**Influence of different anoxic time exposures on active biomass, protozoa and filamentous bacteria
in activated sludge**

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Abstract

Medium size wastewater treatment plants are considered too small to implement anaerobic digestion technologies and too large for extensive treatments. A promising option as sewage sludge reduction method for medium size wastewater treatment plants is the inclusion of anoxic time exposures. In the present study, three different anoxic time exposures of 12, 6 and 4 hours have been studied to reduce sewage sludge production. The best anoxic time exposure was observed under anoxic/oxic cycles of 6 hours, which reduced 29.63% of the biomass production compared with the oxic control conditions. The sludge under different anoxic time exposures, even with a lower active biomass concentration than the oxic control conditions, showed a much higher metabolic activity than the oxic control conditions. Microbiological results suggested that both protozoa density and abundance of filamentous bacteria decrease under anoxic time exposures compared to oxic control conditions. The anoxic time exposures 6/6 showed the highest reduction in both protozoa density, 37.5%, and abundance of filamentous bacteria, 41.1%, in comparison to the oxic control conditions. The groups of crawling ciliate, carnivorous ciliates and filamentous bacteria were highly influenced by the anoxic time exposures. Protozoa density and abundance of filamentous bacteria have been shown as a promising bioindicators to biomass production reduction.

Keywords: biomass production, anoxic periods, active biomass, protozoa population, filamentous bacteria, biomass activity.

1. Introduction

The activated sludge process is the most commonly used sewage treatment worldwide (Zhou *et al.*, 2015). Handling, treatment, and disposal of sewage sludge represent between 50-60% of the operating cost of a secondary treatment plant (Guo *et al.*, 2013). In 2012, the annual sewage sludge production in the European Union (EU) was over 10.9 million tons Dry Solids (DS) (Cieřlik *et al.*, 2015). Implementation of the Urban Wastewater Treatment directive (91/271/EC) (CEC, 1991) is causing a significant increase of annual sewage sludge production in EU, expecting to exceed 13 million tons DS in 2020 (Kelessidis and Stasinakis, 2012). According to the Waste Framework directive (2008/98/EC) (CEU, 2008), sludge reduction is one of the first priorities in waste management hierarchy (Kelessidis and Stasinakis, 2012).

Anaerobic digestion (AD) of the sewage sludge has been successfully applied for large installations (Cieřlik *et al.*, 2015). Extensive wastewater treatment technologies, such as ponds, constructed wetlands, soil trenches or compound media filter bed combined systems have been successfully implemented for small-scale treatments (Wu *et al.*, 2013). However, medium size installations, i.e. around 50000 equivalent population, are too small to implement AD technologies and too large for extensive treatments. Several technologies are currently developed to reduce sewage sludge production when AD is not applied, 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 inclusion of anoxic time exposures in the activated sludge process. Anoxic time exposures induce a decrease in the biomass production (Guo *et al.*, 2013). Anoxic time exposure as biomass production reduction method show many advantages, such as no chemical requirements, easy application and operation and low economic costs (Guo *et al.*, 2013).

Previous studies (Saby *et al.*, 2003; Chudoba *et al.*, 1992; Ye *et al.*, 2008) have indicated that an appropriate control in parameters such as oxidation reduction potential (ORP), reaction temperature and anoxic time might be the most significant parameters to implement anoxic time exposures as biomass production reduction method.

According to Saby *et al.*, (2003) and Chen *et al.*, (2003), ORP value below 100 mV could already reduce biomass production significantly. The latest authors claim that the highest biomass production reductions were observed at ORP values around -250 mV.

Previous studies have been mostly operated at controlled temperatures (20-25 °C) or ambient temperatures (Chudoba *et al.*, 1992; Saby *et al.*, 2003; Chen *et al.*, 2003; Ye *et al.*, 2008; Coma *et al.*,

2013; Yang *et al.*, 2011). Yang *et al.*, (2011) indicated that a significant biomass production reduction could occur at 29°C. However, a high temperature such as 29°C could impact biological activity, activated sludge properties and increase costs (Semblante *et al.*, 2014).

Ye *et al.*, (2008) set different anoxic times, such as 5.5 hours, 7.6 hours, and 11.5 hours in a pilot plant scale experience, indicating the range 6-7 hours as optimal anoxic time. Other anoxic times exposures have been studied, such as Chudoba *et al.*, (1992), who set 3 hours anoxic time, or Chen *et al.*, (2003) and Saby *et al.*, (2003) who set 10.4 hours anoxic time.

The aim of this study was the evaluation of different anoxic time exposures for biomass production reduction. To improve the thorough understanding of the mechanisms behind biomass production reduction is necessary the detailed analysis of active biomass. Therefore, the impact of the anoxic time exposures on the active biomass amount, protozoa population and filamentous bacteria were analysed. Statistical analysis was calculated by Pearson correlation test to show the role of the microbiological population as possible bioindicators.

2. Materials and methods

2.1. Inoculum and culture medium

The inoculum was taken from the secondary sludge recirculation line of a municipal wastewater treatment plant in Seville, Spain. The characteristics of the inoculum used were: 2500 ± 100 mg L⁻¹ of Volatile Suspended Solids (VSS) concentration and 85 ± 10 nmol mL⁻¹ of the Suspended Lipid Phosphate (SLP) concentration.

The culture medium was prepared with a mixture of inoculum and glucose, as sole added organic material, in a mineral medium as shown in Table 1. The pH was adjusted to an initial value of 7 and was kept with phosphate buffer solution. The mean initial concentrations of the Chemical Oxygen Demand (COD) and Total Suspended Solids (TSS) were 1045 ± 57 mg L⁻¹ and 2298 ± 76 mg L⁻¹, respectively.

2.2. Experimental procedure

Taken into account the results in previous studies (Ye *et al.*, 2008; Saby *et al.*, 2003) three different anoxic time exposures were studied together with an oxic control condition. An anoxic time exposure appointed 12/12 with two periods, an anoxic period of 12 hours and an oxic period of 12 hours. An anoxic time exposure appointed 6/6 with four periods, two anoxic periods of 6 hours and two oxic periods of 6 hours. Finally, an anoxic time exposure appointed 4/4 with six periods, three anoxic periods of 4 hours and three oxic periods of 4 hours. All experiments lasted 24 hours (Fig. 1). The air supply rate, when

supplied, was fixed at 1.5 L min^{-1} and the reaction temperature was the ambient temperature c.a. 25 ± 5 °C.

Figure 1. Oxidic and Anoxic periods versus time, ■ ORP values expressed in mV and ▲ DO values expressed in mg L⁻¹, under oxidic control conditions, anoxic time exposures 12/12, 6/6 and 4/4.

Each tested condition was conducted nine times in batch assays (125 mL). The aeration system was composed by an air bubbling pump and a one way valve to allow gas to escape.

2.3. Analytical methods

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 as describes (Zhong *et al.*, 2015). Active biomass measured as SLP are a bacterial cell wall component, which comprise up to 90-98% of bacterial cell wall and do not form part of cell reserves. Phospholipids measured only includes active biomass. The Dissolved Oxygen (DO) concentration was measured using a DO meter (HQ30D Flexi, Hach-Lange), the ORP and pH were measured by digital pH meter (GLP22, Crison). All measurements were made in triplicate.

2.4. Population measurement

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 (Vanysacker *et al.*, 2014; Moreira *et al.*, 2015).

Protozoa density and small flagellates were measured using a counting technique described by Madoni (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.*(1991; 1992; 1994; 1995) and Curds *et al.*(2008).

Abundance of filamentous bacteria was measured using counting technique described by Salvado *et al.* (1995) 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 to10, 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).

The protozoa richness and diversity were quantified by the number of total species and the Shannon diversity index H' (Shannon and Weaver, 1963).

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

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 role of the microbiological population as bioindicators (Lee *et al.*, 2004; Canals *et al.*, 2013). TSS, VSS and SLP concentrations were correlated with density, diversity, filamentous bacteria and ciliates population.

3. Results and Discussion

3.1. Organic matter removal

The initial soluble COD value was 1045 mg L⁻¹ in all batch experiments. The final soluble COD values in the oxic control conditions and batch conditions under different anoxic time exposures 12/12, 6/6 and 4/4 were 115 ± 7, 113 ± 8, 113 ± 6 and 115 ± 7 mg L⁻¹, respectively. Similar final soluble COD values revealed that conditions under anoxic time exposures did not influence the organic matter removal in comparison with the oxic control conditions.

Taking into account that glucose is an easy degradable substrate (Gebremariam *et al.*, 2012), the final soluble COD value was not expected to be glucose. Change from complex substrate such the real wastewater to an easy degradable substrate such as glucose could produce an acclimation of the microbiological population to the new substrate and the extracellular polymeric substances (EPS) production might increase gradually (Ye *et al.*, 2011). The final soluble COD values might be made up of EPS released by the different metabolic pathways of glucose.

According to the similar final soluble COD values, the different anoxic time exposures did not influence the pathways of glucose catabolism compared to results from oxic control conditions.

In oxic control conditions, the average ORP value was +141.45 mV throughout the experiment (Fig. 1). The average ORP value in the batch experiments under the different anoxic times exposures 12/12, 6/6 and 4/4 were -80.29, -67.57 and -48.11 mV, respectively (Fig. 1). Anoxic conditions have an ORP value up to -150 mV and anaerobic conditions have an ORP value below -150 mV (Semblante *et al.*, 2014). Therefore, in this study the range of ORP values (Fig. 1) in the batch experiments under different anoxic time exposures might favour facultative aerobic biomass, but not anaerobic activity.

3.2. Biomass production

The inclusion of the different anoxic time exposures caused a reduction in the biomass production compared with the oxic control conditions (Fig. 2). The final TSS concentration was measured at the end of the experiments. The final TSS concentration under the oxic control and batch conditions under the different anoxic time exposures 12/12, 6/6 and 4/4 were 2.7 ± 0.1 , 2.3 ± 0.2 , 1.9 ± 0.1 and 2.1 ± 0.1 mg TSS mL⁻¹, respectively (Fig. 2). The anoxic time exposures 6/6 showed the best results in the biomass production reduction, i.e. 29.63% lower than oxic control conditions. Ye *et al.*(2008) indicated an anoxic period of 6-7 h as optimal anoxic time to minimize biomass production, similar to observed in the present study.

Figure 2. Biomass production. Initial and final values of TSS and VSS expressed in mg mL⁻¹, under oxic control conditions, anoxic time exposures 12/12, 6/6 and 4/4. ---Initial TSS;Initial VSS; □ Final TSS and ■ Final VSS.

The ORP values observed in the anoxic periods (Fig. 1) and final TSS values (Fig. 2) suggested that ORP was not the sole parameter that influenced biomass production, but also the anoxic time exposures and number of anoxic periods were important parameters for biomass production reduction as indicated previously by other authors (Saby *et al.*, 2003; Chudoba *et al.*, 1992).

The reduction in biomass production observed in batch experiments under different anoxic time exposures (Fig. 2), could be due to oxidation of internal storage products as indicated by Chudoba *et al.*(1992) The energy use for cell maintenance without cell growth, and the predation by protozoa could

have influenced biomass production as other authors reported previously (Chen *et al.*, 2003; Zhou *et al.*, 2014).

3.3 Biomass activity

3.3.1 VSS TSS⁻¹ ratio

Many authors shown that increase in the VSS TSS⁻¹ ratio indicated a low biomass mineralization and an optimal biological activity (Ouyang and Liu, 2009; Krzeminski *et al.*, 2012). On the other hand, a decrease in the VSS TSS⁻¹ ratio indicates inert material accumulation, sludge stabilization, and a biological activity decrease (Ouyang and Liu, 2009; Krzeminski *et al.*, 2012).

The initial VSS TSS⁻¹ ratio was equal to 0.54 ± 0.07 in all experiments (Fig. 3). Final VSS TSS⁻¹ ratio throughout conditions were similar to the initial value, but anoxic time exposures 6/6. The anoxic time exposures 6/6 showed an increase to 0.88 ± 0.03 (Fig. 3). The final VSS TSS⁻¹ ratio observed (Fig. 3) suggested that the anoxic time exposures 12/12 and 4/4 did not decrease the biomass activity in comparison to oxic control conditions. The increase in the VSS TSS⁻¹ ratio in the anoxic time exposures 6/6 can mainly be caused by two reasons: the increase in number of microorganisms and/or the metabolic activity increase. The final TSS values (Fig. 2) showed a biomass reduction in the anoxic time exposures 6/6, so it seems reasonable that if biomass was reduced, the biological activity increase should be due to the increase in metabolic activity. Therefore, it can be concluded that anoxic time exposure might produced an increase in metabolic activity, which showed higher results for the anoxic time exposures 6/6. Chen *et al.*(2001) reported that substrate utilization rate was accelerated after an oxygen insufficient period which could explain the metabolic activity increase observed in this study.

Figure 3. Biomass activity. Initial and final VSS TSS⁻¹ ratios and initial and final SLP concentrations expressed in nmol mL⁻¹, under oxalic control conditions, anoxic time exposures 12/12, 6/6 and 4/4. —··— Initial VSS TSS⁻¹; Initial SLP; —■— Final SLP and —▲— Final VSS TSS⁻¹.

3.3.2. SLP

The final SLP values in the oxalic control conditions and batch experiments under anoxic time exposures 12/12, 6/6 and 4/4 were 58.4 ± 0.4 , 49.1 ± 0.2 , 46.3 ± 0.5 and 48.4 ± 0.4 nmol SLP mL⁻¹, respectively (Fig. 3). The active biomass reduction under anoxic time exposures 12/12, 6/6 and 4/4 in comparison with the oxalic control conditions were 15.91, 20.72 and 17.11%, respectively. Saby *et al.*, (2003) who studied the influence of ORP value decreasing from +100 to -250 mV, found a reduction of the active biomass expressed as respiring bacteria; although the total cell appeared to be 40 to 50% higher than in the control. Moreover, Chudoba *et al.*(1992) found an increment about 50% of poly-P bacteria in comparison with the control in their pilot plant study with a 3 hours anoxic time.

It is worth to notice that the anoxic time exposures 6/6, with a lower active biomass than the oxalic control conditions (Fig. 3), showed a higher metabolic activity in their active biomass as shown in the VSS TSS⁻¹ ratio (Fig. 3).

3.4. Microbiological population

3.4.1. Inoculum

Table 2 summarized microbiological results observed. The sessile ciliate group and the crawling ciliate group were clearly codominant in the inoculum. The crawling ciliates identified were *Aspidisca* spp. (*Ciliophora, Hypotrichia*), *Acineria* spp. (*Ciliophora, Haptoria*), *Euplotes* spp. (*Ciliophora, Hypotrichia*) and *Chilodonella* spp. (*Ciliophora, Phyllopharyngia*). The sessile ciliates identified were *Epistylis* spp. (*Ciliophora, Peritrichia*), *Zoothamnium* spp. (*Ciliophora, Peritrichia*), *Charchesium* spp. (*Ciliophora, Peritrichia*), *Vorticella* spp. (*Ciliophora, Peritrichia*) and *Opercularia* spp. (*Ciliophora, Peritrichia*). The swimming and carnivorous ciliates identified were *Paramecium* spp. (*Ciliophora, Peniculida*) and *Tokophrya* spp. (*Ciliophora, Suctoria*), respectively. The heterotrophic flagellates identified were *Peranema* spp. and *Euglena* spp. Naked amoebae, rotifers and nematodes were rarely identified.

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 1140000 ± 57000 ind L⁻¹, 4 bits and 127.2 ± 6.36 m mL⁻¹, respectively.

3.4.2. Continuous oxic control conditions

The main microbiological findings in the continuous oxic control conditions were compiled in Table 2. The sessile ciliate group and the crawling ciliate group were codominant in the activated sludge under the oxic control conditions. The crawling ciliates identified were *Aspidisca* spp. (*Ciliophora, Hypotrichia*), *Acineria* spp. (*Ciliophora, Haptoria*) and *Chilodonella* spp. (*Ciliophora, Phyllopharyngia*). The sessile ciliates identified were *Epistylis* spp. (*Ciliophora, Peritrichia*), *Vorticella* spp. (*Ciliophora, Peritrichia*) and *Opercularia* spp. (*Ciliophora, Peritrichia*). The swimming and carnivorous ciliates identified were *Paramecium* spp. (*Ciliophora, Peniculida*) and *Tokophrya* spp. (*Ciliophora, Suctoria*), respectively. The heterotrophic flagellates identified were *Peranema* spp. and *Euglena* spp. Naked amoebae, rotifers and nematodes were rarely identified. The protozoa density and abundance of filamentous bacteria were 1280000 ± 86800 ind L⁻¹ and 149.2 ± 10.4 m mL⁻¹, respectively.

SBI was 9 (Class I) according to Madoni (1994), keeping an similar value in comparison with inoculum. Shannon index decreased to 3.63 in comparison with inoculum. The oxic control conditions could induce a species selection which produced a decrease in sessile ciliates group (from 5 to 3 taxa) and crawling ciliates group (from 4 to 3 taxa).

3.4.3. Different anoxic time exposures

The influence of anoxic time exposures on microbiological population are shown in Table 2. The sessile ciliate group and the crawling ciliate group were codominant in the activated sludge in batch experiments studied under different anoxic time exposures. The crawling ciliates identified were *Acineria* spp. (*Ciliophora*, *Haptoria*) in the three anoxic time exposures studied, *Aspidisca* spp. (*Ciliophora*, *Hypotrichia*) in the anoxic time exposures 12/12 and 4/4 and *Chilodonella* spp. (*Ciliophora*, *Phyllopharyngia*) in the anoxic time exposure 4/4. The sessile ciliates identified were *Epistylis* spp. (*Ciliophora*, *Peritrichia*), *Vorticella* spp. (*Ciliophora*, *Peritrichia*) and *Opercularia* spp. (*Ciliophora*, *Peritrichia*) in the three anoxic time exposures studied. The swimming ciliates identified was *Paramecium* spp. (*Ciliophora*, *Peniculida*) in the three anoxic time exposures studied. The carnivorous ciliates identified was *Tokophrya* spp. (*Ciliophora*, *Suctorina*) in the anoxic time exposure 4/4. The heterotrophic flagellates identified were *Peranema* spp. and *Euglena* spp. in the three anoxic time exposures studied. Naked amoebae were rarely identified in anoxic time exposures studied. Rotifers were rarely identified in the anoxic time exposures 12/12 and 4/4, but in 6/6. Nematodes were rarely identified in the anoxic time exposures 4/4. The protozoa density in batch experiments under anoxic time exposures 12/12, 6/6 and 4/4 were 980000 ± 49200 ; 800000 ± 60000 and 920000 ± 46000 ind L⁻¹, respectively. While, the abundance of filamentous bacteria in batch experiments under anoxic time exposure 12/12, 6/6 and 4/4 were 100.5 ± 6.1 , 87.9 ± 6.9 and 109.9 ± 4.4 m mL⁻¹, respectively. Batch experiments under different anoxic time exposures presented lower protozoa density and abundance of filamentous bacteria than oxic control conditions.

Final SBI values in the three anoxic time exposures studied were similar to oxic control conditions, i.e. 9 (Class I) according to Madoni (1994). The activated sludge was very well colonized, stable with excellent biological activity and very good performance according to Madoni (1994) in all experimented conditions. Shannon index decreased in the anoxic time exposures 12/12 and 6/6 in comparison to control, but it was similar to control in the anoxic time exposure 4/4.

3.5. Implications of different anoxic time exposures 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 exposures could be related with biomass production reduction. Furthermore, Zhou *et al.*(2015) reported an evolution of microbial species to slow growers with low sludge yield. Moreover, 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 batch experiments under anoxic time exposures caused the decrease in both protozoa density and abundance of filamentous bacteria. The anoxic time exposures 6/6 showed the highest reduction in both protozoa density, 37.5%, and abundance of filamentous bacteria, 41.1%, in comparison to the oxic control conditions. Also, in the anoxic time exposures 6/6 was determined the highest reduction of taxa in comparison to the oxic control conditions.

The groups of crawling ciliate and carnivorous ciliate, especially *Euplotes* spp., *Chilodonella* spp. and *Tokophrya* spp. were highly influenced by the anoxic time exposures 12/12 and 6/6, which suggested a simplification of microbiological population in the activated sludge. Moreover, the abundance of filamentous bacteria was highly affected by different anoxic time exposures studied, especially in *Nocardia* spp. and Type 1851. The decrease observed in the abundance of filamentous bacteria suggested that the macrostructure of activated sludge could be reduced, which is an advantage in the control of filamentous bacteria growth and further settling properties of activated sludge.

3.6. Statistical study: bioindicators

A positive correlation ($\alpha < 0.01$) to biomass production was observed for protozoa density, diversity, abundance of filamentous bacteria, ciliates population, swimming ciliate group and sessile ciliate group. The crawling ciliates did not show a significant correlation ($\alpha > 0.01$) with biomass production (Table 3). The protozoa density, abundance of filamentous bacteria and ciliates population had higher significant correlations to biomass production in comparison to the other microbiological parameters (Table 3).

The high correlations observed (Table 3) suggested that protozoa density, abundance of filamentous bacteria and ciliates population could be used as bioindicators to reduction of biomass production.

Diversity has not been identify as a suitable bioindicator to the reduction of biomass production (Table 3).

The anoxic time exposure 4/4 showed a reduction in biomass production (Fig. 2) in comparison to oxic control conditions, but similar diversity (Table 3). Moreover, a functional group, i.e. swimming ciliates, sessile ciliates and/or crawling ciliates, has not been identified as bioindicator due to low values of Pearson's correlation (Table 3).

4. Conclusions

Microbiological results revealed that batch experiments under anoxic time exposures decrease in both protozoa density and abundance of filamentous bacteria. Protozoa density, abundance of filamentous

bacteria and ciliates population have been shown as a promising bioindicators of biomass production reduction. The anoxic time exposure 6/6 achieved the highest reduction of biomass production, 29.63%, reducing the active biomass, 20.72%, protozoa density, 37.5%, and abundance of filamentous bacteria, 41.1% compared to oxic control conditions. The anoxic time exposures 6/6, even with a lower active biomass than the oxic control conditions, showed a higher metabolic activity in their active biomass than the oxic control as shown in the VSS TSS⁻¹ ratio.

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