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Influence of an oxic settling anoxic system on biomass activity, protozoa and filamentous bacteria

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1 **Abstract**

2 An oxic settling anoxic system coupled with an activated sludge process has been
3 studied to reduce sewage sludge production. The reduction showed in the experimented
4 system for sludge yield, excess sludge production and active biomass yield were 51.7%,
5 52.9% and 67.1%, respectively, compared with the control system. The oxic reactor of
6 the OSA system, even with a lower active biomass concentration than the oxic reactor
7 of control system, showed a higher metabolic activity in their active biomass. Up to our
8 knowledge, no studies have been done about the influence of the experimented system
9 on the active biomass, protozoa and filamentous bacteria population as done in the
10 present study. Diversity and crawling ciliates group have been shown as promising
11 bioindicators of active biomass yield reduction. The identification of floc-forming
12 bacteria in the control system suggested that OSA system most likely will improve
13 settling properties compared to a CAS process.

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22 Keywords: oxic settling anoxic system; sludge yield; excess sludge; active biomass; protozoa
23 population; filamentous bacteria.

1. Introduction

The Conventional Activated Sludge (CAS) process has been extensively applied to wastewater treatment (Zhou *et al.*, 2015). The large amount of sewage sludge produced from CAS process is a waste management problem. Handling, treatment and disposal costs of the sewage sludge are important concerns for most countries. The increase of the annual sewage sludge worldwide production is expecting to exceed 13 million tons Dried Solids (DS) in 2020 (Kelessidis and Stasinakis, 2012). In view of this, the reduction of sewage sludge production has become one of the first priorities in waste management hierarchy (CEU, 2008).

Many technologies are currently developed to reduce and/or treat sewage sludge in large installations (Cieřlik *et al.*, 2015) and small-scale installations (Wu *et al.*, 2013). However, medium size installations, i.e. around 50000 equivalent population are still a challenge in order to efficiently reduce sludge production. Several technologies are currently developed to reduce sludge production in medium size installations such as the sludge drying bed, worm treatment, thermal processes, drying systems, incineration or co-incineration (Cieřlik *et al.*, 2015).

One promising option as sewage sludge reduction method is the application of the Oxidative Settling Anoxic (OSA) system, which consist in the implementation of an anoxic reactor in the sludge recirculation line of a CAS process. The OSA system shows many advantages such as easy application, avoids undesirable products and seems sustainable in the long term (Coma *et al.*, 2013; Guo *et al.*, 2013; Zhou *et al.*, 2015, 2014). The OSA system is easy to implement into existing wastewater treatment processes and its operation should not be more complex than the previously existing wastewater treatment process. The OSA system has been show as a feasible solution of sewage sludge reduction in medium size installations (Coma *et al.*, 2013).

1 The alternating of the oxic/anoxic/anaerobic environments in wastewater treatment
2 processes has been studied to reduce sewage sludge production in several
3 configurations. The OSA system implementation in a CAS process has been studied by
4 the inclusion of an anoxic reactor in the sludge recirculation line (An and Chen, 2008;
5 Chudoba et al., 1992; Ye et al., 2008). The inclusion of a settler after the Membrane
6 Bioreactor (MBR) and an anaerobic reactor in the sludge recirculation line has been
7 studied as well (Chen *et al.*, 2003; Saby *et al.*, 2003). Also, the inclusion of an anoxic,
8 anaerobic and aerobic reactor after the Moving Bed Biofilm Reactor (MBBR) has been
9 studied (Li *et al.*, 2014). Furthermore, in an anaerobic, anoxic and aerobic reactor has
10 been studied by the inclusion of an anaerobic Side-Stream Reactor (SSR) in the sludge
11 recirculation line (Coma *et al.*, 2013). The inclusion of an anaerobic SSR in the sludge
12 recirculation line of an aerobic Sequencing Batch Reactor (SBR) has been studied as
13 well (Yagci *et al.*, 2015). Also, inclusion of an anaerobic reactor in the sludge
14 recirculation line in an anoxic, oxic reactor has been studied (Zhou *et al.*, 2015, 2014).
15 All of these authors has been reported that the alternating of the oxic/anoxic/anaerobic
16 environments is a highly promising option for sewage sludge reduction.
17 Up to our knowledge, few studies have focused on microbiological dynamics in the
18 OSA system. The anoxic environment have been shown to impact in the cellular
19 metabolism, sludge ecology, protozoa and filamentous bacteria (Rodriguez-Perez and
20 Fermoso, 2015), but it has not been far investigated in a continuous lab scale process.
21 The study of protozoa and filamentous bacteria could be important for the further
22 understanding of the process, due to its role as potential bioindicator as was suggested
23 by Rodriguez-Perez and Fermoso (2015).
24 The aim of this study was the implementation of the OSA system in a continuous CAS
25 process, focusing on the biological sludge minimization. The impact of the OSA system

1 application in the cellular metabolism, protozoa population and filamentous bacteria
2 were investigated. Protozoa population was analysed to determine its role as a potential
3 bioindicators.

4 **2. Materials and methods**

5 **2.1. Inoculum and influent**

6 The inoculum was taken from the secondary sludge recirculation line of a municipal
7 wastewater treatment plant in Seville, Spain. The characteristics of the inoculum used
8 were: 3.4 g L⁻¹ of Total Suspended Solids (TSS) concentration, 2.3 g L⁻¹ of Volatile
9 Suspended Solids (VSS) concentration and 85 ± 10 nmol mL⁻¹ of the Suspended Lipid
10 Phosphate (SLP) concentration.

11 The influent of the continuous systems was prepared with a mixture of glucose, as sole
12 added organic matter, in a mineral medium described by Rodriguez-Perez and Feroso
13 (2015). The pH was adjusted to an initial value of 7.25 ± 0.25 and was kept with
14 phosphate buffer solution. The mean concentration of the influent Chemical Organic
15 Demand (COD) was 282 ± 7 mg COD L⁻¹.

16 **2.2. Experimental procedure**

17 *2.2.1. Lab scale reactor*

18 Two identical lab scale reactors were designed, built and operated in parallel. One of
19 them was used as a control system and operated as a CAS process. The other one was a
20 modified CAS process, which included an anoxic reactor in the sludge recirculation
21 line, i.e the OSA system. The work volumes of oxic reactors and settlers were 2.5 L and
22 3.7 L, respectively, in both lab scale reactors. Bubble diffusers located at the bottom of
23 the oxic reactors provided the aeration at 1.5 L min⁻¹. The OSA system was designed
24 with a hydraulic retention time anoxic/oxic ratio equal to 0.27. The anoxic reactor was
25 designed with a volume of 1.35 L.

2.2.2. Experimental conditions

The lab scale reactors were fed with the same influent flow rate, 6.4 L d^{-1} . The returned sludge ratio was set to 200% in both lab scale reactors. Hydraulic Retention Time (HRT), Dissolved Oxygen (DO) concentration and Mixed Liquor Suspended Solids (MLSS) concentration in both oxic reactors were set at 9 hours, 6 mg L^{-1} and 2.5 g L^{-1} , respectively. HRT, DO concentration and MLSS concentration, in the anoxic reactor included in the OSA system, were set at 2.5 hours, 0.23 mg L^{-1} , 5 g L^{-1} and, respectively. HRT in the control system was 23 hours, while HRT in the OSA system was 25.5 hours. The sludge age for the activated sludge in the control system and OSA system were 4 days, and 14 days, respectively. The reaction temperature was the ambient temperature, c.a. $25 \pm 5 \text{ }^{\circ}\text{C}$. The lab scale reactors operation lasted 100 days.

2.3. Chemical analysis

Soluble COD, TSS and VSS concentrations were determined according to Standard Methods of analysis (Eaton *et al.*, 2005). Active biomass was measured by SLP concentration following the procedure by Arnaiz *et al.* (2006). Phospholipids are the major lipids in bacteria, being 90-98% of the bacterial membrane and not forming part of cell reserves, which make it reasonable to use the SLP concentration as active biomass (Arnaiz *et al.*, 2006). The DO concentration was measured using a DO meter (HQ30D Flexi, Hach-Lange), the Oxidation-Reduction Potential (ORP) and pH were measured by digital pH meter (GLP22, Crison). All measurements were made in triplicate.

2.3. Sludge production

The sludge yield (Y_s) was calculated to evaluate the sludge production. Since solids concentrations in the systems changed, cumulative terms during the period of study were used (Eq. (1)). For this reason, COD and VSS were quantified daily.

$$Y_s = \frac{\Sigma VSS \text{ produced}}{\Sigma COD \text{ removed}} = \frac{\Sigma(F_w * VSS_w + F_e * VSS_e + \Delta VSS_{sys})}{\Sigma(F_i * (COD_i - COD_e))} \quad (1)$$

where F_w , F_i and F_e correspond to the waste, influent and effluent flows ($m^3 d^{-1}$), respectively; COD_i and COD_e correspond to the soluble organic matter in the influent and effluent ($g \text{ COD } m^{-3}$), respectively; VSS_w and VSS_e correspond to the volatile suspended solids in the waste and effluent ($g \text{ VSS } m^{-3}$), respectively; and ΔVSS_{sys} corresponds to the biomass accumulated in the system ($g \text{ VSS } d^{-1}$).

Y_s was determined by regression of the cumulative sludge production versus the cumulative organic matter removal as describes Coma *et al.* (2013). The slope of the regression line is considered the mean Y_s (Fig. 1A).

The active biomass yield (Y_{ab}) was calculated to evaluate the active biomass production.

Since active biomass concentrations in the systems changed, cumulative terms during the period of study were used (Eq. (2)). For this reason, COD and SLP were quantified daily.

$$Y_{ab} = \frac{\Sigma SLP \text{ produced}}{\Sigma COD \text{ removed}} = \frac{\Sigma(F_w * SLP_w + F_e * SLP_e + \Delta SLP_{sys})}{\Sigma(F_i * (COD_i - COD_e))} \quad (2)$$

where F_w , F_i and F_e correspond to the waste, influent and effluent flows ($m^3 d^{-1}$), respectively; COD_i and COD_e correspond to the soluble organic matter in the influent and effluent ($g \text{ COD } m^{-3}$), respectively; SLP_w and SLP_e correspond to the suspended lipid phosphate in the waste and effluent ($mmol \text{ SLP } m^{-3}$), respectively; and ΔSLP_{sys} corresponds to the active biomass accumulated in the system ($mmol \text{ SLP } d^{-1}$).

Y_{ab} was determined by regression of the cumulative active biomass production versus the cumulative organic matter removal similar to Y_s determination described by Coma *et al.* (2013). The slope of the regression line was considered the mean Y_{ab} (Fig. 1B).

2.5. Population dynamics

Protozoa and filamentous bacteria in the activated sludge were monitored by a Nikon microscope E200 Type R equipped with a Progres CT3-USB digital camera following methods used in previous studies (Canals et al., 2013; Moreira et al., 2015).

Protozoa density and small flagellates were measured using a counting technique described by Madoni *et al.* (1994), and expressed as individuals per millilitre (ind mL⁻¹). Protozoa and small flagellates counts were carried out analysing two 25 µL replicates *in vivo* at 100x or 400x magnification by phase contrast and identified as described by Foissner *et al.* (1995, 1994, 1992, 1991) and Curds *et al.* (2008).

Abundance of filamentous bacteria was measured using counting technique described by Canals *et al.* (2013), and expressed as meters per millilitre (m mL⁻¹). Filamentous bacteria counts were carried out at 400x magnification by phase contrast and identified as described by Jenkins *et al.* (2004) and Eikelboom (2006).

The Sludge Biotic Index (SBI) defines the biological quality of the sludge using numerical values from 0 to 10, and categorizing SBI values into four quality classes. From class I that describes a stable high quality activated sludge to class IV that describes an unstable low quality activated sludge (Madoni, 1994). SBI was determined in based on both structure and abundance of the microfauna in relation to the environmental and operational conditions of the plant (Madoni, 1994).

Species richness and diversity were quantified by the Shannon diversity index H' (Shannon and Weaver, 1963).

$$H' = - \sum_{i=1}^R p_i \ln p_i \quad (3)$$

where proportion of species i relative to the total number of species (p_i) is calculated, and then multiplied by the natural logarithm of this proportion ($\ln p_i$).

2.5. Statistical analysis

Statistical data analysis has been performed with the statistical software package Microsoft Excel 2013 for Windows. The Pearson correlation test and its statistical significance (α) was calculated to show the possible role of the protozoa population as bioindicators (Canals *et al.*, 2013; Lee *et al.*, 2004). Sludge yield and active biomass yield were tested for correlation with protozoa density, diversity and filamentous bacteria.

3. Results and discussion

3.1. Organic matter removal and effluent quality

Both lab scale reactors were operated with a continuous soluble COD concentration in the influent of 282 ± 7 mg COD L⁻¹. Effluent soluble COD concentration in the control and OSA systems were 90.34 ± 7.89 mg L⁻¹ and 99.01 ± 9.49 mg L⁻¹ (Fig. 2), respectively. The OSA system showed slightly higher effluent soluble COD concentration in comparison with the control system (Fig. 2).

Even with a slightly higher effluent soluble COD concentration compared with control system, the OSA system showed an effluent soluble COD concentration below 125 mg L⁻¹, i.e. the limit value required in the Urban Waste Water Treatment directive (91/271/EC) (CEC, 1991).

Neither the effluent soluble COD concentration from the control system nor the effluent soluble COD concentration from OSA system were expected to be glucose, due to glucose is an easy degradable substrate (Gebremariam *et al.*, 2012). Changes of the carbon sources characteristics from wastewater to an easy degradable carbon source as glucose most likely produce an increase of the extracellular polymeric substances (EPS) production as described by Ye *et al.*, (2011). The slightly higher effluent soluble COD concentration determined in the OSA system compared with control system might be due to the higher soluble COD load observed in the OSA system compared with control

1 system (Fig. 2). The soluble COD generated in the anoxic reactor fed the oxic reactor
2 through sludge recirculation line. The OSA system was fed with around 10% soluble
3 COD higher than control system (Fig. 2).

4 Up to our knowledge, no previous studies have been done about biomass solubilisation
5 in the anoxic reactor focused in the microbiology. Samples taken from the sludge
6 recirculation line showed biomass in solubilization state in the OSA system, such as
7 breaking points in the membrane (Fig. 3 A, C), but not in the control system (Fig. 3 B,
8 D).

9 OSA system has been shown to improve sludge settling (Chudoba et al., 1992; Saby *et*
10 *al.*, 2003; Ye *et al.*, 2008). Chudoba *et al.* (1992) reported that the OSA system showed
11 a better sludge settling than control system. Saby *et al.* (2003), who studied the
12 influence of ORP value decreasing from +100 to -250 mV, reported that the OSA
13 system coupled with an MBR process improved the sludge settling. Saby *et al.* (2003)
14 suggested that the polymers released by the solubilization in the anoxic reactor could
15 have acted as flocculant. Ye *et al.*, (2008), who studied the influence of SRT in the
16 anoxic reactor, reported that even the SRT influenced the sludge settling, the OSA
17 system generally showed better sludge settling than control system. Ye *et al.*, (2008)
18 found that an SRT of 7.6 hours showed the best sludge settling. The sludge settling
19 might associated with the filamentous bacteria growth and/or diversity and high EPS
20 concentration (Semblante et al., 2014). Up to our knowledge, filamentous bacteria
21 characterization was not performed in the previous mentioned studies, which make
22 difficult to relate their role in the sludge settling improve. In the present study,
23 characterization and abundance of filamentous bacteria in the OSA system was studied
24 in comparison with control.

The effluent TSS concentration in the control and OSA systems were 31 ± 4 mg TSS L⁻¹ and 30 ± 5 mg TSS L⁻¹, respectively. Similar effluent TSS concentrations were observed (Fig. 2), indicating that the OSA system did not deteriorate the effluent quality.

3.2. Biomass production

3.2.1. Excess sludge production

In the control system, the excess sludge production rate was 0.51 g TSS d⁻¹, while in the OSA system the rate dropped to 0.24 TSS d⁻¹, i.e. reduced by 52.94%. The operation data showed that the OSA system effectively reduced excess sludge production. The reduction of excess sludge production observed in this study was similar than previous studies (Chen *et al.*, 2003; Saby *et al.*, 2003; Ye and Li, 2010; Ye *et al.*, 2008).

Chen *et al.* (2003) and Saby *et al.* (2003) reported that the reduction of excess sludge production in a modified OSA system (MBR-OSA) under 10.4 hours in the anaerobic reactor increased from 23% to 51% when ORP level decreased from +100 to -250 mV. Ye *et al.* (2008) reported a 33% reduction of excess sludge production when the HRT was around 6-7 hours in the anoxic reactor. Ye and Li (2010) reported that the reduction of excess sludge production in an OSA system combined with the addition of 3,3',4',5-tetrachlorosalicylanilide (TCS) and under 6.75 hours in the anoxic reactor increased from 21% to 56%.

3.2.2. Sludge yield

The OSA system caused a reduction in the sludge yield, measured as g TSS g COD⁻¹ removed, compared with the control system (Fig. 1A). The sludge yields of the oxic reactor in control and OSA systems were 0.42 g TSS g COD_r⁻¹ and 0.20 g TSS g COD_r⁻¹, respectively (Fig. 1A). The OSA system showed a sludge yield reduction 51.7% lower than control system.

The sludge yield reduction achieved in this study corresponds to the previous studies of the OSA system (14-87%) (Chen *et al.*, 2003; Chudoba *et al.*, 1992; Coma *et al.*, 2013; Saby *et al.*, 2003; Yagci *et al.*, 2015; Zhou *et al.*, 2014). Chudoba *et al.* (1992) reported a 39% of reduction of sludge yield under 3 hours in the anoxic reactor and high load operation conditions. Chen *et al.* (2003) and Saby *et al.* (2003) reported that the reduction of sludge yield in a modified OSA system (MBR-OSA) under 10.4 hours in the anaerobic reactor increased from 20% to 55% when ORP level decreased from +100 to -250 mV. Coma *et al.* (2013) reported that the reduction of sludge yield in an anaerobic, aerobic reactor coupled with an anoxic SSR under -150 mV of ORP, increase from 0.2% to 18.3% when HRT decreased from 34.5 hours to 5.9 hours. Zhou *et al.* (2014) achieved a 32% of reduction of sludge yield in an anoxic, aerobic reactor coupled with an anaerobic reactor under 6 hours. Yagci *et al.*, (2015) reported a 87% sludge yield reduction in an oxic SBR coupled with an anaerobic SSR when the iron level, in the influent, was increased to 16.05 mg L⁻¹. According to the previous studies, ORP value was important parameter for reduction of sludge yield. The mean ORP value in the oxic reactor from control system, oxic reactor from OSA system and anoxic reactor were +224 ± 9, +231 ± 6, and -128 ± 10 mV, respectively. The oxic reactors from both systems showed similar ORP values, despite the anoxic conditions of the anoxic reactor in the OSA system. In general, the oxic conditions has an ORP value higher than +50 mV, the anoxic conditions have an ORP value up to -150 mV and anaerobic conditions have an ORP value below -150 mV (Semblante *et al.*, 2014). In this study, both lab scale reactors showed similar oxic conditions which favour the sludge yield. The anoxic reactor operated under ORP conditions which might favour the selection of facultative aerobic biomass.

1 The oxidation of internal storage products without cell growth, metabolic selection, and
2 the predation could have influence in the reduction of sludge yield as other authors
3 reported previously (Chudoba *et al.*, 1992; Saby *et al.*, 2003; Zhou *et al.*, 2015, 2014).

4 3.2.3. Active biomass yield

5 The active biomass yield shows evidences about not only the internal storage products
6 oxidation without cell growth shown by the sludge yield, but also about sludge
7 metabolic activity. The OSA system caused a reduction in the active biomass yield
8 compared with the control system (Fig. 1B). The active biomass yields of the oxic
9 reactor in control and OSA systems were 2886.8 nmol SLP g COD_r⁻¹ and 945.5 nmol
10 SLP g COD_r⁻¹ (Fig. 1B), respectively. The OSA system showed a reduction of active
11 biomass yield 67.1% lower than control system.

12 The OSA system showed a lower active biomass yield (Fig. 1B) than control system,
13 but similar organic matter removal (Fig. 2) even with a higher soluble COD load. The
14 lower active biomass yield in the activated sludge under the OSA system conditions
15 may be due to a higher metabolic activity than those in the control system. The OSA
16 system induced catabolic metabolic routes, no cell growth against anabolic routes, and
17 cell growth (Chudoba *et al.*, 1992; Saby *et al.*, 2003).

18 The OSA system caused a reduction in the active biomass concentration into the oxic
19 reactor compared with oxic reactor of the control system. The active biomass
20 concentrations of the oxic reactor in control and OSA systems were 23.5 ± 2.3 nmol
21 SLP mL⁻¹ and 18.2 ± 2.4 nmol mL⁻¹, respectively. The OSA system showed a 22.53%
22 reduction of active biomass concentration compared with the control system. Saby *et al.*
23 (2003), who studied the influence of ORP value decreasing from +100 to -250 mV,
24 found a reduction of the active biomass expressed as respiring bacteria; although the
25 total cell appeared to be 40 to 50% higher than in the control. Chudoba *et al.* (1992)

1 found an increment about 50% of poly-P bacteria with 3 hours anoxic time in
2 comparison with the control.

3 The OSA system showed a lower active biomass concentration and a higher metabolic
4 activity in their active biomass than the control system as demonstrated with a similar
5 organic matter removal (Fig. 2), despite a lower sludge yield (Fig. 1 A) and active
6 biomass yield (Fig. 1 B).

7 3.5. Microbiological dynamics

8 3.5.1. Inoculum

9 The sessile ciliate group and the crawling ciliate group were clearly codominant in the
10 inoculum (Table 1). The crawling ciliates identified were *Aspidisca* sp. (*Ciliophora*,
11 *Hypotrichia*), *Acineria* sp. (*Ciliophora*, *Haptoria*), *Euplotes* sp. (*Ciliophora*,
12 *Hypotrichia*) and *Chilodonella* sp. (*Ciliophora*, *Phyllopharyngia*) (Table 1). The sessile
13 ciliates identified were *Epistylis* sp. (*Ciliophora*, *Peritrichia*), *Zoothamnium* sp.
14 (*Ciliophora*, *Peritrichia*), *Charchesium* sp. (*Ciliophora*, *Peritrichia*), *Vorticella* sp.
15 (*Ciliophora*, *Peritrichia*) and *Opercularia* sp. (*Ciliophora*, *Peritrichia*) (Table 1). The
16 swimming and carnivorous ciliates identified were *Paramecium* sp. (*Ciliophora*,
17 *Peniculida*) and *Tokophrya* sp. (*Ciliophora*, *Suctoria*), respectively (Table 1). The
18 heterotrophic flagellates identified were *Peranema* sp., *Euglena* sp. and *Entosiphon* sp.
19 (Table 1). Naked amoebae, rotifers and nematodes were rarely identified (Table 1).

20 SBI was 10 (Class I) according to Madoni (1994), indicating that the activated sludge
21 was very well colonized, stable with excellent biological activity and very good
22 performance. The protozoa density, Shannon index and abundance of filamentous
23 bacteria were 1140000 ± 57000 ind L⁻¹, 4 bits and 127.2 ± 6.36 m mL⁻¹, respectively.

24 3.5.2. Control system

The sessile ciliate group and the crawling ciliate group were codominant in the activated sludge under the control system (Table 1). The crawling ciliates identified were *Oxytricha* sp. (*Ciliophora*, *Hypotrichia*) and *Acineria* sp. (*Ciliophora*, *Haptoria*) (Table 1). The sessile ciliates identified were *Epistylis* sp. (*Ciliophora*, *Peritrichia*), *Vorticella* sp. (*Ciliophora*, *Peritrichia*) and *Opercularia* sp. (*Ciliophora*, *Peritrichia*) (Table 1). The swimming ciliates identified were *Paramecium* sp. (*Ciliophora*, *Peniculida*) and *Cinetochilium* sp. (*Ciliophora*, *Scuticociliatia*). The carnivorous ciliate identified was *Litonotus* sp. (*Ciliophora*, *Haptoria*) (Table 1). The heterotrophic flagellate identified was *Peranema* sp. (Table 1). Naked amoebae, rotifers and nematodes were rarely identified (Table 1). The abundance of small flagellates was kept below 50000 flag mL⁻¹.

SBI was 9 (Class I) according to Madoni (1994), keeping an similar value in comparison with the inoculum. The protozoa density and Shannon index were 1398300 ± 53400 ind L⁻¹ and 3.91 ± 0.06 bits. The mean abundance of filamentous bacteria were defined in 2 periods, in the first period, i.e. days 1-52 was 99 ± 6 m mL⁻¹ and in the second period, i.e. days 53-100 was 66 ± 4 m mL⁻¹.

The decreasing trend in the control system could be explained considering the use of synthetic wastewater with glucose as sole carbon source, which most likely did not allow a conventional development of the microflora (Dubber and Gray, 2011). The decrease of filamentous bacteria produced the disintegration of the flocs and the increase of the floc-forming bacteria such as *Zooglea ramigera* (Fig. 4 A, C). The floc-forming bacteria are usually observed in the start-up of reactors when the settling properties are not optimal (Koivuranta *et al.*, 2015). In the control system were identified Type 0041, *Sphaerotilus natans*, *Haliscomenobacter hydrossis* and *Zooglea ramigera* (Fig. 4 A, C) as dominant and secondary filamentous bacteria. The dominant

microflora and the presence of *Zooglea ramigera* (Fig. 4 A, C) suggest slow sludge settling.

3.5.2. OSA system

The sessile ciliate group and the crawling ciliate group were codominant in the activated sludge under the OSA system (Table 1). The crawling ciliates identified were *Acinertia* sp. (Table 1). The sessile ciliates identified were *Vorticella* sp. (*Ciliophora*, *Peritrichia*) and *Opercularia* sp. (*Ciliophora*, *Peritrichia*) (Table 1). The swimming ciliates identified were *Paramecium* spp. (*Ciliophora*, *Peniculida*) and *Cinetochilium* sp. (*Ciliophora*, *Scuticociliatia*). The carnivorous ciliate identified were *Tokophrya* sp. (*Ciliophora*, *Suctorina*) and *Litonotus* sp. (*Ciliophora*, *Haptoria*) (Table 1). The heterotrophic flagellate identified were *Peranema* sp. and *Entosiphon* sp. (Table 1). Naked amoebae, rotifers and nematodes were rarely identified (Table 1). The abundance of small flagellates was kept below 50000 flag mL⁻¹.

SBI was 10 (Class I) according to Madoni (1994), indicating that the activated sludge was very well colonized, stable with excellent biological activity and very good performance. The protozoa density, Shannon index and abundance of filamentous bacteria were 3.73 ± 0.05 bits, 1163500 ± 39400 ind L⁻¹ and 80 ± 4 m mL⁻¹, respectively.

In the OSA system were identified *Sphaerotilus natans*, *Nostocodia limicola*, Type 0675 and Type 021N as dominant and secondary filamentous bacteria. The dominant microflora suggests efficiency to metabolize sugars as carbon sources under low oxygen and nutrients concentrations. The absence of floc-forming bacteria and the bonding process by filamentous bacteria (Fig. 4 B, D) suggest an improved in the sludge settling in comparison with the control system.

3.5.3. Implications of OSA system on microbiological population

Zhou *et al.* (2015) observed that the enrichment of fermentative, hydrogenogenic and acidogenic bacteria in their pilot plant study with different anoxic/anaerobic time expositions could be related with biomass production reduction. Zhou *et al.* (2015) determined the *phyla* *Proteobacteria*, *Bacteroidetes*, *Chlorobi*, *Actinobacteria* and *Caldiserica* as the predominant *phyla* of the bacteria population of activated sludge. However, it was difficult to draw firm conclusion on the role of predatory bacteria because of its small relative abundance and limited microbiological information.

In the present study, microbiological results suggested that the groups of crawling ciliate and sessile ciliate, especially *Oxytricha* spp. and *Epistylis* spp. were highly reduced in the OSA system. Moreover, the OSA system favoured colonisation by carnivorous species, such as *Tokophrya* spp. (*Ciliophora*, *Suctorina*) and *Litonotus* sp. (*Ciliophora*, *Haptoria*). *Acineria* spp. (*Ciliophora*, *Haptoria*) and *Opercularia* spp. (*Ciliophora*, *Peritrichia*) were the species most identified, these two species are usually correlated with increase in the organic matter load and the absence of DO concentration in the system.

The OSA system produced decrease in both protozoa density and diversity in comparison to the control system (Table 1). Despite of lower protozoa density and diversity in the OSA system than in the control system, it was not observed a fall in the SBI under the OSA system in comparison to control system (Table 1).

The OSA system favoured the stable development of filamentous bacteria, which is an advantage in the control of settling properties. In the control system was observed an increase in floc-forming bacteria both in flocs and inter-flocs spaces (Fig. 4 A, C). In the OSA system floc-forming bacteria were not observed (Fig. 4 B, D), which supports the potential improve of the settling properties notified by other authors (Chudoba *et al.*, 1992; Saby *et al.*, 2003; Yagci *et al.*, 2015; Ye *et al.*, 2008).

3.6. Statistical study: bioindicators

The protozoa density showed the highest positive correlation to sludge yield in comparison to the other microbiological parameters in the control system (Table 2). In the OSA system, the swimming ciliates group was found as the highest positive correlation to sludge yield in comparison to the other microbiological parameters (Table 2). The swimming ciliates group could be used as bioindicators to sludge yield in the OSA system.

The diversity and abundance of filamentous bacteria showed the highest correlations to active biomass yield in comparison to the other microbiological parameters in the control system (Table 2). Diversity showed a negative correlation, while abundance filamentous bacteria showed a positive correlation. In the OSA system, the diversity, crawling ciliates group and swimming ciliates group were found as the highest significant correlations to active sludge yield in comparison to the other microbiological parameters (Table 2). Diversity and crawling ciliates group showed a negative correlation, while swimming ciliates group showed a positive correlation. The swimming ciliates group could be used as the bioindicator to active biomass yield in the OSA system. Diversity and crawling ciliates group could be used as the bioindicators of the active biomass yield reduction in the OSA system.

4. Conclusions

The OSA system biomass decreases in both protozoa density and diversity. Diversity and crawling ciliates group have been shown as promising bioindicators of active biomass yield reduction. The OSA system achieved a 51.7% reduction of sludge yield, reducing the excess sludge production 52.9% and active biomass yield 67.1%, compared with the control system. The oxic reactor of the OSA system showed a higher metabolic activity in their active biomass than the oxic reactor of control system. The

- 1 identification of floc-forming bacteria in the control system suggested that OSA system
- 2 likely will improve settling properties compared to a CAS process.

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Table 1

Table 1. The microbial functional groups, protozoa, SBI (Madoni Class), density (ind L⁻¹), Shannon index (bits) and Abundance of filamentous bacteria (m mL⁻¹).

Inoculum		CAS process		OSA system
Crawling ciliates				
<i>Euplotes</i> sp.	•			
<i>Oxytricha</i> sp.	•	•		
<i>Chilodonella</i> sp.	•			
<i>Aspidisca cicada</i>	•			
<i>Acineria uncinata</i>	•	•		•
Sessile ciliates				
<i>V. microstoma</i>	•	•		•
<i>V. striata</i>	•	•		•
<i>V. alpestris</i>	•	•		•
<i>Epistylis</i> sp.	•	•		
<i>Opercularia</i> sp.	•	•		•
<i>Charchesium</i> sp.	•			
Swimming ciliates				
<i>Cinetochilium</i> sp.		•		•
<i>Paramecium aurelia</i>	•	•		•
<i>Paramecium bursaria</i>	•			
Carnivorous ciliates				
<i>Tokophrya</i> sp.	•			•
<i>Litonotus</i> sp.	•	•		•
Flagellates				
Pequeños flagelados	•	•		•
<i>Peranema</i> sp.	•	•		•
<i>Entosiphon</i> sp.	•			•
<i>Euglena</i> sp	•			
Amoebae				
Amebas desnudas	•	•		•
Metazoa				
<i>Nematoda</i>	•	•		•
<i>Rotaria</i> sp.	•	•		•
SBI (Madoni Class)	10 (I)	9 (I)		10 (I)
Density (ind L ⁻¹)	1300000 ± 45000	1398300 ± 53400		1163500 ± 39400
Shannon index (bits)	4.49± 0.04	3.91 ± 0.06		3.73 ± 0.05
Abundance of filamentous bacteria (m mL ⁻¹)	109.14 ± 4.16	Period I (1-52 days) 99 ±6	Period II (53-100 days) 66 ± 4	80 ± 4

Table 2. Pearson's correlation coefficients between microbiological characteristics and biomass yield parameters.

Control system				
	Sludge yield	P ($\alpha=0.01$)	Active biomass yield	P ($\alpha=0.01$)
Density	0.71	< 0.01	0.30	< 0.01
Diversity	0.15	< 0.01	-0.63	< 0.01
Filamentous bacteria	-0.01	< 0.01	0.61	< 0.01
Crawling ciliates	0.22	< 0.01	-0.38	< 0.01
Sessile ciliates	0.18	< 0.01	0.35	< 0.01
Swimming ciliates	-0.04	< 0.01	-0.38	< 0.01
Carnivorous	-0.34	< 0.01	-0.08	< 0.01
OSA system				
	Sludge yield	P ($\alpha=0.01$)	Active biomass yield	P ($\alpha=0.01$)
Density	-0.27	< 0.01	-0.36	< 0.01
Diversity	-0.16	< 0.01	-0.67	< 0.01
Filamentous bacteria	-0.16	< 0.01	0.37	< 0.01
Crawling ciliates	-0.25	< 0.01	-0.61	< 0.01
Sessile ciliates	0.36	< 0.01	0.49	< 0.01
Swimming ciliates	0.51	< 0.01	0.74	< 0.01
Carnivorous	0.16	< 0.01	0.003	< 0.01

P: p-value; α : Degrees of freedom or Significance level

Figure 1

Figure 1. A) Sludge yield. ■ Control system and ▲ OSA system. B) Active biomass yield.

■ Control system and ▲ OSA system.

Figure 2

Figure 2. COD concentrations and the effluent TSS concentrations. ▲ Control effluent TSS; △ OSA system effluent TSS; ■ Control effluent COD; □ OSA system effluent COD; ● COD control system input and ○ COD OSA system input.

Figure 3

Figure 3. A) Contrast phase picture 400x in the OSA system, arrows show the breaking points membrane in *Oxytricha fallax*; B) Contrast phase picture 200x in the control system, arrows show *Oxytricha fallax* without breaking points membrane; C) Contrast phase picture 400x in the OSA system, arrows show the breaking points membrane in *Chilodonella uncinata*, 400x; D) Contrast phase picture 400x in the control system, arrows show *Chilodonella uncinata*, without breaking points membrane.

Figure 4

Figure 4. A) Contrast phase picture 200x in the control system, arrows show Floc-forming bacteria, *Zooglea ramigera*, in the inter-floc space; B) Contrast phase picture 200x in the OSA system, arrows shows inter-floc space without the presence of floc-forming bacteria; C) Contrast phase picture 200x in the control system, arrows shows Floc and floc-forming bacteria *Zooglea ramigera*, into the floc; D) Contrast phase picture 200x in the OSA system, arrows shows flocs and inter-floc space without the presence of floc-forming bacteria.