CONCERNING THE ROLE OF CELL LYSIS-CRYPTIC GROWTH IN ANAEROBIC
SIDE-STREAM REACTORS: THE SINGLE-CELL ANALYSIS OF Viable, DEAD
AND Lysed BACTERIA

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Abstract
In the Anaerobic Side-Stream Reactor (ASSR), part of the return sludge undergoes alternating aerobic and
anaerobic conditions with the aim of reducing sludge production. In this paper, viability, enzymatic activity,
death and lysis of bacterial cells exposed to aerobic and anaerobic conditions for 16 d were investigated at
single-cell level by flow cytometry, with the objective of contributing to the understanding of the mechanisms of
sludge reduction in the ASSR systems.

Results indicated that total and viable bacteria did not decrease during the anaerobic phase, indicating that
anaerobiosis at ambient temperature does not produce a significant cell lysis. Bacteria decay and lysis occurred
principally under aerobic conditions. The aerobic decay rate of total bacteria (b_{TB}) was considered as the rate of
generation of lysed bacteria. Values of b_{TB} of 0.07-0.11 d^{-1} were measured in anaerobic+aerobic sequence. The
enzymatic activity was not particularly affected by the transition from anaerobiosis to aerobiosis. Large
solubilisation of COD and NH_{4}^{+} was observed only under anaerobic conditions, as a consequence of hydrolysis
of organic matter, but not due to cell lysis.

The observations supported the proposal of two independent mechanisms contributing equally to sludge
reduction: (1) under anaerobic conditions: sludge hydrolysis of non-bacterial material, (2) under aerobic conditions: bacterial cell lysis and oxidation of released biodegradable compounds.

Keywords: Sludge reduction; anaerobic side-stream reactor; flow cytometry; cell lysis; cryptic growth.

1. INTRODUCTION

Activated sludge is an efficient and reliable process in treating wastewaters; however, the process produces a large amount of excess sludge, incurring high costs for treatment and disposal. Various technologies have been proposed for the reduction of excess sludge production directly within the wastewater treatment plant (WWTP), based on mechanical, physical-chemical or biological processes (Foladori et al., 2010a). Among the biological techniques, the Oxic-Settling-Anaerobic (OSA) process is based on an anaerobic reactor operating at ambient temperature inserted in the return sludge line between the secondary settler and the aeration tank (Chudoba et al., 1992a; Chudoba et al., 1992b). In this process, part of the return sludge undergoes alternating oxic (in the activated sludge reactor) and anaerobic (in the additional anaerobic reactor) conditions. Various modifications of the original OSA process have recently been proposed, in which the settled sludge is fed in the anaerobic side-stream reactor (ASSR) intermittently rather than continuously, as in the OSA process (Semblante et al., 2014). The OSA and ASSR systems are promising techniques for reducing sludge production, also having additional benefits such as low operational costs, good process stability and easy management (Wang et al., 2008). Reduction of sludge production of up to 60% was found, but the highest reductions have been obtained in lab-scale plants using synthetic wastewater (Foladori et al., 2010a; Semblante et al., 2014), while the plants fed with real wastewater demonstrate lower sludge reduction (Coma et al., 2013).
Despite the increasing interest in the application of OSA or ASSR systems, the present level of understanding of the mechanisms underlying sludge reduction in these processes is still limited (Semblante et al., 2014). Some hypotheses have been proposed in the literature to explain the possible mechanisms of sludge reduction, such as uncoupling metabolism (Chudoba et al., 1992b; Troiani et al., 2011) or cell lysis-cryptic growth (Wei et al., 2003; Quan et al., 2012), but these processes have not been fully demonstrated to date. In the study of An and Chen (2008) sludge decay in the anaerobic reactor was indicated as the main mechanism of the OSA system. However, sludge is a complex matrix composed of both bacterial biomass and non-bacterial material and sludge decay is the result of how each part is affected.

Microbiological aspects of sludge seem to play an important role in the OSA and ASSR systems. It is well known that cultivation-dependent analysis of microbial populations in sludge produces partial and heavily biased results and therefore this approach has never been applied. To obtain a more accurate view of bacteria populations and dynamics, molecular methods would be advised, but the application of these approaches in the OSA and ASSR systems is still being researched.

Amongst these methods, flow cytometry (FCM) is a powerful single-cell analysis that allows for obtaining a rapid and precise quantification of bacteria in environmental samples (inter alia Steen 2000; Tracy et al., 2010). When coupled with the fluorescent molecular staining of cells, various functions of bacterial cells can be investigated in just few minutes at single-cell level.

This paper aims to investigate the viability, activity, death and lysis of bacterial cells exposed to aerobic and anaerobic conditions, with the objective of contributing to the understanding of the mechanisms of sludge reduction in the ASSR systems. Viability, activity, death and lysis
of bacterial cells were investigated in this research by FCM according to the physiological status presented in Figure 1.

Viability was assessed by membrane integrity, which demonstrates the protection of constituents in intact cells classified as viable cells (Nebe von Caron et al., 2000). Cells without an intact membrane are considered as permeabilised and are classified as dead cells. Viable and dead cells can be identified simultaneously by applying Propidium Iodide (a dye able to enter only permeabilised cells) and SYBR-Green I (able to enter all cells) (Ziglio et al., 2002).

As the structures of dead cells are freely exposed to the environment, they will eventually undergo subsequent cell lysis and decomposition of constituents (Nebe-von-Caron et al., 2000). Lysed cells are no longer detectable by FCM, because their components are released in the bulk liquid; therefore they can be quantified by difference of total cells at two different points in time (Figure 1).

Cellular activity is a more restrictive condition than membrane integrity (Figure 1), because it requires that cells be intact and able to demonstrate one of the following functions: biosynthesis, pump activity, membrane potential or enzyme activity. Among these, enzymatic activity can be identified by using the fluorogenic substrate BCECF-AM (Ziglio et al., 2002).

This study was conducted on the sludge taken from a full-scale municipal WWTP integrated with an ASSR system aimed at sludge reduction. To our knowledge, the single-cell analysis of the physiological status of bacteria in the sludge taken from a full-scale ASSR system has not yet been reported in the scientific literature. Thus this paper focuses for the first time on
the changes of enzymatic activity, viability, death and lysis of bacterial cells measured during aerobic and anaerobic batch tests with a duration comparable to the typical HRT in the ASSR tank. The aim is to gain a better insight of the role of cell lysis in sludge reduction in the ASSR systems, evaluating aerobic and anaerobic environmental conditions and the effect of transition from anaerobiosis to aerobiosis.

2. MATERIALS AND METHODS

2.1. Configuration of WWTP and ASSR

The municipal WWTP of Levico (Italy) treats a mean population equivalent of 48,000 and is based on an activated sludge process (volume of reactors of 7,000 m$^3$) as shown in Figure 2. The ASSR tank (Cannibal®, Siemens, introduced in 2008) has a volume of 2,293 m$^3$ and treats a part of the sludge (330 m$^3$/d) separated from the return flow before being returned to the activated sludge reactors. Intermittent mixing was provided in the ASSR tank to ensure homogenous conditions, while treated sludge was discharged during no-mixing periods. The theoretical hydraulic retention time (HRT) in the ASSR tank was about 7 d, coinciding in practice with the sludge retention time (SRT) in the anaerobic tank, due to the scarce settleability of anaerobic sludge as a consequence of the high solids concentrations. The HRT in the ASSR tank is in the same order of magnitude of 10 d applied in other ASSR studies (Novak et al., 2007; Johnson et al., 2008; Troiani et al., 2011; Kim et al., 2012). ORP and pH inside the ASSR were below -250 mV (but reached -150 mV during feeding of sludge) and in the range 6-6.5, respectively.
The excess sludge production in the WWTP was calculated from 2005 to 2013 by means of the observed sludge yield ($Y_{\text{obs}}$), which was calculated as the slope of the linear regression curve obtained from the data of the cumulative Total Suspended Solids (TSS) produced versus the cumulative Chemical Oxygen Demand (COD) removed, according to Chon et al. (2011b) and Coma et al. (2013). $Y_{\text{obs}}$ of $0.350\pm0.004$ kgTSS/kgCOD was measured in the presence of the ASSR system, resulting significantly lower than the $0.442\pm0.002$ kgTSS/kgCOD measured before the introduction of the ASSR in 2008. The excess sludge reduction due to the ASSR system can thus be estimated at 20%, similar to other observations in ASSR systems treating real wastewater (Coma et al., 2013).

2.2. Sludge sampling points

Sludge was collected from the full-scale WWTP and thus the sludge was considered as already acclimatised to cyclic conditions of aerobiosis and anaerobiosis. About 25 litres of sludge were taken at the following three points of the WWTP (Figure 2) and used in the batch tests:

1) activated sludge collected from the oxidation tank;
2) return sludge collected from the return flow before being fed into the ASSR;
3) sludge collected from the ASSR.

2.3. Chemical analyses

Concentrations of Total Suspended Solids (TSS), COD and NH$_4^+$ were measured according to Standard Methods (APHA, 2012). Soluble COD was measured after filtration of the sample on 0.45-$\mu$m-membrane (Pall-Gelman).
2.4. Set-up of the batch tests

Three bench-scale reactors of 25-L volume were filled with each type of sludge collected as indicated in Section 2.2 and batch tests were carried out according to Table 1. In the “AS-aerobic” test, the activated sludge collected from the oxidation tank underwent full aeration and it was used as a control. The “RS-anaerobic-aerobic” and “SS-anaerobic-aerobic” tests were aimed to evaluate the modifications in the sludge during the transition from anaerobic to aerobic conditions. In the “RS-anaerobic-aerobic” test, the sludge collected from the return flow was gently and discontinuously mixed to form anaerobic conditions for 12 d, with the aim of simulating the process in the ASSR tank and then underwent aeration to simulate the return in the oxidation tank for 4 d. Similarly, in the “SS-anaerobic-aerobic” test, the sludge collected from the full-scale ASSR underwent mixing to form anaerobic conditions for 12 d and then underwent aeration for 4 d.

All the batch tests were managed without feeding and at a temperature of around 20°C. Water was added to the batch reactors during the monitoring period to replace the water lost through evaporation. The TSS concentration at the beginning of each batch test is indicated in Table 1. A higher TSS concentration was measured in the “RS-anaerobic-aerobic” and “SS-anaerobic-aerobic” tests due to the introduction of settled sludge. Due to the difference in TSS concentration in the three batch tests, results of bacteria enumeration will be expressed per unit of TSS. During the 12-d batch tests, the TSS concentration did not decrease significantly, probably because of the long sludge retention time (36 days) of the sludge in the full-scale WWTP.
For chemical and microbiological analyses, samples of sludge were taken during each batch test at a frequency of 1 or 2 days. Samples were conferred to the lab immediately after sampling.

Data interpolations were performed using Origin software (OriginLab) and all the parameters resulting from the interpolations are presented with 95% confidence interval.

2.5. Pre-treatment of sludge samples before flow cytometry analysis

Pre-treatment of sludge was applied to obtain a free cell suspension suitable for FCM analysis, according to Foladori et al. (2007). Briefly, samples underwent dilution and sonication (Branson 250 Digital Ultrasonifier, 20 kHz) at a transferred specific energy of 80 kJ L\(^{-1}\) in order to obtain the complete disaggregation of flocs maintaining cell integrity. The obtained free cells suspension was diluted to 1:400 v/v in Phosphate-Buffered-Saline (PBS) so as to reach 10\(^6\)–10\(^7\) bacteria per mL. A coarse filtration on 20-\(\mu\)m membranes (Celltrics, Partec) was performed to eliminate coarse particles which could clog the nozzle of the flow cytometer (particles excluded amounted to less than 3% of the initial floc area, according to Foladori et al., 2010b).

2.6. Fluorescent staining of bacteria

Viable and dead bacteria were determined after staining with the fluorescent dyes SYBR-Green I (SYBR-I, 1:30 dilution of commercial stock in dimethyl sulfoxide; provided by Invitrogen, USA; \(\lambda_{\text{ex}}=495\) nm, \(\lambda_{\text{em}}=525\) nm) and Propidium Iodide (PI, stock solution concentration 1 mg mL\(^{-1}\); provided by Invitrogen, USA; \(\lambda_{\text{ex}}=530\) nm, \(\lambda_{\text{em}}=620\) nm). An amount of 10 \(\mu\)L of both dyes was added to 1 mL of the cell suspension containing about 10\(^6\)-10\(^7\) cells/mL. Samples were then incubated at room temperature for 15 min in the dark. In permeabilised cells, the presence of both dyes activates the fluorescence resonance energy

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transfer phenomenon, so that the green fluorescence emission of SYBR-I is no longer visible; thus permeabilised cells appear only as fluorescent red, while intact cells appear fluorescent green. The coefficients of variation (CV) calculated on the five independent replicated samples were about 9% and 15% for viable and dead cells, respectively (Ziglio et al., 2002). For the analysis of enzymatically active bacteria, the fluorogenic substrate BCECF-AM (Invitrogen, USA) was used, which is hydrolysed inside the cell by intracellular non-specific esterases to produce fluorescein ($\lambda_{\text{ex}}$=490 nm, $\lambda_{\text{em}}$=535 nm). Fluorescein is hydrophilic and retained by intact and active cells. An amount of 10 $\mu$L of BCECF-AM 0.2 mM solution in dimethyl sulfoxide (DMSO, Merck, Germany) was added to 1 mL of bacteria suspension containing about $10^6$-$10^7$ cells/mL. Incubation was carried out at 36±1°C for 30 minutes in the dark. A CV of about 10% was estimated for enzymatically active cells.

2.7. Flow cytometry

FCM analyses were performed with an Apogee-A40 flow cytometer (Apogee Flow Systems, UK) equipped with an Ar laser (488 nm). Data acquisition gates were set on green and red fluorescence distribution so as to eliminate non-fluorescent debris. Green and red fluorescences were collected with logarithmic gain. At least 10,000 cells were analysed for each sample within a few minutes, providing good statistical data. Results of green and red fluorescences measured for each stained cell were graphically represented in a dot plot (Figure 3). In the cytograms, populations of dead and viable cells can be well distinguished and quantified.

< Insert Figure 3. FCM cytogram of viable and dead cells identified by staining with SYBR-I and PI. >
2.8. Calculation of COD produced from cell lysis

When bacteria undergo cell lysis, their intracellular material is released in the bulk liquid and it can be measured as soluble COD. The soluble COD, which could be potentially released, coincides with the COD of the entire bacterial cell. Using an approximated estimation, the following mass of COD can be calculated for one bacterial cell in sludge:

\[
\text{COD[mg/cell]} = V \cdot C_s \cdot 10^{-12} \cdot 1.42/0.53 = 191 \cdot 10^{-12} \text{ mg/cell}
\]

where \( V \) is the bacterial biovolume, here assumed as equal to 0.23 \( \mu m^3 \), as previously determined for activated sludge (Foladori et al., 2010b). \( C_s \) is the carbon content per unit of cell volume assumed here as equal to 310 fgC \( \mu m^{-3} \) (Fry, 1990). The carbon content is 53% of the dry weight of cells (derived from the empirical formula of bacteria composition, \( C_5H_7NO_2 \)) and the coefficient 1.42 is used to convert the dry weight of cells into COD.

An equivalent mass of soluble COD of 191 fg per bacterial cell which undergoes cell lysis was calculated from the expression indicated above.

The result is expressed as soluble COD because the intracellular compounds released in the bulk liquid have a size smaller than 0.45 \( \mu m \), the size used in the literature to distinguish between soluble and particulate COD.

3. RESULTS

3.1. Profiles of total bacteria during the batch tests

Total bacteria were calculated as the sum of viable and dead bacteria according to the scheme represented in Figure 1. The total bacteria (concentration ± standard deviation) were (5.9±0.6)\( \times 10^{12} \) cells/L in the activated sludge, (10.6±1.0) \( \times 10^{12} \) cells/L in the return sludge and (12.1±1.2) \( \times 10^{12} \) cells/L in the ASSR. The total bacteria concentration in the activated sludge samples was of the same order of magnitude as previously obtained for activated
sludge (Foladori et al., 2010b).

The profiles of total bacteria expressed per unit of TSS during the three batch tests are shown in Figure 4. The “RS-anaerobic-aerobic” test (RS-test) and “SS-anaerobic-aerobic” test (SS-test) are the focus of this study, that is to evaluate the effect of transition in the environmental conditions from anaerobiosis to aerobiosis, whilst the “AS-aerobic” test was continuously aerated and thus it served as a control.

From Figure 4, the difference in behaviour between the “AS-aerobic” test and the tests carried out under anaerobic conditions (RS-test and SS-test) appears immediately.

In the “AS-aerobic” test, the number of total bacteria decreased progressively over time ($t$) according to the following exponential curve:

$$TB = TB_0 \cdot e^{-b_{TB}t}$$

where $TB_0$ is the initial number of total bacteria (at $t=0$) and $b_{TB} = 0.07 \pm 0.01 \text{ d}^{-1}$ is the decay rate of total bacteria. Conversely, the number of total bacteria did not decrease in the anaerobic phase of the RS-test and SS-test. A linear interpolation of the experimental points gives slopes of $(1.9\pm2.4) \times 10^{10} \text{ cells gTSS}^{-1} \text{ d}^{-1}$ for the RS-test and $(2.5\pm2.4) \times 10^{10} \text{ cells gTSS}^{-1} \text{ d}^{-1}$ for the SS-test, which were not significantly different from a horizontal line. This indicates that the amount of cells do not decrease significantly during the analysis time interval.

After the recovery of aerobic conditions in the RS-test and SS-test, the number of total cells demonstrated a significant decrease. During the aerobic phase in the SS-test, the total bacteria concentration passed from $1.42\times10^{12} \text{ cells/gTSS}$ (at day 12) to $1.15\times10^{12} \text{ cells/gTSS}$ after 2 days of aeration, with a net loss of $0.27\times10^{12} \text{ cells/gTSS}$ (a reduction of 19%). Similarly, a reduction of 15% was observed in the first two days of the aerobic phase in the RS-test. The decay exponential factors of total bacteria are: $b_{TB} = 0.11\pm0.08 \text{ d}^{-1}$ in the RS-test and $b_{TB} = 0.08\pm0.02 \text{ d}^{-1}$ in the SS-test. Although no statistically significant difference was observed between the decay rates in the three tests, it is evident in all the tests the role of aerobiosis in
the development of a net decrease of total bacteria.

< Insert Figure 4. Total bacteria: profiles in the batch tests to compare aerobic conditions (AS-aerobic test) and the transition from anaerobic to aerobic conditions (RS-test and SS-test). >

3.2. Profiles of viable bacterial cells during the batch tests

The variations of viable bacteria during the three batch tests are shown in Figure 5. Profiles of viable cells are similar to those of total cells as presented in Figure 4, because viable cells constitute the largest fraction of total cells in all the tests. The viable bacteria concentrations at the beginning of the batch tests were in the range of $1.0 \times 10^{12}$ to $1.4 \times 10^{12}$ cells/gTSS, corresponding to 87-92% of total bacteria.

In the “AS-aerobic” test the number of viable bacteria decreased progressively over time according to the following exponential curve:

$$ VB = VB_0 \cdot e^{-b_{VB} t} $$

where $VB_0$ is the initial number of viable bacteria (at $t=0$) and $b_{VB}$ is the decay rate of viable bacteria which resulted as $0.07 \pm 0.02$ d$^{-1}$ and is thus very similar to the value found for total bacteria ($b_{TB} = 0.07 \pm 0.01$ d$^{-1}$).

In the RS-test and SS-test, the viable cells did not decrease during the anaerobic phase. A linear interpolation of the experimental points gives slopes of $(1.4 \pm 1.9) \times 10^{10}$ cells gTSS$^{-1}$ d$^{-1}$ for the RS-test and $(2.1 \pm 1.9) \times 10^{10}$ cells gTSS$^{-1}$ d$^{-1}$ for the SS-test, which were not significantly different from a horizontal line.

After the recovery of the aerobic condition in the RS-test and SS-test, the number of viable cells decreased gradually and the following decay rates were calculated during aerobic
conditions from exponential curves: $b_{VB} = 0.10 \pm 0.07 \text{ d}^{-1}$ in the RS-test and $b_{VB} = 0.07 \pm 0.02 \text{ d}^{-1}$ in the SS-test. Analogously to total cells, no statistically significant difference was observed between the decay rates of viable cells in the three tests. However, the data in all the tests confirmed the role of aerobiosis in the net decrease of viable bacteria.

< Insert Figure 5. Viable bacteria: profiles in the batch tests to compare aerobic conditions (AS-aerobic test) and the transition from anaerobic to aerobic conditions (RS-test and SS-test). >

### 3.3. Profiles of dead bacterial cells during the batch tests

The profiles of dead cells during the three batch tests are shown in Figure 6. The dead bacteria concentrations at the beginning of the batch tests were in the range of $1.3-1.5 \times 10^{11}$ cells/gTSS. The concentration of dead bacteria in the “AS-aerobic” test was very similar to the $1.5 \times 10^{11}$ cells/gTSS found in activated sludge in previous works (Foladori et al., 2010b). A marked difference was observed by comparing the profiles of dead bacteria under aerobic and anaerobic conditions.

The dead cells in the “AS-aerobic” test decreased slightly, passing from $1.4 \times 10^{11}$ cells/gTSS (at t=0) to $1.0 \times 10^{11}$ cells/gTSS at 12 d. The decrease is described by the following exponential curve:

$$DB = DB_0 \cdot e^{-b_{DB}t}$$

where $DB_0$ is the initial number of dead bacteria (at t=0) and $b_{DB}$ is the decay rate of dead bacteria. A value of $b_{DB}$ of $0.02 \pm 0.02 \text{ d}^{-1}$ was found, resulting as much lower than the decay rate found for both total bacteria and viable bacteria.

Conversely, a significant increase in dead cells was observed during the anaerobic phases in
the RS-test and SS-test. A linear interpolation of the experimental points gives slopes of 

\((5.4 \pm 4.5) \times 10^9 \text{ cells gTSS}^{-1} \text{ d}^{-1}\) for the RS-test and 

\((3.3 \pm 3.3) \times 10^9 \text{ cells gTSS}^{-1} \text{ d}^{-1}\) for the SS-test. Analogously to total bacteria (Figure 4) and viable bacteria (Figure 5), the slope of dead 

bacteria in the SS-test did not differ significantly from a horizontal line. Conversely, the RS-test evidenced a significant increase in the concentration of dead cells, with a significance 

level of 95%, if compared to zero slope. In the RS-test dead cells passed from \(1.4 \times 10^{11}\) at \(t=0\) 

to \(2.0 \times 10^{11}\) cells/gTSS at 12 d (+43% increase). The concentration of dead cells in the RS-test 

under anaerobic conditions increased significantly, because the return sludge was fresher and 

reactive, in contrast to the SS sludge derived directly from the ASSR tank with retention time 

of 7 days. The increase in dead cells in the RS-test demonstrates that cells damaged under 

anaerobic conditions accumulate in the sludge while maintaining their cellular structure 

without undergoing cell lysis.

When aerobic conditions were restored in the RS-test and SS-test, the concentration of dead 

bacteria decreased immediately, reaching \(1.1 \times 10^{11}\) cells/gTSS after just 1 day of aerobiosis (a 

reduction of about 30% of dead cells) as a result of the rapid lysis of a fraction of dead cells. 

The rapid loss of dead bacteria resulted in a remarkable value of the decay rate of dead 

bacteria \(b_{DB}\) under aerobic conditions. In particular, the exponential factor is 

\(b_{DB} = 0.24 \pm 0.12 \text{ d}^{-1}\) in the RS test and 

\(b_{DB} = 0.21 \pm 0.08 \text{ d}^{-1}\) in the SS test. Therefore, the transition 

from anaerobic to aerobic conditions resulted as being much more effective towards the decay 

of dead cells, which resulted significantly higher than under continuous aeration, where the 

decay was described by an exponential factor \(b_{DB}=0.02 \pm 0.02 \text{ d}^{-1}\).

Despite the high decay rate of dead cells in the anaerobic-aerobic tests, the contribution of 

dead cells in the sludge reduction has only a limited role, since dead cells account for only 8- 

13% of total cells in the sludge samples.
3.4. Enzymatically active cells during the batch tests

Enumeration of enzymatically active cells performed by BCECF-AM revealed a large percentage of bacteria with esterase activity, corresponding approximately to one half of viable cells. This result is in agreement with previous works, where active cells in activated sludge were 45% of the viable ones (Foladori et al., 2010b).

Although the anaerobic environment might be considered stressful for bacterial cells, the enzymatic (esterase) activity did not change significantly during the anaerobic conditions in either the RS-test or SS-test. The ratio between enzymatically active cells and viable cells (Figure 7) remained quite constant in all tests, also when the conditions passed from anaerobiosis to aerobiosis. Therefore the enzymatic activity was not particularly affected by the transition from anaerobic to aerobic conditions and remained as high as in the continuously aerated test.

3.5. Solubilisation of COD and nitrogen during the batch tests

At the beginning of the batch tests (t=0), the concentration of soluble COD was 44 mg/L and 51 mg/L in the activated sludge and return sludge respectively, while it was 294 mg/L in the ASSR sludge due to its long permanence under anaerobic conditions.

The application of anaerobic conditions (RS-test and SS-test) produced a progressive increase in soluble COD which reached 549 and 586 mg/L in the RS-test and SS-test respectively,
whilst the concentration remained quite constant in the AS-aerobic test (Figure 8).

In addition, the concentration of NH$_4^+$-N increased greatly under anaerobic conditions in the RS-test and SS-test, reaching 94 and 105 mgN/L in the RS-test and SS-test respectively (Figure 8). Conversely, the ammonia concentration remained consistently low in the AS-aerobic test.

These observations are in agreement with Novak et al. (2007) who observed the solubilisation of iron-bound organic matter, particularly proteins, in the ASSR tank, and with Park et al. (2006) who observed soluble protein generation and ammonium production in anaerobically digested sludge. The solubilisation of COD and nitrogen under anaerobic conditions demonstrated that the sludge, maintained for a long time in the ASSR, conserved a remarkable capacity of performing hydrolysis in a similar manner to the enzymatic hydrolysis detected inside bacterial cells (measured by hydrolysis of the BCECF-AM substrate in section 3.4).

Subsequently, the recovery of aerobic conditions in the RS-test and SS-test caused a rapid consumption of the solubilised compounds in just one day: the soluble COD was oxidised reaching concentrations below 80 mg/L, while ammonia underwent nitrification and dropped below 1 mgN/L (Figure 8).

Thus, in the OSA-like processes, solubilised compounds are expected to degrade under aerobic conditions when the sludge passes from the ASSR to the main aerobic activated sludge reactor.

Novak et al. (2007) indicate that iron-bound organic matter cannot be degraded under fully aerobic conditions even if at a long SRT (extended aeration processes or aerobic digestion),
but it requires undergoing anaerobic conditions where solubilisation occurs. Chon et al. (2011b) observed that some organic matter cannot be degraded via separate anaerobic digestion, while requiring a sequence of aerobic-anaerobic conditions to enhance the biodegradability of sludge. The high biodegradability of solubilised COD was observed by Novak et al. (2007) while measuring the oxygen uptake rate of the ASSR supernatant.

4. DISCUSSION

4.1. Bacterial cells do not decrease under anaerobic conditions

The number of total cells and viable cells did not decrease significantly during the anaerobic tests (Figures 4 and 5).

It would be erroneous to consider the ASSR system as being completely devoid of any substrate where bacteria undergo famine. Conversely, a large amount of soluble and biodegradable organic substrates and nitrogen are released from sludge and are thus available in the anaerobic reactor in an easily utilisable form. However, anaerobic conditions does not allow bacteria to grow significantly, as demonstrated by the slow or negligible increase in viable bacteria (Figure 5). The evolution of microbial species to slow growers with low sludge yield under anaerobic conditions was proved by pyrosequencing (Zhou et al., 2015). Among slow-growing bacteria, enrichment of fermenters was observed in plants equipped with aerobic–anaerobic zones, with ability to degrade flocs and accelerate sludge decay (Goel and Noguera, 2006; Quan et al., 2012; Li et al., 2014).

The number of dead cells increased significantly during the 12-day anaerobic tests by 43% in the RS-test (Figure 6). Although indicating that some sort of damage to cells occurs naturally under anaerobic conditions, the damaged bacteria did not undergo cell lysis.
4.2. Bacterial cells decay under aerobic conditions

When aerobic conditions were applied in the RS-test and SS-test after the 12-day anaerobic phase, decay of total cells, viable cells and dead cells was observed. Table 2 demonstrates a summary of the decay rates calculated under aerobiosis in the RS-test and SS-test compared to the continuously aerated “AS-aerobic” test. Decay rates were calculated according to the exponential curve, which is the common relationship used to describe endogenous decay in activated sludge models (inter alia Henze et al., 1999). The decay rates indicated in Table 2 refer to the decay of bacterial cells, and thus they may differ from other values referred to in the literature regarding, in general, the decay rate of sludge which includes bacterial cells and organic matter.

During cell decay, cryptic growth could also take place. Therefore, the values of the decay rate indicated in Table 2 could be underestimated with respect to real values; thus they here assume the meaning of a net decay rate. The values indicated in Table 2 may not immediately be comparable with the literature data on the endogenous decay rate if this parameter was measured with different methods. Troiani et al. (2011) observed that different methodologies applied for measuring the decay rate may give different values and further investigation is therefore needed to understand comparison between literature data. In addition, higher decay coefficient under aerobic conditions in comparison with anaerobic conditions was also demonstrated for ammonia oxidising bacteria (Munz et al., 2011).

< Insert Table 2. Decay rates of total bacteria, viable bacteria and dead bacteria under aerobic conditions in the three batch tests (95% confidence interval). Legend: n.d. = no decay. >
Comparing the decay rates of total bacteria ($b_{TB}$) measured in the aerobic phases of the three tests, no significant difference was found among the tests and values of $b_{TB}$ were in the range of 0.07-0.11 d$^{-1}$.

With regards to viable bacteria, the decay rates ($b_{VB}$) measured in the aerobic phases of the batch tests were very similar to decay rates of total bacteria, assuming values in the range of 0.07-0.10 d$^{-1}$. Troiani et al. (2011) confirmed that the endogenous decay rate was not particularly improved in the ASSR tank, while a higher decay rate was observed in the oxidation tank, but the authors highlighted the need for further research in this field.

The decay rates of dead cells ($b_{DB}$) measured in the aerobic phase assumed the following relationship: RS-test $\sim$ SS-test $\gg$ AS-aerobic test. The values of $b_{DB}$ in the RS-test and SS-test were one order of magnitude higher than in the AS-aerobic test (Table 2). Therefore, the transition from anaerobic to aerobic conditions permitted an increase in the aerobic decay rate of dead cells leading definitively to an enhancement of cell lysis.

In conclusion, in the transition from anaerobiosis to aerobiosis (or the application of a recirculation from aerobic to anaerobic tanks in full-scale plants), only the aerobic phase contributes to bacteria reduction due to an appreciable aerobic decay rate.

The importance of the sludge recirculation between the external ASSR and the main aerobic bioreactors has been confirmed in the literature (Semblante et al., 2014): it has been underlined that the biomass reduction is maximised when sludge is returned to the aerobic main reactor, where decay occurs (Chon et al., 2011a,b; Kim et al., 2012).

Kim et al. (2012), working with an ASSR with a 10% interchange rate, which corresponds to HRT of 10 d, observed that the main activated sludge reactor and the ASSR were characterised by different bacterial communities although these stages were connected via continuous sludge recirculation. The sludge remains in the ASSR for a relatively long HRT (around 10 d), which is enough to establish modifications in the structure and composition of
Chon et al. (2011a) observed that some unique anaerobic microorganisms were enriched in the ASSR with a continuous recirculation. About the fate of anaerobic organisms under the subsequent aerobic conditions, Li et al. (2014) observed that some fermentative bacteria, such as *Clostridium* and *Stenotrophomonas*, intensified in anaerobic zone, then disappeared in the aerobic zone. These observations support the assumption that anaerobic microorganisms developed in the ASSR will undergo stress or cell lysis when aerobic conditions are restored after the recirculation of the anaerobic sludge in the activated sludge reactor. Moreover, the role of obligated aerobic organisms cannot be excluded, which may not survive the anaerobic conditions and may be eventually lysed when they return to the aerobic conditions. However, further research is needed to confirm these assumptions and to distinguish the fate of aerobic and anaerobic microorganisms in the OSA-like processes.

### 4.3. The quantitative role of cell lysis

Cell lysis is a process in which the bacterial structure is completely disrupted and the intracellular compounds are released into the bulk liquid. Knowing the number of lysed cells calculated as the loss of total cells (Section 3.1), the amount of soluble COD originated by cell lysis was theoretically estimated using the approximate calculation described in Section 2.8. Although this simple calculation could not be perfectly accurate, and some uncertainties remain regarding carbon content or biovolume, it allows us to obtain at least a coarse approximation of the COD produced from cell lysis, and therefore contributes to an understanding of sludge reduction.

For example, the concentration of lysed bacteria in the RS-test after two days of aerobic conditions (calculated using the decay rate of total cells) was 0.30×10^{12} cells/gTSS, which approximately corresponds to the amount of 57 mgCOD/gTSS or 464 mgCOD/L produced from cell lysis. A summary of the soluble COD produced from cell lysis during the aerobic
4.4. Proposal of the concept of anaerobic sludge hydrolysis and aerobic cell lysis

The observation of COD solubilisation occurring under anaerobic conditions (Figure 8) and cell lysis occurring under aerobic conditions (Figure 4) in the RS-test and SS-test can be explained as the result of two classes of reactions:

(1) under anaerobic conditions: hydrolysis of the non-bacterial portion of the organic matter such as iron-bound compounds or fermentation of high molecular weight substances into volatile fatty acids and alcohols, resulting in an accumulation of soluble biodegradable COD, but no cell lysis;

(2) under aerobic conditions: oxidation of the anaerobically solubilised COD, occurrence of cell lysis and oxidation of biodegradable compounds in the lysate.

COD solubilisation during anaerobic conditions for 12 d accounted for 549-586 mgCOD/L in the RS-test and SS-test respectively (Figure 8), and was associated with sludge solubilisation without cell lysis (Table 3). With regards to the composition of the organic fraction of sludge, the mass of viable or dead bacteria accounts respectively for a fraction of about 20% and 2% of particulate COD, while the remaining part (78% of particulate COD of sludge) is considered non-bacterial material.

The cell lysis occurring during the subsequent aerobic conditions in the RS-test and SS-test produced a similar amount of soluble COD (393-464 mgCOD/L) after 2 days of aerobiosis (Table 3).

In this concept, the sludge undergoes “anaerobic” sludge hydrolysis and “aerobic” cell lysis, which are two independent mechanisms occurring in different stages of the plant but contributing similarly to sludge reduction.
The important role of COD solubilisation under anaerobic conditions has been confirmed in the literature (Novak et al., 2003; Novak et al., 2007; Chon et al., 2011a), suggesting that this is one of the most important mechanisms of sludge reduction. The solubilisation under anaerobic conditions was explained with the release of iron and/or aluminium-associated organic matter into sludge solution or with the fermentation of particulate matter, whilst bacteria cell lysis was not accounted for. Our data supports these observations, since sludge solubilisation occurred in the ASSR without any cell lysis, thus confirming the role of non-bacterial material.

The equally important role of cell lysis during the aerobic phase, which causes the release of a comparable amount of soluble COD, was demonstrated in this research for the first time. In both these phenomena (anaerobic sludge hydrolysis and aerobic cell lysis), the degradation of the soluble COD occurs in the oxidation tanks where the biodegradable fraction is lost and converted to respiration products such as $\text{H}_2\text{O}$, $\text{CO}_2$, etc., thus contributing to sludge reduction.

Chon et al. (2011b) observed that about one half of the overall sludge reduction occurred in the oxidation tanks, while the other half was directly achieved in the ASSR. These proportions are in agreement with the concept of anaerobic sludge hydrolysis and aerobic cell lysis observed in the present research.

Chon et al. (2011a) concluded that the sequence of aerobic and anaerobic conditions (in activated sludge and ASSR) was much more effective than conventional activated sludge or conventional digestion alone (aerobic or anaerobic used separately) in terms of sludge reduction.
Coma et al. (2013), in a pilot-scale ASSR, stated that sludge reduction can be enhanced by rapid passages through the anaerobic and aerobic conditions.

The transition from anaerobiosis to aerobiosis thus appears as one of the key points of sludge reduction in the OSA-like processes and the repeated alternation between aerobic and anaerobic conditions can reduce the overall biomass even if the cryptic-growth is included.

5. CONCLUSION

The physiological status (viability, activity, death and lysis) of bacteria in the sludge and the changes when exposed to aerobic and anaerobic conditions were investigated by FCM to obtain new information about the mechanisms of sludge reduction in the ASSR systems.

The main outcomes are the following:

1) anaerobic conditions at ambient temperature did not produce a significant cell lysis;
2) cell decay and lysis occurred principally under aerobic conditions;
3) the sludge conserved a good enzymatic hydrolysis (measured using the substrate BCECF-AM) even under anaerobic conditions; thus the conservation of good hydrolysis both inside and outside bacterial cells can be reasonably expected;
4) anaerobic conditions favoured the hydrolysis and solubilisation of non-bacterial material with a large increase in soluble biodegradable COD and NH$_4^+$ in the anaerobically treated sludge.

On the basis of these findings, the two independent mechanisms contributing equally to sludge reduction were: anaerobic sludge hydrolysis and aerobic cell lysis. The transition from anaerobiosis to aerobiosis thus appears one of the key points of sludge reduction in the OSA-like processes and the repeated alternation between aerobic and anaerobic conditions could reduce the overall biomass even if the cryptic-growth is included.
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REFERENCES


Table 1. Operational conditions in the three batch tests.

<table>
<thead>
<tr>
<th>Type of sludge</th>
<th>AS-aerobic</th>
<th>RS-anaerobic-aerobic</th>
<th>SS-anaerobic-aerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditions during</td>
<td>Aerobic (12 d)</td>
<td>Anaerobic (12 d)</td>
<td>Anaerobic (12 d)</td>
</tr>
<tr>
<td>the test (duration)</td>
<td></td>
<td>Aerobic (4 d)</td>
<td>Aerobic (4 d)</td>
</tr>
<tr>
<td>TSS concentration</td>
<td>3.8 kgTSS/m³</td>
<td>8.1 kgTSS/m³</td>
<td>9.8 kgTSS/m³</td>
</tr>
<tr>
<td>at the beginning</td>
<td>of the test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>of the test</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Decay rates of total bacteria, viable bacteria and dead bacteria under aerobic conditions in the three batch tests (95% confidence interval). Legend: n.d. = no decay.

<table>
<thead>
<tr>
<th>AS-aerobic</th>
<th>RS-anaerobic-aerobic</th>
<th>SS-anaerobic-aerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>Decay of total cells b &lt;i&gt;T_B&lt;/i&gt;</td>
<td>0.07±0.01 d&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>n.d.</td>
</tr>
<tr>
<td>Decay of viable cells b &lt;i&gt;V_B&lt;/i&gt;</td>
<td>0.07±0.02 d&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>n.d.</td>
</tr>
<tr>
<td>Decay of dead cells b &lt;i&gt;D_B&lt;/i&gt;</td>
<td>0.02±0.02 d&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
Table 3. Release of soluble COD in the three batch tests due to sludge hydrolysis or cell lysis. Legend: n.a. = not appreciable.

<table>
<thead>
<tr>
<th>COD solubilisation due to sludge hydrolysis (excluded cell lysis)</th>
<th>AS-aerobic</th>
<th>RS-anaerobic-aerobic</th>
<th>SS-anaerobic-aerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD solubilisation due to cell lysis</td>
<td>mgCOD/L</td>
<td>mgCOD/L</td>
<td>mgCOD/L</td>
</tr>
<tr>
<td>n.a.</td>
<td>+549</td>
<td>+586</td>
<td></td>
</tr>
<tr>
<td>(+after 12 d)</td>
<td>-</td>
<td>(+after 12 d)</td>
<td></td>
</tr>
<tr>
<td>+483</td>
<td>+250</td>
<td>+194</td>
<td></td>
</tr>
<tr>
<td>(+after 12 d)</td>
<td>-</td>
<td>(+after 1 d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+464</td>
<td>+393</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(+after 2 d)</td>
<td>(+after 2 d)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Physiological status of bacterial cells and test criteria for identification. Lysed cells are quantified by difference of total cells at two different times (0, t).
Figure 2. Flow sheet of the WWTP including the anaerobic side stream reactor (ASSR) for sludge reduction. The points of sampling of sludge used in the batch tests are indicated.
Figure 3. FCM cytogram of viable and dead cells identified by staining with SYBR-I and PI.
Figure 4. Total bacteria: profiles in the batch tests to compare aerobic conditions (AS-aerobic test) and the transition from anaerobic to aerobic conditions (RS-test and SS-test).
Figure 5. Viable bacteria: profiles in the batch tests to compare aerobic conditions (AS-aerobic test) and the transition from anaerobic to aerobic conditions (RS-test and SS-test).
Figure 6. Dead bacteria: profiles in the batch tests to compare aerobic conditions (AS-aerobic test) and the transition from anaerobic to aerobic conditions (RS-test and SS-test).
Figure 7. Ratio of enzymatically active bacteria in the batch tests.
Figure 8. Profiles of soluble COD (A) and NH$_4^+$-N (B) in the batch tests.