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# An innovative respirometric method to assess the autotrophic active fraction: Application to an alternate oxic–anoxic MBR pilot plant

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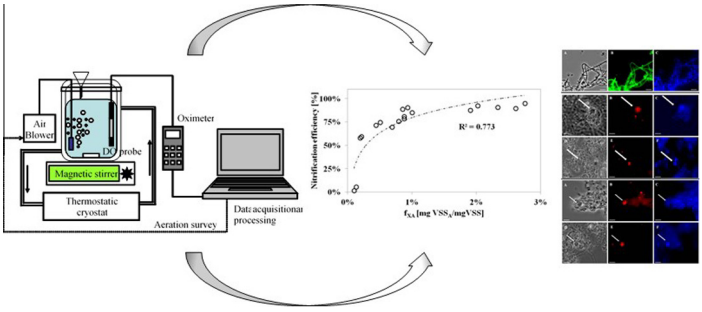
## HIGHLIGHTS

- Heterotrophic metabolic activity was not affected by the duration of aerated phase.
- The aerated phase increase resulted in a higher autotrophic biomass development.
- A novel respirometric method to estimate the autotrophic active fraction was proposed.
- Microbiological FISH analyses revealed the validity of proposed method.

## GRAPHICAL ABSTRACT

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## ABSTRACT

An innovative respirometric method was applied to evaluate the autotrophic active fraction in an alternate anoxic/oxic membrane bioreactor (MBR) pilot plant. The alternate cycle (AC) produces a complex microbiological environment that allows the development of both autotrophic and heterotrophic species in one reactor. The present study aimed to evaluate autotrophic and heterotrophic active fractions and highlight the effect of different aeration/non aeration ratios in a AC-MBR pilot plant using respirometry. The results outlined that the autotrophic active fraction values were consistent with the nitrification efficiency and FISH analyses, which suggests its usefulness for estimating the nitrifying population. Intermittent aeration did not significantly affect the heterotrophic metabolic activity but significantly affected the autotrophic biomass development. Finally, the heterotrophic active biomass was strongly affected by the wastewater characteristics, whereas the resultant autotrophic biomass was considerably affected by the duration of the aerated phase.



## 1. Introduction

In recent years, the demand for high-quality effluents and the scarcity of available areas to upgrade existing wastewater treatment plants (WWTPs) have driven the interest of the scientific community towards new and innovative solutions, which are more compact and efficient than conventional activated sludge (CAS) systems.

Among these new and innovative solutions, the alternated-cycle (AC) processes, which are characterized by alternating oxic/anoxic conditions in the same reactor using intermittent aeration (IA), and membrane biological reactors (MBRs) represent two of the most suitable alternatives to address the aforementioned issues.

The current MBR is a consolidated process that combines a biological process with a filtration unit (usually a microfiltration/ultra

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**Nomenclature**

| Symbol | Parameter |
|---|---|
| AC-MBR | alternated cycled membrane bioreactor |
| IA | intermittent aeration |
| FISH | fluorescence in situ hybridization |
| F/M | food on microorganisms |
| TSS | total suspended solids (mg $L^{-1}$) |
| VSS | volatile suspended solids (mg $L^{-1}$) |
| $BOD_5$ | biochemical oxygen demand (mg $L^{-1}$) |
| COD | chemical oxygen demand (mg $L^{-1}$) |
| $NH_4$-N | nitrogen as ammonium (mg $L^{-1}$) |
| AOB | ammonia-oxidizing bacteria |
| OUR | oxygen uptake rate (mg $O_2$ $L^{-1}$ $h^{-1}$) |
| SOUR | specific oxygen uptake rate (mg $O_2$ $g^{-1}$VSS $h^{-1}$) |
| $Y_H$ | heterotrophic yield coefficient (mgVSS $mg^{-1}$COD) |
| $Y_{STO}$ | heterotrophic storage yield coefficient (mgVSS $mg^{-1}$COD) |
| $b_H$ | heterotrophic decay rate ($d^{-1}$) |
| $\mu_H$ | heterotrophic growth rate coefficient ($d^{-1}$) |
| $v_{H,max}$ | maximum substrate removal rate (mg COD $mg^{-1}$VSS $d^{-1}$) |
| $Ks$ | half-Saturation coefficient for heterotrophic (mg COD $L^{-1}$) |
| $X_{B,H}$ | heterotrophic active biomass (mg $SSV_{active}$ $L^{-1}$) |
| $f_{XH}$ | active fraction of heterotrophic biomass |
| $Y_A$ | autotrophic yield coefficient (mgVSS $mg^{-1}$COD) |
| $\mu_A$ | autotrophic Growth rate coefficient ($d^{-1}$) |
| $v_{H,max}$ | maximum substrate removal rate (mg $NH_4$-N $mg^{-1}$VSS $d^{-1}$) |
| $K_A$ | half-Saturation coefficient for autotrophic (mg $NH_4$-N $L^{-1}$) |
| $b_A$ | autotrophic decay rate ($d^{-1}$) |
| $X_{B,A}$ | autotrophic active biomass (mg $SSV_{active}$ $L^{-1}$) |
| $f_{XA}$ | active fraction of autotrophic biomass |
| $i_{xb}$ | mass of nitrogen within the biomass at the beginning |
| $i_{xp}$ | mass of nitrogen within the biomass at the end |
| $f_p$ | fraction of inert biomass |
| HRT | hydraulic retention time (h) |
| SRT | sludge retention time (d) |
| OLR | organic loading rate (kg COD $m^{-3}$ $d^{-1}$) |

filtration membrane). Compared to CAS systems, MBRs feature higher effluent quality, smaller volumes and a lower amount of excess sludge because of the higher biomass concentration in the bioreactor [1]. In recent years, MBR systems were successfully coupled with the AC process, as reported by many authors [2–5]. The AC processes allow the removal of biological organic matter and nutrients in a single reactor by alternating in time the reactions that occur in different in-series reactors in the BNR traditional systems (University of Cape Town – UCT, Modified University of Cape Town – MUCT, Bardenpho) [6]. Therefore, AC processes may represent a suitable and effective strategy to save energy and improve the nutrient removal efficiency [7].

It is worth mentioning that IA introduces a cyclic stress condition on the biomass and consequently induces a modification of the biomass metabolism and biokinetic parameters, which reduces the sludge production rates [8]. Furthermore, the IA strategy enables aeration cost reduction, which represents more than 50% of the total energy consumption of the entire treatment process [9]. In recent years, the AC process has been successfully applied to upgrade existing WWTPs and highlights excellent performances even for nutrient removal (among others Battistoni et al. [10] and Wang et al. [11]). Moreover, the AC process is a low-cost solution because it only requires an automatic control system.

Nevertheless, because both autotrophic (nitrifying) and heterotrophic (denitrifying) microorganisms will grow in one reactor, many authors (among others Lim et al. [12] and Mota et al. [13]) noted that an incorrect aeration/non aeration ratio could promote an unbalanced growth of heterotrophic and autotrophic bacteria and worsen the removal efficiencies. Referring in particular to nitrogen removal, previous studies revealed a partial nitrification to nitrite under limiting oxygen condition [14,15]. Therefore, the proper balance of the aeration/non aeration ratio in the reaction cycle is a key parameter to achieve high removal efficiencies. Another challenge of AC processes (and BNR systems in general) is the necessity of a reliable evaluation of the biomass biokinetic activity. Indeed, in AC processes, the biomass biokinetic activity can be modified compared to that in the conventional in-series system, as previously discussed. With these considerations, the biokinetic parameters and active biomass fractions must be evaluated to properly operate these systems. In this context, respirometric techniques may be useful for the microbiological analysis of such complex systems [41,44].

The volatile suspended solid (VSS) concentration, which was mostly used in the past to quantify the active biomass in mixed liquors, may not be adequate to express the active biomass fraction. Indeed, in addition to the active microorganisms, VSS includes biodegradable/inert particles and residues from bacterial death and lysis phenomena (endogenous residue) [16]. The latter is not negligible in MBR systems because of the barrier effect that is exerted by the membrane. Because only the biomass active fraction is responsible for the biological removal process [17], its proper quantification is a key issue to understand the biological process and for mathematical modeling purposes. In recent years, many methods were proposed to quantify the active biomass in activated sludge (both heterotrophic and autotrophic), including fluorescence *in situ* hybridization (FISH) and flow cytometry (FCM) [18]. However, these techniques are quite expensive, difficult to implement and do not provide the response in a short time. As a result, the active biomass is generally estimated by applying activated-sludge models (ASMs) and using kinetic and stoichiometric parameters from the literature. Moreover, the heterotrophic active fraction determination could be achieved by applying several standardized methods such as respirometric endogenous batch test [19], whereas the autotrophic active fraction is currently determined by applying only ASMs. To the authors' knowledge, a direct tool to assess the autotrophic active fraction has not been proposed in the technical literature.

Therefore, this paper mainly aims to propose an innovative analytical procedure to evaluate the autotrophic active fraction by applying the ASM1 model and kinetic parameter, which is directly evaluated using respirometric batch tests. Fluorescent *in situ* hybridization (FISH) analyses were simultaneously performed to detect qualitatively the presence of ammonia-oxidizing bacteria (AOB), which are responsible for the oxidation of ammonium to nitrite.

Furthermore, the effects of different aeration/non aeration ratios on the heterotrophic and autotrophic kinetic parameters and active biomass fractions in an MBR pilot plant, which was operated with the AC strategy, are discussed. The effect of wastewater characteristics, in terms of influent N/COD ratio and COD

fractions, on the heterotrophic and autotrophic biomass growth is also discussed.

## 2. Materials and methods

### 2.1. AC-MBR pilot plant and operating condition description

The experimental study was performed on a MBR pilot plant, which was built at Palermo Municipal WWTP (Acqua dei Corsari) and operated with the AC strategy. The reaction tank (mean volume 0.48 $m^3$) of the MBR pilot plant was fed with real municipal wastewater, which was collected downstream of the WWTP screening unit and pumped into a stirred equalization tank after being sieved through a 2 mm screen. The aerobic/anoxic conditions were alternated by controlling the air blower using a programmable logic controller (PLC). The mixed liquor was pumped into the membrane compartment (volume 0.028 $m^3$), which was continuously aerated to reduce membrane fouling. Two submerged hollow fiber membrane modules, Zenon Zeeweed and ZW10 (pore size 0.04 μm and nominal surface 0.93 $m^2$), were installed into this compartment.

Briefly, the MBR pilot plant was operated for 120 days to investigate the effects of the AC strategy on the biological performances of the system. The entire experimental campaign was divided into three different periods by varying both the duration of the aerated and non-aerated phases ($t_A$ and $t_{NA}$, respectively) and the cycle length. In particular, Period 1 (duration: 57 days) was performed with a cycle length of 180 min (60 min of aeration and 120 min of no aeration). Period 2 (duration: 47 days) was performed with the identical cycle length to the former but split into 80 min of aeration and 100 min of no aeration. Finally, Period 3 (duration: 16 days) was operated with a cycle length of 90 min, which was split into 30 min of aeration and 60 min of no aeration. The DO concentrations ranged between 0 (beginning) and a maximum value (end) that depended on the duration of the aeration phase. In particular, the maximum DO concentration ranged between 4 and 5 mg $L^{-1}$ for the whole experimentation. From day 0 to day 22 (Period 1), the MBR pilot plant was operated with regular sludge withdrawals to set the sludge retention time (SRT) close to 5 days. However, because of the low strength of the influent wastewater, by day 34, we decided to operate the MBR pilot plant with a complete sludge retention strategy. Table 1 reports the main features of each cycle as well as the main characteristics of the feeding wastewater and the operating conditions of the MBR pilot plant (average values). For further details on the experimental campaign, analytical procedures and biological performances, the reader is referred to the literature [7].

### 2.2. Respirometric apparatus and batch test description

Biomass samples for the biokinetic-parameter evaluation were obtained from the reaction tank and aerated until endogenous conditions were attained based on the oxygen uptake rate (OUR) monitoring. The collected samples for the batch tests were eventually diluted with pilot plant permeate to obtain a mixed liquor suspended solid (MLSS) concentration of 2–3 gVSS $L^{-1}$. The Respirometric apparatus consisted of a flowing-gas/static-liquid respirometer, which was connected to a thermostatic cryostat to maintain the sample temperature near 20 °C. The batch tests were performed with an intermittent aeration strategy; in detail, mixing was provided using fine-bubble aerators during the aerated phases and magnetic stirrers during the non-aeration phases. The dissolved oxygen concentration was measured through an oxygen probe (WTW CellOX 325), which was coupled to an oximeter (WTW MULTI340i). All batch tests were performed with a dissolved oxygen concentration of 3.5–5.5 $mgO_2$ $L^{-1}$. OUR values were evaluated as the slope of the oxygen profile during the consumption phase by bacteria following the substrate spiking. In the batch tests aimed to evaluate the biokinetic parameters of heterotrophic species, the nitrifying biomass was inhibited by adding Allylthiourea (ATU), where the exogenous oxygen uptake rate (OUR) was enhanced by adding sodium acetate ($CH_3COONa$) as a readily biodegradable organic substrate. Regarding the autotrophic species, the biokinetic parameters were measured with the identical experimental procedure, where ammonium chloride ($NH_4Cl$) was spiked as the substrate. Then, the biokinetic parameters were achieved by processing the respirogram charts. For further details on the adopted procedure, the reader is referred to the literature [20].

### 2.3. Heterotrophic biomass characterization

The heterotrophic active biomass ($X_{BH:}$ $mgVSS_{active}$ $L^{-1}$) was evaluated by applying the method proposed by Fall et al. [19]. The endogenous decay coefficient ($b_H$) was evaluated according to the single batch test, which was proposed *inter alia* by Vanrolleghem et al. [21]. The yield coefficient ($Y_H$), the storage yield coefficient ($Y_{sto}$), maximum removal rate ($v_{H,max}$), maximum growth rate ($\mu_{H,max}$) and half-saturation coefficient ($K_S$) for organic matter were evaluated according to procedures reported in Di Trapani et al. [22].

### 2.4. Autotrophic biomass characterization

Similarly to heterotrophic species, to estimate the autotrophic biomass kinetic parameters, the biomass active fraction in the sample must be evaluated. Based on the ASM1 "death-regeneration" model, the endogenous OUR was related to the autotrophic active biomass through the following expression [16]:

$$OUR_{end,Aut} = (i_{xb-f_p \cdot i_{xp}}) \cdot (4.57 - Y_A) \cdot b_A \cdot X_{BA} + (1 - f_p) \cdot (1 - Y_H) \cdot b_A \cdot X_{BA} \quad (1)$$

where

- $i_{xb}$: mass of nitrogen contained within the biomass at the beginning (default value 0.086);
- $i_{xp}$: nitrogen mass in the biomass at the end (default value: 0.06);
- $f_p$: fraction of inert biomass residues (default value 0.2);
- $Y_H$: heterotrophic yield coefficient (obtained from the heterotrophic batch test as an average value);
- $Y_A$: autotrophic specific yield coefficient (obtained from the autotrophic batch test);

**Table 1**
Main characteristics of aeration cycles, operating conditions, feeding wastewater and reactor biological performances.

| Period | Cycle length (min) | $T_A/T_{NA}$ – | COD,in (mg $L^{-1}$) | $BOD_5$,in (mg $L^{-1}$) | TN,in (mg $L^{-1}$) | OLR (kgCOD $m^{-3}d^{-1}$) | F/M (kgCOD $kg^{-1}VSSd^{-1}$) | ηCOD ($d^{-1}$) | ηTN (-) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 180 | 0.5 | 380 | 221 | 42 | 0.21 | 0.08 | 86 ± 12 | 76 ± 11 |
| 2 | 180 | 0.8 | 483 | 245 | 36 | 0.26 | 0.06 | 89 ± 9 | 75 ± 14 |
| 3 | 90 | 0.5 | 414 | 201 | 28 | 0.19 | 0.05 | 91 ± 1 | 55 ± 21 |

- $b_A$: autotrophic decay rate;
- $OUR_{end,Aut}$: endogenous uptake rate of the autotrophic biomass at the beginning of the batch test;
- $X_{BA}$: autotrophic active biomass concentration in the sample ($mgVSS_{active}\ L^{-1}$).

When turning to logarithms, Eq. (1) becomes Eq. (2):

$$\ln OUR_{end,Aut} = Ln\lfloor (i_{xb} - f_p \cdot i_{xp}) \cdot (4.57 - Y_A) \cdot b_A \cdot X_{BA} + (1 - f_p) \cdot (1 - Y_H) \cdot b_A \cdot X_{BA} \rfloor \quad (2)$$

The autotrophic decay rate ($b_A$) and endogenous uptake rate at the beginning of the batch test ($OUR_{end,Aut}$) were evaluated following the "multiple-batch test" procedure proposed by Avcioglu et al. [23]. In short, each multiple-batch test had a duration of 4–6 days and involved using a continuously aerated reactor to maintain the autotrophic biomass in starvation conditions throughout the test (digestion reactor). At daily intervals, a sample from this reactor was fed into the batch respirometer, and the autotrophic biomass activity was monitored through a single-batch test by ammonium chloride spiking. Progressively, the maximum OUR decreased because of the autotrophic biomass decay, as shown in the chart in Fig. 1a, which is provided as an example.

We plotted the $\ln OUR_{(t)}/\ln OUR_{(t0)}$ ratio *vs* time, where $\ln OUR_{(t)}$ is the difference between the exogenous and endogenous OUR values at time $t$, and $\ln OUR_{(t0)}$ is the difference in at time $t_0$. Then, $OUR_{end,Aut}$ and $b_A$ were obtained as the intercept and slope of the regression line, respectively (Fig. 1b). Therefore, the autotrophic active biomass concentration in the sample was obtained simply by applying Eq. (2). The reliability of the proposed method was evaluated by comparing the active fraction values with the nitrification efficiency and FISH analyses, which is better outlined as follows.

As aforementioned, other autotrophic kinetic parameters (e.g., maximum removal rate ($v_{N,max}$) and half-saturation coefficient ($K_N$) for the ammonium substrate) were estimated by applying the identical procedures for the heterotrophic biomass with the exception of the ATU dosage and spiked substrate (ammonium chloride). The autotrophic specific yield coefficient $Y_A$ was evaluated according to Chandran and Smets [24].

### 2.5. FISH (fluorescence in situ hybridization) analysis and microscopy-image analysis

FISH analysis was performed on three samples, which were collected in the three experimental periods. The samples were taken on days 27 (Period 1), 92 (Period 2) and 119 (Period 3) to analyze the effects of different operating conditions. Moreover, samples with different estimates of the active fraction were selected to support the method reliability.

FISH with 16S rRNA targeted probes was performed according to the described procedure in Amann et al. [25] on the samples (three replicates each), which were collected from the reaction tank. The ammonia-oxidizing $\beta$-proteobacteria ($\beta$-AOB) were identified using the fluorescent-labeled probe NSO1225. Equimolar mixtures of the probes EUB338 (specific for most members of the domain Bacteria), EUB338II (specific for Planctomycetales) and EUB338III (specific for Verrucomicrobiales) were used to detect almost all members of the domain Bacteria. The specific sequences for each probe and formamide percentage were obtained from the probeBase database [26]. The NSO1225 probe and EUB338 mixture probe were synthesized with the Cy3 label and FITC label, respectively and purchased from MWG AG Biotech (Germany). All hybridizations with the AOB specific probe were also performed with DAPI staining to detect the total biomass, which was directly added to the hybridization buffer at a final concentration of $l\ \mu g\ mL^{-1}$. Replicates were observed using an epifluorescence microscope (Olympus BX51) at 1000 × magnification with filters for FITC (excitation, 470–490 nm; emission, 520 nm) and CY3 (excitation, 546 nm; emission, 590 nm). The images were taken with a digital camera (Olympus XM-10). The ImageJ software package (version1.37v, Wayne Rasband, National Institute of Health, Bethesda, MD, USA, available in the public domain at http://rsb.info.nih.gov/ij/index.html) was used for image analyses. Furthermore, it is important to stress that FISH results were not used with the aim to quantify the autotrophic active fraction, but only to detect their presence/absence, thus supporting the respirometric results.

## 3. Results and discussion

### 3.1. Autotrophic and heterotrophic kinetic parameters

As previously discussed, respirometric batch tests enabled us to evaluate the biomass kinetic parameters. The average results for both heterotrophic and autotrophic species are shown in Table 2, and the literature values are reported in Table 3 for comparison with the results of the present study.

Regarding the heterotrophic biomass, the lowest specific yield coefficient $Y_H$ was obtained in Period 1 (0.38 gVSS $g^{-1}$COD), when the $t_A/t_{NA}$ ratio was 0.5. However, $Y_H$ increased to 0.42 and 0.43 gVSS $g^{-1}$COD (average values) in Periods 2 and 3, when the $t_A/t_{NA}$ ratio was 0.8 and 0.5, respectively. These values were slightly lower than those obtained in other AC plants [28], possibly because of the lower organic loading rates; nevertheless, they were consistent with previous data in MBR systems [33].

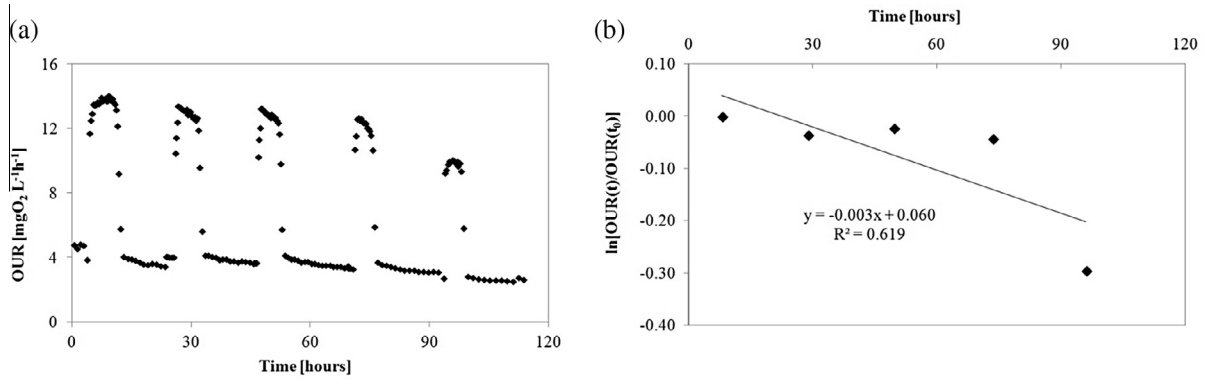


**Fig. 1.** Multiple-batch test for autotrophic endogenous decay rate estimation (a), logarithmic OUR profile vs. time (b).

**Table 2**
Average values of biokinetic parameters for both heterotrophic and autotrophic biomass in this experimentation.

| Period | $Y_H$ (mgVSS mg$^{-1}$COD) | $Y_{sto}$ (mgVSS mg$^{-1}$COD) | $\mu_H$ (d$^{-1}$) | $K_s$ (mg L$^{-1}$) | $v_H$ (mgCOD mg$^{-1}$VSSd$^{-1}$) | $b_H$ (d$^{-1}$) | $f_{XH}$ (-) | $SOUR_{max}$ (mg$O_2$ g$^{-1}$VSSh$^{-1}$) | $SOUR_{end}$ (mg$O_2$ g$^{-1}$VSSh$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.38 | 0.49 | 1.65 | 3.12 | 4.03 | 0.64 | 0.18 | 26.57 | 3.91 |
| 2 | 0.42 | 0.50 | 1.51 | 2.92 | 3.50 | 0.82 | 0.09 | 10.38 | 1.68 |
| 3 | 0.43 | 0.51 | 1.17 | 1.87 | 2.49 | 1.40 | 0.07 | 5.70 | 1.08 |
| Period | $Y_A$ (mgVSS mg$^{-1}$N) | | $\mu_A$ (d$^{-1}$) | $K_A$ (mg L$^{-1}$) | $v_A$ (mg$NH_4$-N mg$^{-1}$VSSd$^{-1}$) | $b_A$ (d$^{-1}$) | $f_{XA}$ (–) | $SOUR_{max}$ (mg$O_2$ g$^{-1}$VSSh$^{-1}$) | |
| 1 | 0.26 | | 1.45 | 0.25 | 4.61 | 0.07 | 0.0114 | 10.92 | |
| 2 | 0.27 | | 1.67 | 1.67 | 8.92 | 0.09 | 0.0079 | 14.84 | |
| 3 | 0.19 | | 0.28 | 0.28 | 1.99 | 0.08 | 0.0078 | 5.35 | |

**Table 3**
References values of biokinetic parameters for both heterotrophic and autotrophic biomass.

| Heterotrophic References | Process | $Y_H$ (mgVSS mg$^{-1}$COD) | $\mu_H$ (d$^{-1}$) | $v_H$ (mgCOD mg$^{-1}$VSSd$^{-1}$) | $b_H$ (d$^{-1}$) |
|---|---|---|---|---|---|
| [22] | UCT-MBR | 0.36–0.46 | 0.4–2.4 | – | 0.3–0.56 |
| [27] | | 0.46 | 3–6 | – | 0.2–0.62 |
| [28] | AC–CAS | 0.455 | 13.29 | 29.21 | 0.53 |
| [28] | CAS with pre denitrification | 0.469 | 9.48 | 20.21 | – |
| [29] | AC–CAS | 0.412 | – | – | – |
| [29] | CAS with pre denitrification | 0.468 | – | – | – |
| [30] | MEBPR | 0.5 | 8.36 | 16.72 | 0.24 |
| [30] | CEBPR | 0.59 | 11.9 | 20.17 | 0.31 |
| [31] | CAS | 0.46 | 2 | 4.35 | 0.1 |
| [32] | MBR | 0.28–0.55 | – | – | 0.05 |
| [33] | MBR | 0.48 | 0.93 | 1.94 | 0.11 |
| Autotrophic | | $Y_A$ (mgVSS mg$^{-1}$$NH_4$-N) | $\mu_A$ (d$^{-1}$) | $v_A$ (mg$NH_4$-N mg$^{-1}$VSSd$^{-1}$) | $b_A$ (d$^{-1}$) |
| [6] | | 0.10–0.15 | 0.2–0.9 | 2–6 | 0.05–0.15 |
| [22] | UCT-MBR | 0.18 | 0.27 | 1.5 | 0.074 |
| [34] | MBR with pre denitrification | 0.24 | 0.15 | 0.625 | 0.08 |
| [33] | MBR | 0.263 | 0.05 | 0.18 | 0.09 |
| [35] | CAS | 0.22 | 0.264 | 1.2 | – |
| [36] | CAS | 0.19–0.26 | 0.26–0.38 | 1.39–1.48 | – |
| [42] | Batch tests | n.a. | 0.65–1.40 | n.a. | n.a. |
| [43] | AC–SBR | n.a. | 0.8–1.6 | 2–7.5 | 0.04–0.22 |

The highest value of the maximum growth rate $\mu_{H,max}$ (3.04 d$^{-1}$) was obtained at the beginning of Period 1, when sludge withdrawals were operated. Nevertheless, this value rapidly decreased to an average value of 1.65 d$^{-1}$, when sludge withdrawals were interrupted after experimental day 34. In Periods 2 and 3, when a complete sludge retention strategy was operated, the $\mu_{H,max}$ values slowly decreased in the experiments from 1.65 to 1.51 d$^{-1}$ in Period 2 and from 1.51 to 1.1 d$^{-1}$ in Period 3. However, the endogenous decay coefficient $b_H$ showed an opposite trend: from the average value of 0.64 d$^{-1}$ in Period 1, it slightly increased to 0.82 d$^{-1}$ and 1.04 d$^{-1}$ (as average) in Periods 2 and 3, respectively.

The decrease of $\mu_{H,max}$ and a contextual increase of the decay rate $b_H$ are likely related to the decrease in the food/microorganism (F/M) ratio. In fact, under these conditions, competition among the heterotrophic microorganisms may arise for the organic substrate availability, and its scarcity promotes a limiting condition for biomass growth. Moreover, the increase in $b_H$ may be a result of the "indefinite SRT" strategy during Periods 2 and 3, which promoted biomass ageing and consequently affected the rate of cell lysis and endogenous respiration.

The biomass respiratory activity, which is expressed in terms of the specific oxygen uptake rate (SOUR), did not show a clear relationship with the $t_A/t_{NA}$ ratio. SOUR progressively decreased throughout the experiments, even when the aerated fraction was 0.8. This behavior has been noticed by other authors [22,37] in pilot plants that are operated with continuous aeration and complete sludge retention. In such conditions, the SOUR decrease can be imputable to biomass ageing, which involves a progressive reduction of its metabolic activity.

The achieved results suggest that the $t_A/t_{NA}$ ratio did not significantly affect the net growth rate of the heterotrophic population and the synthesis of new biomass, as expected. However, it is important to stress that the results of the present study were likely affected by the low organic loading rates. This condition, which results in a low F/M ratio, leads to slow oxygen depletion in the bioreactor; therefore, aerobic conditions were also maintained during a portion of the non-aerated phase [7]. Thus, under these peculiar operating conditions, the absence of aeration is not a stress condition for the heterotrophic biomass metabolism, and the effect of the alternate cycle on the kinetic parameters of heterotrophic species cannot be properly verified.

Regarding the autotrophic biomass, the kinetic parameters were significantly affected by the fluctuations in the active autotrophic fraction, as better outlined in the following. Indeed, because of sludge withdrawals in Period 1 and a temporary plant shutdown in the middle of Period 2, the autotrophic active biomass fraction did not reach steady-state conditions. Accordingly, the kinetic parameters did not show a regular trend during the experiments. In Period 1, because of the low SRT value, the autotrophic

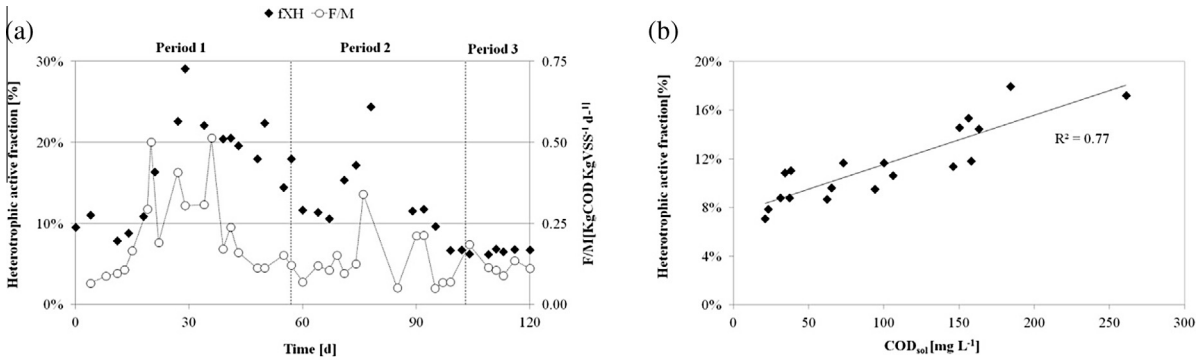


**Fig. 2.** Trend of the heterotrophic active fraction and influent OLR (a); correlation between the heterotrophic active fraction and influent soluble COD (b).

biomass was subjected to a rapid washout. Consequently, the active fraction quickly decreased to nearly zero. However, after sludge withdrawals were interrupted on day 34, the autotrophic microorganisms began to accumulate again in the bioreactor. In Period 1, because of sludge withdrawals, the autotrophic active fraction from the inoculum value, which was 4.33%, collapsed to 0% on experimental day 27. After day 34, because the plant was operated with complete sludge retention, the active fraction increased to 0.21% in the last days of Period 1. Consequently, the maximum specific growth rate $\mu_{A,max}$ showed high fluctuations of 0.104–5.56 $d^{-1}$; the latter value corresponds to the minimum autotrophic active fraction. Conversely, in Periods 2 and 3, the maximum specific growth rate $\mu_{A,max}$ (average values) decreased to 1.67 $d^{-1}$ and 0.28 $d^{-1}$, respectively. The maximum specific growth rates resulted higher compared literature data (Table 3). However, the achieved values were in line with previous data obtained in reactors working with intermittent aeration [43], suggesting that this kind of systems enable the growth of autotrophic biomass with higher metabolic activity. The autotrophic endogenous decay rate showed a similar trend: it was at a maximum in Period 1 (0.27 $d^{-1}$) because of the low SRT value and almost constant in Periods 2 and 3 (0.09 $d^{-1}$ and 0.08 $d^{-1}$, respectively). It is worth noting that the $\mu_{A,max}$ value in Period 1 cannot be compared with the others. Indeed, in this period, the system operating conditions led to unlimited growth of autotrophic microorganisms, which may explain the observed high values. This result is consistent with the reported result of Pollice et al. [15] for an intermittent aeration system. Pollice and co-workers found that a lower sludge age resulted in a decrease in autotrophic biomass concentration, which increased the ammonium availability and led to a higher specific growth rate. Comparing the $\mu_{A,max}$ values in Periods 2 and 3, which are characterized by similar autotrophic active fractions, it can be noticed that the highest values were obtained when the $t_A/t_{NA}$ ratio was 0.8. This result suggests that the increase in the $t_A/t_{NA}$ ratio will result in higher autotrophic biomass growth. Similar observations are drawn for the autotrophic yield coefficient $Y_A$ and SOUR values. In particular, the heterotrophic and autotrophic SOUR values were notably similar, once sludge withdrawals were interrupted, which enabled the growth of autotrophic microorganisms. Therefore, both intermittent aeration and complete sludge retention enable a balanced development of autotrophic and heterotrophic metabolic activity in the bioreactor.

### 3.2. Heterotrophic and autotrophic active fractions

Referring to the heterotrophic active fraction, several batch tests were performed in the experiments to evaluate the effects of different $t_A/t_{NA}$ ratios on this parameter. In more detail, Fig. 2a shows the obtained heterotrophic active fractions that were coupled to the organic loading rate (OLR) values fed to the pilot plant, whereas Fig. 2b shows the relationship between soluble COD ($COD_{sol}$) and heterotrophic active fraction.

Fig. 2a shows that the $t_A/t_{NA}$ ratio does not clearly affect the heterotrophic active fraction; in detail, with the exception of Period 3, the heterotrophic active fraction significant fluctuated throughout the experiment from 8% to 30% and reached a notable steady-state condition only at the end of the experiments. This behavior was likely because of the significant variations of the influent wastewater characteristics; in particular, as noticed from Fig. 2b, the heterotrophic active fraction is strongly affected by the influent $COD_{sol}$ (correlation coefficient: $R^2$ = 0.77), which is almost representative of the readily biodegradable organic fraction of the wastewater. Therefore, the influent wastewater characteristics, particularly the low OLR, affect the heterotrophic active fraction more than the $t_A/t_{NA}$ ratio. In this context, the effect of the aerobic and anoxic phase duration on the heterotrophic active fraction was not properly highlighted.

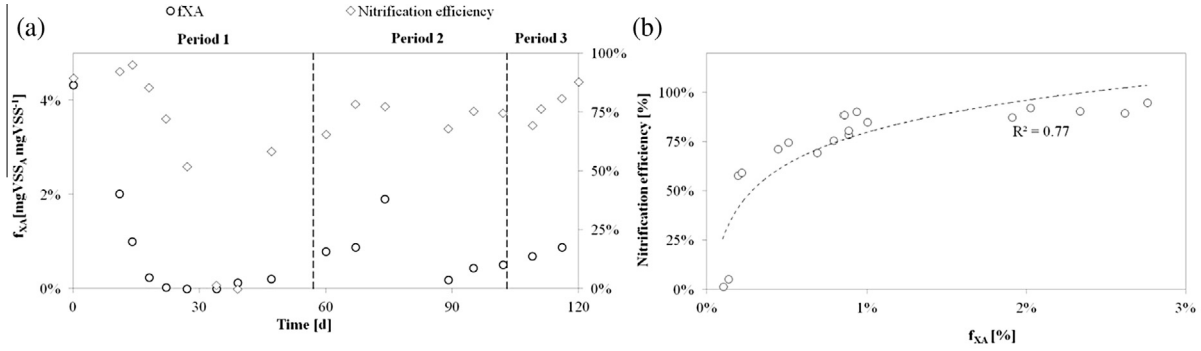


**Fig. 3.** Autotrophic active fraction and nitrification efficiency trend (a); relation between two parameters (b).

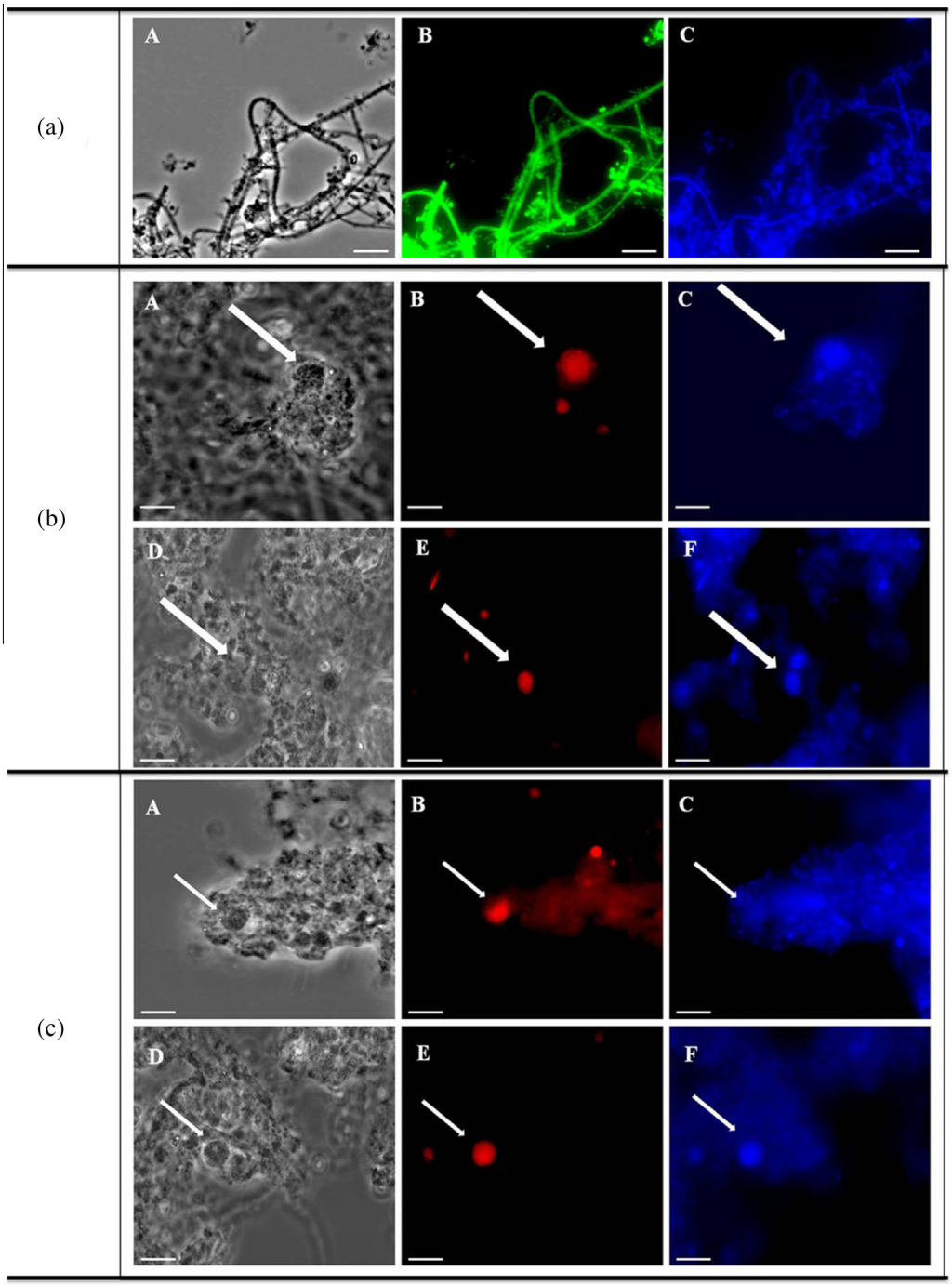


**Fig. 4.** *In situ* epifluorescence micrographs of $\beta$-AOB communities in the samples of the first (a), second (b) and third periods (c), as detected with FISH. (A) and (D) contrast phase micrographs of floc; (B) and (E) rod-shaped $\beta$-AOB bacteria associated in aggregates in the flocs, which were targeted with probe NSO1225 (red signal); (C) and (F) total cells stained with DAPI (blue signal). Bar: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Multiple-batch tests were performed on autotrophic bacteria to evaluate the autotrophic active fraction in each experimental period. The autotrophic active fraction was obtained as the ratio between the active biomass concentration ($X_{BA}$) and the VSS concentrations. The obtained results are shown in Fig. 3a with the nitrification efficiency trend, and Fig. 3b shows the relationship between $f_{XA}$ and the nitrification efficiency.

The data in Fig.e 3a show two different trends: an initial sharp decrease of the autotrophic active biomass from 4.3% (inoculum sludge) to 0% in less than 20 days because of the autotrophic biomass washout, which was determined by the low applied SRT. Therefore, after sludge withdrawals were stopped, the $f_{XA}$ values slowly increased at the end of Period 1. In Periods 2 and 3, two different trends can be observed, which are related to the complete sludge retention strategy that was interrupted by a plant failure in the middle of Period 2.

In general, the autotrophic kinetic parameters resulted higher compared to literature ones. The autotrophic metabolic activity is generally higher in alternating aerobic/anoxic conditions and the results obtained in the present study are in line with those reported by Munz et al. [43]. This result could be likely related to the fact that alternating oxic/anoxic conditions allow to maintain a good balance between heterotrophic and autotrophic microorganisms, decreasing the competition for oxygen. Indeed, the

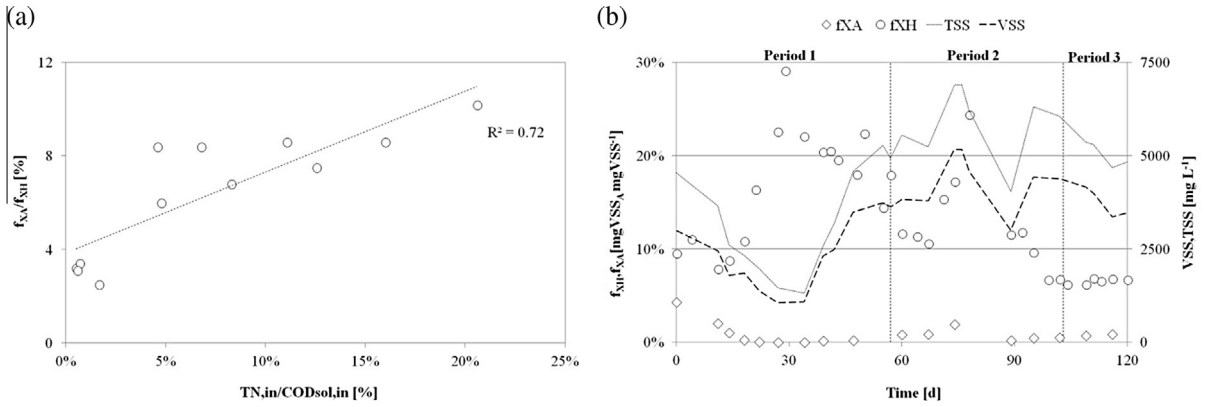


**Fig. 5.** Correspondence between the heterotrophic/autotrophic active fraction ratio and the $NH_4$-N/COD ratio of the feeding wastewater (a); trend of autotrophic and heterotrophic active biomass fractions and VSS-TSS (b).

heterotrophic growth under anoxic/anaerobic conditions is significantly lower compared to aerobic conditions, thus promoting higher autotrophic growth rates.

### 3.3. FISH and microscopy image analyses

The FISH analyses, which were performed on three samples taken in different experimental periods, show the absence of AOB populations in the sample in Period 1 (Fig. 4a), which is consistent with the results of the respirometric batch tests. As previously discussed, the short applied SRT caused a rapid washout of autotrophic species. Therefore, these conditions were incompatible with their growth in the bioreactor. Conversely, the $\beta$-AOB cells were found in the samples of Period 2 (Fig. 4b) and Period 3 (Fig. 4c), when the biomass respirometric activity was detected, and appeared as rod-shaped bacteria in aggregates with variable dimensions and patchy distribution in the flocs. The image analysis reveals high variability in AOB density among different microscopic fields, which is consistent with previous studies [38], and they were mainly localized at the core of the flocs/biofilms. The $\beta$-AOB cells were tightly packed and formed 5–15 μm aggregates. The image analysis also showed voids in the clusters, which likely facilitated the exchange of nutrients and gases between the surface and deep regions of the clusters. The size and morphology of the investigated AOB populations are consistent with those reported in the literature [38,39], although AOB clusters can also be present in larger aggregates depending on the sample age [39,40].

Microbiological analyses reveal the presence/absence of active autotrophic microorganisms, which is consistent with what is observed in respirometric batch tests. The consistency of the achieved $f_{XA}$ values with the biological performance was also assessed by comparing the $f_{XA}$ values with the observed nitrification efficiency throughout the experiments. Indeed, as shown in Fig. 3a, the nitrification efficiency and active fraction showed similar trends throughout the experimental campaign, which suggests an effective consistency of $f_{XA}$ values that were assessed with the proposed methodology. Moreover, the correspondence between active fraction and nitrification is clearly highlighted in Fig. 3b: the results showed a significant relationship ($R^2 = 0.77$) between the two parameters, which suggests that the $f_{XA}$ assessment provides consistent information about the biological process under study. Therefore, the autotrophic active fraction that was evaluated with the proposed method is a valid indication to estimate the nitrifying population in the bioreactor. As previously discussed, the procedure to evaluate $f_{XA}$ was proposed to better understand the biological process with reference to plant management and mathematical modeling.

Furthermore, it must be stressed that with a low ammonia loading rate, an active fraction of approximately 1÷1.5% is sufficiently high to provide a nitrification efficiency above 90%. Indeed, the nitrification efficiency was almost independent of the $f_{XA}$ values down to 1%; for lower values, a sharp decrease in nitrification efficiency was observed. Higher SRT values did not significantly affect the nitrification efficiency, even if they enabled high percentages of autotrophic active fraction, but they induced lower heterotrophic metabolic activity because of sludge ageing [37].

Similarly to the result of the heterotrophic biomass, a clear relationship between $f_{XA}$ and $t_A/t_{NA}$ was not observable. This circumstance can be ascribed to the fact that steady-state conditions were not attained at the end of each experimental period. Although a slightly higher active fraction was observed in Period 2 (2%), 17 days after it began, in Period 3, the active fraction was halved (0.9%) after the identical time length. Therefore, the $t_A/t_{NA}$ ratio may play a role in the development of the autotrophic active fraction. In particular, regardless of the sludge age, a higher duration of the aerated phase in the cycle may increase the autotrophic biomass development and active biomass amount. However, further studies in steady-state conditions are necessary to confirm this hypothesis.

Furthermore, Fig. 5a shows a significant correspondence between the heterotrophic/autotrophic active fraction ($f_{XH}/f_{XA}$) ratio and the $NH_4$-N/COD ratio of the feeding wastewater.

The data in Fig. 5a are only relative to complete sludge retention conditions. These results show that in the presence of high N/C values, both autotrophic and heterotrophic microorganisms can grow without significant competition for ammonia substrate. Therefore, a good balance between two species can be maintained in the bioreactor under these conditions.

Fig. 5b compares the VSS, TSS and active fractions values for the entire experiments. The autotrophic active biomass and SS had similar trends, whereas the heterotrophic active fraction significantly differs from the VSS trend. These results highlight that VSS is not a reliable parameter to properly describe the heterotrophic active biomass amount, particularly when the system is under dynamic conditions. Conversely, although the VSS measurement does not induce significant error in the estimate of $f_{XA}$ (considering average values in the range of 1–2%), the results of the present study suggest that the operating conditions can significantly affect $f_{XA}$, and a proper estimate is essential to improve the comprehension of the biological process under study.

## 4. Conclusions

The results outlined that the $t_A/t_{NA}$ ratio did not affect the heterotrophic metabolic activity because of the low strength of the influent wastewater. However, the increase in $t_A/t_{NA}$ resulted in

high autotrophic biomass development. This study demonstrates that the heterotrophic fraction is independent from the $t_A/t_{NA}$ ratio but depends on the influent $COD_{sol}$. A higher duration of the aerated phase leads to a higher autotrophic active fraction regardless of the sludge age. The autotrophic active fractions are consistent with the nitrification efficiency and FISH analyses. Therefore, the proposed method is a valid indication to estimate the active amount of the nitrifying population.

## Acknowledgements

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