB and 7.5 for NOB, while the estimates for AB were shifted towards higher optima (8.8 for HB, 9.5 for AOB and 7.8 for NOB), suggesting that bacteria adapted in the AB system to more alkaliphilic conditions. These higher pH optima in AB compared to AS can be indeed explained by the higher pH values promoted by photosynthetic CO_2 uptake in algae-based wastewater treatment processes (according to the results reported in Table 1, the pH in the RWP was on average 7.1, with peaks up to 7.9). Previous studies have confirmed that in AS bacterial consortia, a pH ranging from 6.8 to 8.5 resulted in a high microbial activity and rate of biodegradation (Zhou et al., 2019). For HB, high consumption rates could be reached in a wide range of pH values (from 3

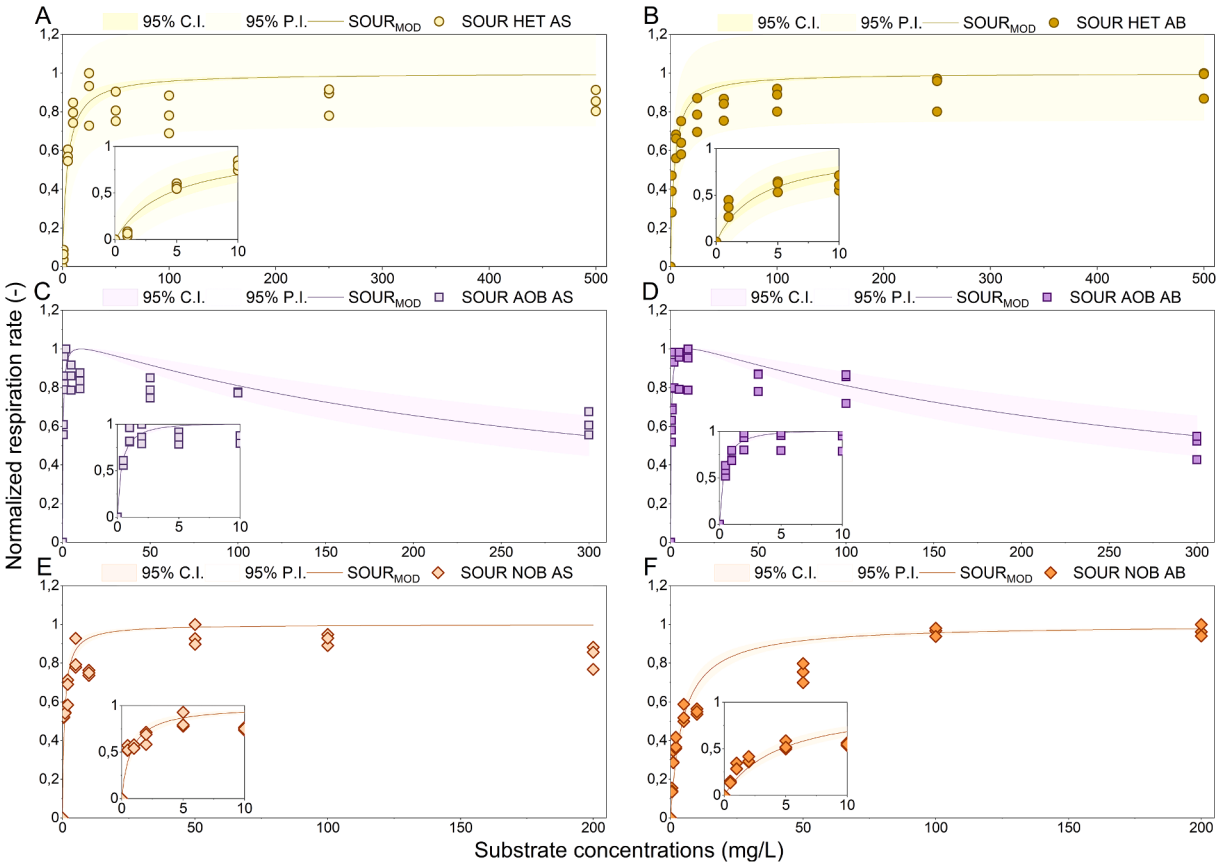

Fig. 4. Effect of substrates on bacterial populations in activated sludge (AS) and algae-bacteria (AB) samples: heterotrophic bacteria in AS (A), heterotrophic bacteria in AB (B), ammonia-oxidizing bacteria in AS (C), ammonia-oxidizing bacteria in AB (D), nitrite-oxidizing bacteria in AS (E), nitrite-oxidizing bacteria in AB (F).

to 9), with a very sharp drop at higher pH. Also, the pH dependence curve for nitrifiers is quite flat-topped, showing similarly high activities at pH values from 7 to 9.5 and with very little activity, or complete inactivation, only below pH 6 and above pH 10 (Shammas, 1986).

Concerning AB consortia, only a few studies have recently reported the use of the CPM to describe the effect of pH on bacterial populations. Results from Sánchez Zurano et al. (2021) and the ALBA model (Casagli et al., 2021b) were fairly consistent with the parameter estimates given in Table 3. Similarly, calibrated values adopted in the BIO_ALGAE model (Solimeno et al., 2019) are very close to the estimates proposed in this study. In these studies, describing the pH dependence for bacterial populations in AB, the pH_{MIN}, pH_{OPT}, and pH_{MAX} assumed values in the following ranges: 2.0–6.0, 7.0–9.0 and 11.0–13.4, respectively, further confirming the ability of bacteria to grow in a wide range of pH conditions.

3.3. Effect of dissolved oxygen

As depicted in Fig. 3, DO have a relevant effect on bacterial respiration rates. DO had an important inhibitory effect on HB and AOB sampled from AB, while almost no inhibition was observed at high DO concentrations, for all AS bacterial populations and for NOB in both systems. As an explanation for this fact, possible inhibitory effects of DO concentrations far above air saturation were previously reported to occur because of the DO diffusion through the membranes and to cause oxidative stress in cells (Baez and Shiloach, 2014). However, this inhibitory effect only seems to be related to long-term exposure times to DO oversaturation. Therefore, the differences observed between the two treatment systems could be because in AS, the microorganisms are rarely exposed to high DO concentrations, while in AB cultures the frequent exposition to high DO concentrations (caused by the algal photosynthetic activity), could have led to long-term cell stress (Baez and Shiloach, 2014). Estimated half-saturation constants for DO in AS samples were 1.2 and 2.1 mg $DO{\cdot}L^{-1}$, respectively, for HB and NOB, and the optimal DO for AOB was 6.6 mg $DO{\cdot}L^{-1}$. In AB samples, a similar half-saturation coefficient was found for NOB (2.3 mg $DO{\cdot}L^{-1}$), and the optimal DO concentrations for HB and AOB were 1.3 and 3.3 mg $DO{\cdot}L^{-1}$, respectively (Table 3). By comparing the DO dependence for the three populations in both AS and AB, it was confirmed, as previously reported for AS samples (Daebel et al., 2007), that the affinity for oxygen in HB was generally higher than for nitrifiers. Moreover, the AS had floccular nature, while AB grew as suspended cells. The presence of bacterial aggregates can cause a higher resistance to diffusion in AS, that could explain the lower affinity for DO observed in AS. The lower oxygen affinity for NOB compared to AOB was also described in several studies, and this was often adopted as a selective strategy in partial nitritation reactors, allowing to wash out NOB and to achieve stable accumulation of NO_2^- (Blackburne et al., 2008).

3.4. Effect of substrate limitation / inhibition

The effects of nutrient concentrations on bacterial populations of AS and AB are reported in Fig. 4. For HB, the half-saturation constants for COD were quite similar in both AS and AB (Fig. 4A and B, respectively), only showing a slightly lower substrate affinity in AS (4.4 mg $COD{\cdot}L^{-1}$), compared to AB (3.5 mg $COD{\cdot}L^{-1}$). Half-saturation constants found in this study for AS were very close to previous studies (Orhon, 2015), though a wide range of values is available in the literature, reaching up to one order of magnitude more than those found in this work, i.e. up to 20–45 mg $COD{\cdot}L^{-1}$, depending on process characteristics (Esquivel-Rios et al., 2014). Regarding AB, as no experimental determination of the half-saturation values is available in the literature for HB, mathematical

models describing the growth of AB consortia generally assume a value of 20 mg COD·L^{-1} from the ASMs (Sánchez-zurano et al., 2021; Solimeno et al., 2019), or more similar values to those experimentally found in this study (4 mg COD·L^{-1}), as reported by Casagli et al. (2021b).

Regarding the effect of nutrients for nitrifying bacteria, the results for AOB are given in Fig. 4C (for AS) and D (for AB), while the results for NOB are reported in Fig. 4E (for AS) and F (for AB). When looking at the parameter estimates for AOB, inhibitory effects of NH_4^+ occurred in both AS and AB, as further emphasized by the low optimal ammonia concentrations ($NH_{4,OPT}$ = 10.5 mg N·L^{-1} and 10.8 mg N·L^{-1} for AS and AB, respectively). This was coherent with the findings reported in previous studies for AS bacteria (Kim et al., 2006) and might be due to the generation of small amounts of free ammonia (FA), even if the pH was kept at 7.5 to minimize this effect. Indeed, FA is a strong growth/activity inhibitor for several types of microorganisms (Rossi et al., 2020b). However, no evidence is directly available for bacterial populations in AB consortia, and the dependence of bacterial growth on ammoniacal nitrogen is generally evaluated in AB models based on a Monod-type function, i.e., not considering substrate inhibition. The findings reported in the present study suggest that a deeper investigation should be conducted, to define whether the inhibitory effect is to be attributed to FA or the ammonium ions. These results were coherent with the experimental determinations of the half-saturation constants for ammoniacal nitrogen in AS: the ASMs and other studies reported values ranging from 0.4 to 5.2 mg N·L^{-1} (Henze et al., 2015; Iacopozzi et al., 2007; Leyva-Díaz et al., 2020), though a large variability was again found for this parameter, reaching in some cases values up to 9–40 mg N·L^{-1} (Terada et al., 2013). Very similar results were also obtained for AOB in AB consortia Due to the unavailability of experimental studies on bacterial activities, the only possible comparisons can be made with AB models, in which the assumed half-saturation constant for ammoniacal nitrogen range from 0.5 mg N·L^{-1} (Casagli et al., 2021b; Reichert et al., 2001; Solimeno et al., 2019), up to 1 mg N·L^{-1} (Sánchez-zurano et al., 2021).

Regarding NOB, a similar effect was observed for the AS and AB samples, since in both ecosystems a Monod-type curve could fit well the experimental values. However, results suggested that NOB populations in AS had a higher substrate affinity (K_{NO2} = 0.8 mg N·L^{-1}) compared to AB (K_{NO2} = 4.6 mg N·L^{-1}). Such values are highly consistent with recent respirometric studies reporting kinetic parameters of NOB for AS samples (Iacopozzi et al., 2007; Jiménez et al., 2012), along with recent AB models considering two-step nitrification (Casagli et al., 2021b; Solimeno et al., 2019). It should be finally noticed that NO_2^- concentrations in the AB cultivation system reached up to 144 mg N·L^{-1}. On the other hand, no relevant NO_2^- accumulation was ever recorded in the AS tank, due to the high nitrite-oxidizing activity, that always resulted in complete nitrification, regardless of the operational conditions. However, no explanation regarding the phenomenon of incomplete nitrification in AB reactors could be provided, based on the results of this study. Therefore, it is suggested that further experimental work is conducted, to evaluate other possible factors which could result in limited NOB growth, such as the half-saturation constants for NOB on other nutrients than nitrite (e.g., for inorganic carbon, or phosphorus). Results of kinetic parameter identification for nutrients are reported in Table 3.

4. Conclusions

The tested environmental and operational conditions were demonstrated to strongly impact each bacterial population (HB, AOB and NOB). Furthermore, bacteria in activated sludge and AB were differentially sensitive to the tested conditions, especially with respect to pH, dissolved oxygen, and nitrite concentration. This finding demonstrates that kinetic models developed and calibrated on the activated sludge process cannot be directly extended to AB processes. Respirometric techniques proved to be an essential tool to identify and calibrate proper kinetic models for the AB process, to be eventually applied as an optimization tool to improve the efficiency and stability of AB-based wastewater treatment.

CRediT authorship contribution statement

A. Sánchez-Zurano: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **S. Rossi:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **J.M. Fernández-Sevilla:** Validation, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **G. Acién-Fernández:** Validation, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **E. Molina-Grima:** Validation, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **E. Ficara:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Acknowledgements

This research was funded by Fondazione CARIPLO (project: “Il polo delle microalghe”) and by EU H2020 Framework Programme (project: PRODIGIO, 101007006). A. Sánchez-Zurano would like to thank the Spanish Ministry of Education (FPU16/05996).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.127116.

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