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).

< *Insert 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).* >

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

105 the changes of enzymatic activity, viability, death and lysis of bacterial cells measured during
106 aerobic and anaerobic batch tests with a duration comparable to the typical HRT in the ASSR
107 tank. The aim is to gain a better insight of the role of cell lysis in sludge reduction in the
108 ASSR systems, evaluating aerobic and anaerobic environmental conditions and the effect of
109 transition from anaerobiosis to aerobiosis.
110
111 **2. MATERIALS AND METHODS**
112
113 **2.1. Configuration of WWTP and ASSR**
114 The municipal WWTP of Levico (Italy) treats a mean population equivalent of 48,000 and is
115 based on an activated sludge process (volume of reactors of 7,000 m^3) as shown in Figure 2.
116 The ASSR tank (Cannibal®, Siemens, introduced in 2008) has a volume of 2,293 m^3 and
117 treats a part of the sludge (330 m^3/d) separated from the return flow before being returned to
118 the activated sludge reactors. Intermittent mixing was provided in the ASSR tank to ensure
119 homogenous conditions, while treated sludge was discharged during no-mixing periods. The
120 theoretical hydraulic retention time (HRT) in the ASSR tank was about 7 d, coinciding in
121 practice with the sludge retention time (SRT) in the anaerobic tank, due to the scarce
122 settleability of anaerobic sludge as a consequence of the high solids concentrations. The HRT
123 in the ASSR tank is in the same order of magnitude of 10 d applied in other ASSR studies
124 (Novak et al., 2007; Johnson et al., 2008; Troiani et al., 2011; Kim et al., 2012). ORP and pH
125 inside the ASSR were below -250 mV (but reached -150 mV during feeding of sludge) and in
126 the range 6-6.5, respectively.
127
128 *< Insert Figure 2. Flow sheet of the WWTP including the anaerobic side stream reactor*
129 *(ASSR) for sludge reduction. The points of sampling of sludge used in the batch tests are*

indicated. >

The excess sludge production in the WWTP was calculated from 2005 to 2013 by means of the observed sludge yield (Y_{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_{obs} of 0.350±0.004 kgTSS/kgCOD was measured in the presence of the ASSR system, resulting significantly lower than the 0.442±0.002 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-μm-membrane (Pall-Gelman).

155

156 **2.4. Set-up of the batch tests**

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

168

169 *< Insert Table 1. Operational conditions in the three batch tests. >*

170

171 All the batch tests were managed without feeding and at a temperature of around 20°C. Water
172 was added to the batch reactors during the monitoring period to replace the water lost through
173 evaporation.

174 The TSS concentration at the beginning of each batch test is indicated in Table 1. A higher
175 TSS concentration was measured in the "RS-anaerobic-aerobic" and "SS-anaerobic-aerobic"
176 tests due to the introduction of settled sludge. Due to the difference in TSS concentration in
177 the three batch tests, results of bacteria enumeration will be expressed per unit of TSS. During
178 the 12-d batch tests, the TSS concentration did not decrease significantly, probably because of
179 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-µm 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; λ_{ex}=495 nm, λ_{em}=525 nm) and Propidium Iodide (PI, stock solution concentration 1 mg mL^{-1}; provided by Invitrogen, USA; λ_{ex}=530 nm, λ_{em}=620 nm). An amount of 10 µL 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

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 (λ_{ex}=490 nm, λ_{em}=535 nm). Fluorescein is hydrophilic and retained by intact and active cells. An amount of 10 µL 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. >

230 **2.8. Calculation of COD produced from cell lysis**

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

235 $COD[mg/cell] = V \cdot C_s \cdot 10^{-12} \cdot 1.42/0.53 = 191 \cdot 10^{-12}$ mg/cell

236 where V is the bacterial biovolume, here assumed as equal to 0.23 μm^3, as previously
237 determined for activated sludge (Foladori et al., 2010b). C_s is the carbon content per unit of
238 cell volume assumed here as equal to 310 fgC μm^{-3} (Fry, 1990). The carbon content is 53% of
239 the dry weight of cells (derived from the empirical formula of bacteria composition,
240 $C_5H_7NO_2$) and the coefficient 1.42 is used to convert the dry weight of cells into COD.
241 An equivalent mass of soluble COD of 191 fg per bacterial cell which undergoes cell lysis
242 was calculated from the expression indicated above.
243 The result is expressed as soluble COD because the intracellular compounds released in the
244 bulk liquid have a size smaller than 0.45 μm, the size used in the literature to distinguish
245 between soluble and particulate COD.
246
247 **3. RESULTS**
248
249 **3.1. Profiles of total bacteria during the batch tests**
250 Total bacteria were calculated as the sum of viable and dead bacteria according to the scheme
251 represented in Figure 1. The total bacteria (concentration ± standard deviation) were
252 $(5.9\pm0.6)\times10^{12}$ cells/L in the activated sludge, $(10.6\pm1.0)\times10^{12}$ cells/L in the return sludge
253 and $(12.1\pm1.2)\times10^{12}$ cells/L in the ASSR. The total bacteria concentration in the activated
254 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:

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

where TB_0 is the initial number of total bacteria (at t=0) and $b_{TB} = 0.07 \pm 0.01$ 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 $(1.9\pm2.4)\times10^{10}$ cells $gTSS^{-1}$ d^{-1} for the RS-test and $(2.5\pm2.4)\times10^{10}$ cells $gTSS^{-1}$ 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 1.42×10^{12} cells/gTSS (at day 12) to 1.15×10^{12} cells/gTSS after 2 days of aeration, with a net loss of 0.27×10^{12} 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: $b_{TB} = 0.11\pm0.08$ d^{-1} in the RS-test and $b_{TB} = 0.08\pm0.02$ 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

280 the development of a net decrease of total bacteria.

281

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

285

286

287 **3.2. Profiles of viable bacterial cells during the batch tests**

288 The variations of viable bacteria during the three batch tests are shown in Figure 5. Profiles of
289 viable cells are similar to those of total cells as presented in Figure 4, because viable cells
290 constitute the largest fraction of total cells in all the tests. The viable bacteria concentrations
291 at the beginning of the batch tests were in the range of 1.0×10^{12} to 1.4×10^{12} cells/gTSS,
292 corresponding to 87-92% of total bacteria.
293 In the "AS-aerobic" test the number of viable bacteria decreased progressively over time
294 according to the following exponential curve:
295 $$VB = VB_0 \cdot e^{-b_{VB} \cdot t}$$
296 where VB_0 is the initial number of viable bacteria (at t=0) and b_{VB} is the decay rate of viable
297 bacteria which resulted as 0.07 ± 0.02 d^{-1} and is thus very similar to the value found for total
298 bacteria ($b_{TB} = 0.07\pm0.01$ d^{-1}).
299 In the RS-test and SS-test, the viable cells did not decrease during the anaerobic phase. A
300 linear interpolation of the experimental points gives slopes of $(1.4\pm1.9)\times10^{10}$ cells $gTSS^{-1}$ d^{-1}
301 for the RS-test and $(2.1\pm1.9)\times10^{10}$ cells $gTSS^{-1}$ d^{-1} for the SS-test, which were not
302 significantly different from a horizontal line.
303 After the recovery of the aerobic condition in the RS-test and SS-test, the number of viable
304 cells decreased gradually and the following decay rates were calculated during aerobic

conditions from exponential curves: b_{VB} = 0.10±0.07 d^{-1} in the RS-test and b_{VB} = 0.07±0.02 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 1.3-1.5×10^{11} cells/gTSS. The concentration of dead bacteria in the "AS-aerobic" test was very similar to the 1.5×10^{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 1.4×10^{11} cells/gTSS (at t=0) to 1.0×10^{11} cells/gTSS at 12 d. The decrease is described by the following exponential curve:

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

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±0.02 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

330 the RS-test and SS-test. A linear interpolation of the experimental points gives slopes of
331 $(5.4\pm4.5)\times10^{9}$ cells $gTSS^{-1}$ d^{-1} for the RS-test and $(3.3\pm3.3)\times10^{9}$ cells $gTSS^{-1}$ d^{-1} for the SS-
332 test. Analogously to total bacteria (Figure 4) and viable bacteria (Figure 5), the slope of dead
333 bacteria in the SS-test did not differ significantly from a horizontal line. Conversely, the RS-
334 test evidenced a significant increase in the concentration of dead cells, with a significance
335 level of 95%, if compared to zero slope. In the RS-test dead cells passed from 1.4×10^{11} at t=0
336 to 2.0×10^{11} cells/gTSS at 12 d (+43% increase). The concentration of dead cells in the RS-test
337 under anaerobic conditions increased significantly, because the return sludge was fresher and
338 reactive, in contrast to the SS sludge derived directly from the ASSR tank with retention time
339 of 7 days. The increase in dead cells in the RS-test demonstrates that cells damaged under
340 anaerobic conditions accumulate in the sludge while maintaining their cellular structure
341 without undergoing cell lysis.
342 When aerobic conditions were restored in the RS-test and SS-test, the concentration of dead
343 bacteria decreased immediately, reaching 1.1×10^{11} cells/gTSS after just 1 day of aerobiosis (a
344 reduction of about 30% of dead cells) as a result of the rapid lysis of a fraction of dead cells.
345 The rapid loss of dead bacteria resulted in a remarkable value of the decay rate of dead
346 bacteria (b_{DB}) under aerobic conditions. In particular, the exponential factor is b_{DB} =
347 0.24 ± 0.12 d^{-1} in the RS test and $b_{DB} = 0.21\pm0.08$ d^{-1} in the SS test. Therefore, the transition
348 from anaerobic to aerobic conditions resulted as being much more effective towards the decay
349 of dead cells, which resulted significantly higher than under continuous aeration, where the
350 decay was described by an exponential factor $b_{DB}=0.02\pm0.02$ d^{-1}.
351 Despite the high decay rate of dead cells in the anaerobic-aerobic tests, the contribution of
352 dead cells in the sludge reduction has only a limited role, since dead cells account for only 8-
353 13% of total cells in the sludge samples.
354

355 *< Insert Figure 6. Dead bacteria: profiles in the batch tests to compare aerobic conditions*
356 *(AS-aerobic test) and the transition from anaerobic to aerobic conditions (RS-test and SS-*
357 *test). >*
358
359 **3.4. Enzymatically active cells during the batch tests**
360 Enumeration of enzymatically active cells performed by BCECF-AM revealed a large
361 percentage of bacteria with esterase activity, corresponding approximately to one half of
362 viable cells. This result is in agreement with previous works, where active cells in activated
363 sludge were 45% of the viable ones (Foladori et al., 2010b).
364 Although the anaerobic environment might be considered stressful for bacterial cells, the
365 enzymatic (esterase) activity did not change significantly during the anaerobic conditions in
366 either the RS-test or SS-test. The ratio between enzymatically active cells and viable cells
367 (Figure 7) remained quite constant in all tests, also when the conditions passed from
368 anaerobiosis to aerobiosis. Therefore the enzymatic activity was not particularly affected by
369 the transition from anaerobic to aerobic conditions and remained as high as in the
370 continuously aerated test.
371
372 *< Insert Figure 7. Ratio of enzymatically active bacteria in the batch tests. >*
373
374 **3.5. Solubilisation of COD and nitrogen during the batch tests**
375 At the beginning of the batch tests (t=0), the concentration of soluble COD was 44 mg/L and
376 51 mg/L in the activated sludge and return sludge respectively, while it was 294 mg/L in the
377 ASSR sludge due to its long permanence under anaerobic conditions.
378 The application of anaerobic conditions (RS-test and SS-test) produced a progressive increase
379 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.

< Insert Figure 8. Profiles of soluble COD (A) and NH_4^+-N (B) in the batch tests. >

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),

405 but it requires undergoing anaerobic conditions where solubilisation occurs. Chon et al.
406 (2011b) observed that some organic matter cannot be degraded via separate anaerobic
407 digestion, while requiring a sequence of aerobic-anaerobic conditions to enhance the
408 biodegradability of sludge. The high biodegradability of solubilised COD was observed by
409 Novak et al. (2007) while measuring the oxygen uptake rate of the ASSR supernatant.
410
411
412 **4. DISCUSSION**
413
414 **4.1. Bacterial cells do not decrease under anaerobic conditions**
415 The number of total cells and viable cells did not decrease significantly during the anaerobic
416 tests (Figures 4 and 5).
417 It would be erroneous to consider the ASSR system as being completely devoid of any
418 substrate where bacteria undergo famine. Conversely, a large amount of soluble and
419 biodegradable organic substrates and nitrogen are released from sludge and are thus available
420 in the anaerobic reactor in an easily utilisable form. However, anaerobic conditions does not
421 allow bacteria to grow significantly, as demonstrated by the slow or negligible increase in
422 viable bacteria (Figure 5). The evolution of microbial species to slow growers with low
423 sludge yield under anaerobic conditions was proved by pyrosequencing (Zhou et al., 2015).
424 Among slow-growing bacteria, enrichment of fermenters was observed in plants equipped
425 with aerobic–anaerobic zones, with ability to degrade flocs and accelerate sludge decay (Goel
426 and Noguera, 2006; Quan et al., 2012; Li et al., 2014).
427 The number of dead cells increased significantly during the 12-day anaerobic tests by 43% in
428 the RS-test (Figure 6). Although indicating that some sort of damage to cells occurs naturally
429 under anaerobic conditions, the damaged bacteria did not undergo cell lysis.

430

431 **4.2. Bacterial cells decay under aerobic conditions**

432 When aerobic conditions were applied in the RS-test and SS-test after the 12-day anaerobic
433 phase, decay of total cells, viable cells and dead cells was observed. Table 2 demonstrates a
434 summary of the decay rates calculated under aerobiosis in the RS-test and SS-test compared
435 to the continuously aerated "AS-aerobic" test. Decay rates were calculated according to the
436 exponential curve, which is the common relationship used to describe endogenous decay in
437 activated sludge models (*inter alia* Henze et al., 1999). The decay rates indicated in Table 2
438 refer to the decay of bacterial cells, and thus they may differ from other values referred to in
439 the literature regarding, in general, the decay rate of sludge which includes bacterial cells and
440 organic matter.
441 During cell decay, cryptic growth could also take place. Therefore, the values of the decay
442 rate indicated in Table 2 could be underestimated with respect to real values; thus they here
443 assume the meaning of a net decay rate. The values indicated in Table 2 may not immediately
444 be comparable with the literature data on the endogenous decay rate if this parameter was
445 measured with different methods. Troiani et al. (2011) observed that different methodologies
446 applied for measuring the decay rate may give different values and further investigation is
447 therefore needed to understand comparison between literature data. In addition, higher decay
448 coefficient under aerobic conditions in comparison with anaerobic conditions was also
449 demonstrated for ammonia oxidising bacteria (Munz et al., 2011).

450

451 *< Insert Table 2. Decay rates of total bacteria, viable bacteria and dead bacteria under*
452 *aerobic conditions in the three batch tests (95% confidence interval). Legend: n.d. = no*
453 *decay. >*

454

455 Comparing the decay rates of total bacteria (b_{TB}) measured in the aerobic phases of the three
456 tests, no significant difference was found among the tests and values of b_{TB} were in the range
457 of 0.07-0.11 d^{-1}.
458 With regards to viable bacteria, the decay rates (b_{VB}) measured in the aerobic phases of the
459 batch tests were very similar to decay rates of total bacteria, assuming values in the range of
460 0.07-0.10 d^{-1}. Troiani et al. (2011) confirmed that the endogenous decay rate was not
461 particularly improved in the ASSR tank, while a higher decay rate was observed in the
462 oxidation tank, but the authors highlighted the need for further research in this field.
463 The decay rates of dead cells (b_{DB}) measured in the aerobic phase assumed the following
464 relationship: RS-test ~ SS-test >> AS-aerobic test. The values of b_{DB} in the RS-test and SS-
465 test were one order of magnitude higher than in the AS-aerobic test (Table 2). Therefore, the
466 transition from anaerobic to aerobic conditions permitted an increase in the aerobic decay rate
467 of dead cells leading definitively to an enhancement of cell lysis.
468 In conclusion, in the transition from anaerobiosis to aerobiosis (or the application of a
469 recirculation from aerobic to anaerobic tanks in full-scale plants), only the aerobic phase
470 contributes to bacteria reduction due to an appreciable aerobic decay rate.
471 The importance of the sludge recirculation between the external ASSR and the main aerobic
472 bioreactors has been confirmed in the literature (Semblante et al., 2014): it has been
473 underlined that the biomass reduction is maximised when sludge is returned to the aerobic
474 main reactor, where decay occurs (Chon et al., 2011a,b; Kim et al., 2012).
475 Kim et al. (2012), working with an ASSR with a 10% interchange rate, which corresponds to
476 HRT of 10 d, observed that the main activated sludge reactor and the ASSR were
477 characterised by different bacterial communities although these stages were connected via
478 continuous sludge recirculation. The sludge remains in the ASSR for a relatively long HRT
479 (around 10 d), which is enough to establish modifications in the structure and composition of

480 sludge. Chon et al. (2011a) observed that some unique anaerobic microorganisms were
481 enriched in the ASSR with a continuous recirculation. About the fate of anaerobic organisms
482 under the subsequent aerobic conditions, Li et al. (2014) observed that some fermentative
483 bacteria, such as *Clostridium* and *Stenotrophomonas*, intensified in anaerobic zone, then
484 disappeared in the aerobic zone. These observations support the assumption that anaerobic
485 microorganisms developed in the ASSR will undergo stress or cell lysis when aerobic
486 conditions are restored after the recirculation of the anaerobic sludge in the activated sludge
487 reactor. Moreover, the role of obligated aerobic organisms cannot be excluded, which may not
488 survive the anaerobic conditions and may be eventually lysed when they return to the aerobic
489 conditions. However, further research is needed to confirm these assumptions and to
490 distinguish the fate of aerobic and anaerobic microorganisms in the OSA-like processes.
491
492 **4.3. The quantitative role of cell lysis**
493 Cell lysis is a process in which the bacterial structure is completely disrupted and the
494 intracellular compounds are released into the bulk liquid. Knowing the number of lysed cells
495 calculated as the loss of total cells (Section 3.1), the amount of soluble COD originated by
496 cell lysis was theoretically estimated using the approximate calculation described in Section
497 2.8. Although this simple calculation could not be perfectly accurate, and some uncertainties
498 remain regarding carbon content or biovolume, it allows us to obtain at least a coarse
499 approximation of the COD produced from cell lysis, and therefore contributes to an
500 understanding of sludge reduction.
501 For example, the concentration of lysed bacteria in the RS-test after two days of aerobic
502 conditions (calculated using the decay rate of total cells) was 0.30×10^{12} cells/gTSS, which
503 approximately corresponds to the amount of 57 mgCOD/gTSS or 464 mgCOD/L produced
504 from cell lysis. A summary of the soluble COD produced from cell lysis during the aerobic

505 phase of the three batch tests is shown in Table 3.

506

507 **4.4. Proposal of the concept of anaerobic sludge hydrolysis and aerobic cell lysis**

508 The observation of COD solubilisation occurring under anaerobic conditions (Figure 8) and
509 cell lysis occurring under aerobic conditions (Figure 4) in the RS-test and SS-test can be
510 explained as the result of two classes of reactions:
511 (1) under anaerobic conditions: hydrolysis of the non-bacterial portion of the organic matter
512 such as iron-bound compounds or fermentation of high molecular weight substances into
513 volatile fatty acids and alcohols, resulting in an accumulation of soluble biodegradable COD,
514 but no cell lysis;
515 (2) under aerobic conditions: oxidation of the anaerobically solubilised COD, occurrence of
516 cell lysis and oxidation of biodegradable compounds in the lysate.
517 COD solubilisation during anaerobic conditions for 12 d accounted for 549-586 mgCOD/L in
518 the RS-test and SS-test respectively (Figure 8), and was associated with sludge solubilisation
519 without cell lysis (Table 3). With regards to the composition of the organic fraction of sludge,
520 the mass of viable or dead bacteria accounts respectively for a fraction of about 20% and 2%
521 of particulate COD, while the remaining part (78% of particulate COD of sludge) is
522 considered non-bacterial material.
523 The cell lysis occurring during the subsequent aerobic conditions in the RS-test and SS-test
524 produced a similar amount of soluble COD (393-464 mgCOD/L) after 2 days of aerobiosis
525 (Table 3).
526 In this concept, the sludge undergoes “anaerobic” sludge hydrolysis and “aerobic” cell lysis,
527 which are two independent mechanisms occurring in different stages of the plant but
528 contributing similarly to sludge reduction.

529

530 *< Insert Table 3. Release of soluble COD in the three batch tests due to sludge hydrolysis or*
531 *cell lysis. Legend: n.a. = not appreciable. >*
532
533 The important role of COD solubilisation under anaerobic conditions has been confirmed in
534 the literature (Novak et al., 2003; Novak et al., 2007; Chon et al., 2011a), suggesting that this
535 is one of the most important mechanisms of sludge reduction. The solubilisation under
536 anaerobic conditions was explained with the release of iron and/or aluminium-associated
537 organic matter into sludge solution or with the fermentation of particulate matter, whilst
538 bacteria cell lysis was not accounted for. Our data supports these observations, since sludge
539 solubilisation occurred in the ASSR without any cell lysis, thus confirming the role of non-
540 bacterial material.
541 The equally important role of cell lysis during the aerobic phase, which causes the release of a
542 comparable amount of soluble COD, was demonstrated in this research for the first time.
543 In both these phenomena (anaerobic sludge hydrolysis and aerobic cell lysis), the degradation
544 of the soluble COD occurs in the oxidation tanks where the biodegradable fraction is lost and
545 converted to respiration products such as H_2O, CO_2, etc., thus contributing to sludge
546 reduction.
547 Chon et al. (2011b) observed that about one half of the overall sludge reduction occurred in
548 the oxidation tanks, while the other half was directly achieved in the ASSR. These
549 proportions are in agreement with the concept of anaerobic sludge hydrolysis and aerobic cell
550 lysis observed in the present research.
551 Chon et al. (2011a) concluded that the sequence of aerobic and anaerobic conditions (in
552 activated sludge and ASSR) was much more effective than conventional activated sludge or
553 conventional digestion alone (aerobic or anaerobic used separately) in terms of sludge
554 reduction.

555 Coma et al. (2013), in a pilot-scale ASSR, stated that sludge reduction can be enhanced by
556 rapid passages through the anaerobic and aerobic conditions.
557 The transition from anaerobiosis to aerobiosis thus appears as one of the key points of sludge
558 reduction in the OSA-like processes and the repeated alternation between aerobic and
559 anaerobic conditions can reduce the overall biomass even if the cryptic-growth is included.
560
561
562 **5. CONCLUSION**
563 The physiological status (viability, activity, death and lysis) of bacteria in the sludge and the
564 changes when exposed to aerobic and anaerobic conditions were investigated by FCM to
565 obtain new information about the mechanisms of sludge reduction in the ASSR systems.
566 The main outcomes are the following:
567 1) anaerobic conditions at ambient temperature did not produce a significant cell lysis;
568 2) cell decay and lysis occurred principally under aerobic conditions;
569 3) the sludge conserved a good enzymatic hydrolysis (measured using the substrate BCECF-
570 AM) even under anaerobic conditions; thus the conservation of good hydrolysis both inside
571 and outside bacterial cells can be reasonably expected;
572 4) anaerobic conditions favoured the hydrolysis and solubilisation of non-bacterial material
573 with a large increase in soluble biodegradable COD and NH_4^+ in the anaerobically treated
574 sludge.
575 On the basis of these findings, the two independent mechanisms contributing equally to
576 sludge reduction were: anaerobic sludge hydrolysis and aerobic cell lysis. The transition from
577 anaerobiosis to aerobiosis thus appears one of the key points of sludge reduction in the OSA-
578 like processes and the repeated alternation between aerobic and anaerobic conditions could
579 reduce the overall biomass even if the cryptic-growth is included.

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REFERENCES

An, K., Chen, G., 2008. Chemical oxygen demand and the mechanism of excess sludge reduction in an oxic-settling-anaerobic activated sludge process. Journal of Environmental Engineering 134(6), 469-477.

APHA, AWWA, WERF, 2012. Standard Methods for the Examination of Water and Wastewater, 22nd ed., Washington, DC.

Chon, D.H., McNamara, R., Kim, H.S., Park, C., 2011a. Investigating the mechanism of sludge reduction in activated sludge with an anaerobic side-stream reactor. Water Sci. Technol. 63, 93–99.

Chon, D.H., Rome, M., Kim, Y.M., Park, K.Y., Park, C., 2011b. Investigation of the sludge reduction mechanism in the anaerobic side-stream reactor process using several control biological wastewater treatment processes. Water Res. 45, 6021–6029.

Chudoba, P., Chudoba, J., Capdeville, B., 1992a. The aspect of energetic uncoupling of microbial growth in the activated sludge process: OSA system. Wat. Sci. Technol. 26 (9–11), 2477–2480.

603 Chudoba, B., Morel, A., Capdeville, B., 1992b. The case of both energetic uncoupling and
604 metabolic selection of microorganisms in the OSA activated sludge system. Environ.
605 Technol. 13, 761–770.
606 Coma, M., Rovira, S., Canals, J., Colprim, J., 2013. Minimization of sludge production by a
607 side-stream reactor under anoxic conditions in a pilot plant. Bioresource Technology, 129:
608 229-235.
609 Foladori, P., Bruni, L., Andreottola, G., Ziglio, G., 2007. Effects of sonication on bacteria
610 viability in wastewater treatment plants evaluated by flow cytometry – fecal indicators,
611 wastewater and activated sludge. Water Res. 41, 235–243.
612 Foladori, P., Andreottola, G., Ziglio, G., 2010a. Sludge Reduction Technologies in
613 Wastewater Treatment Plants. London: IWA Publishing, 2010.
614 Foladori, P., Bruni, L., Tamburini, S., Ziglio, G., 2010b. Direct quantification of bacterial
615 biomass in influent, effluent and activated sludge of wastewater treatment plants by using
616 flow cytometry. Water Res. 44 (13), 3807-3818.
617 Fry, J.C., 1990. Direct methods and biomass estimation. Methods in Microbiology 22, 41-85.
618 Goel, R., Noguera, D., 2006. Evaluation of sludge yield and phosphorus removal in a
619 Cannibal solids reduction process. J. Environ. Eng. 132, 1331–1337.
620 Henze, M., Gujer, W., Mino, T., Matsuo, T., Wentzel, M.C., Marais, G.v.R., van Loosdrecht,
621 M.C.M., 1999. Activated sludge model No. 2D, ASM2D. Wat. Sci. Tech. 39(1), 165 –
622 182.
623 Johnson, B.R., Diagger, G.T., Novak, J.T., 2008. The use of ASM based models for the
624 simulation of biological sludge reduction processes. Water Practice & Technology, 3(3).
625 Doi: 10.2166/WPT.2008074.

626 Kim, Y.M, Chon, D.H., Kim, H.S., Park, C., 2012. Investigation of bacterial community in
627 activated sludge with an anaerobic side-stream reactor (ASSR) to decrease the generation
628 of excess sludge. Water Res. 46, 4292–4300.
629 Li, X., Liu, X., Wu, S., Rasool, A., Zuo, J., Li, C., Liu, G., 2014. Microbial diversity and
630 community distribution in different functional zones of continuous aerobic–anaerobic
631 coupled process for sludge in situ reduction. Chemical Engineering Journal 257: 74–81.
632 Munz, G., Lubello, C., Oleszkiewicz, J.A., 2011. Modeling the decay of ammonium oxidizing
633 bacteria. Water Res. 45, 557-564.
634 Nebe-von-Caron, G., Stephens, P.J., Hewitt, C.J., Powell, J.R., Badley, R.A., 2000. Analysis
635 of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting.
636 Journal of Microbiological Methods 42, 97-114.
637 Novak, J.T., Sadler, M.E., Murthy, S.N., 2003. Mechanisms of floc destruction during
638 anaerobic and aerobic digestion and the effect on conditioning and dewatering of biosolids.
639 Water Res. 37, 3136–3144.
640 Novak, J.T., Chon, D.H., Curtis, B., Doyle, M., 2007. Biological solids reduction using
641 Cannibal process. Water Environ Res. 79(12), 2380-2386.
642 Park, C., Abu-Orf, M.M., Novak, J.T., 2006. The digestibility of waste activated sludges.
643 Water Environ. Res. 78, 59–68.
644 Quan, F., Anfeng, Y., Libing, C., Hongzhang, C., Xing, X.-H., 2012. Mechanistic study of
645 on-site sludge reduction in a baffled bioreactor consisting of three series of alternating
646 aerobic and anaerobic compartments. Biochem. Eng. J. 67, 45–51.
647 Semblante, G.U., Hai, F.I., Ngo, H.H., Guo, W., You, S.-J., Price, W.E., Nghiem, L.D., 2014.
648 Sludge cycling between aerobic, anoxic and anaerobic regimes to reduce sludge production
649 during wastewater treatment: Performance, mechanisms, and implications. Bioresource
650 Technology 155, 395–409.

651 Steen, H.B., 2000. Flow cytometry of bacteria: glimpses from the past with a view to the
652 future. Journal of Microbiological Methods 42, 65-74.
653 Tracy, B.P., Gaida, S.M., Papoutsakis, E.T., 2010. Flow cytometry for bacteria: enabling
654 metabolic engineering, synthetic biology and the elucidation of complex phenotypes.
655 Current Opinion in Biotechnology 21, 85–99.
656 Troiani, C., Eusebi, A.L., Battistoni, P., 2011. Excess sludge reduction by biological way:
657 from experimental experience to a real full scale application. Bioresource Technology 102,
658 10352-10358.
659 Wang, J., Zhao, Q., Jin, W., Lin, J., 2008. Mechanism on minimization of excess sludge in
660 oxic-settling-anaerobic (OSA) process. China, Front. Environ. Sci. Engin, 36-43.
661 Wei, Y., Van Houten, R.T., Borger, A.R., Eikelboom, D.H., Fan, Y., 2003. Minimization of
662 excess sludge production for biological wastewater treatment. Water Research 37, 4453–
663 4467.
664 Zhou, Z., Qiao, W., Xing, C., An, Y., Shen, X., Ren, W., Jiang, L., Wang, L., 2015. Microbial
665 community structure of anoxic–oxic-settling-anaerobic sludge reduction process revealed
666 by 454-pyrosequencing. Chemical Engineering Journal 266, 249–257.
667 Ziglio, G., Andreottola, G., Barbesti, S., Boschetti, G., Bruni, L., Foladori, P., Villa, R., 2002.
668 Assessment of activated sludge viability with flow cytometry. Water Research 36, 460-
669 468.
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671

Table 1. Operational conditions in the three batch tests.

	AS-aerobic	**RS-anaerobic-aerobic**		**SS-anaerobic-aerobic**	
Type of sludge	Activated sludge from the oxidation tank	Return sludge		Sludge from the ASSR	
Conditions during the test (duration)	Aerobic (12 d)	Anaerobic (12 d)	Aerobic (4 d)	Anaerobic (12 d)	Aerobic (4 d)
TSS concentration at the beginning of the test	3.8 kgTSS/m^3	8.1 kgTSS/m^3		9.8 kgTSS/m^3	

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.

		AS-aerobic	**RS-anaerobic-aerobic**		**SS-anaerobic-aerobic**	
		Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic
Decay of total cells	b_{TB}	0.07±0.01 d^{-1}	n.d.	0.11±0.08 d^{-1}	n.d.	0.08±0.02 d^{-1}
Decay of viable cells	b_{VB}	0.07±0.02 d^{-1}	n.d.	0.10±0.07 d^{-1}	n.d.	0.07±0.02 d^{-1}
Decay of dead cells	b_{DB}	0.02±0.02 d^{-1}	n.d.	0.24±0.12 d^{-1}	n.d.	0.21±0.08 d^{-1}

Table 3. Release of soluble COD in the three batch tests due to sludge hydrolysis or cell lysis. Legend: n.a. = not appreciable.

		AS-aerobic	**RS-anaerobic-aerobic**		**SS-anaerobic-aerobic**	
		Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic
COD solubilisation due to sludge hydrolysis (excluded cell lysis)	mgCOD/L	n.a.	+549 (after 12 d)	-	+586 (after 12 d)	-
COD solubilisation due to cell lysis	mgCOD/L	+ 483 (after 12 d)	-	+250 (after 1 d)	-	+194 (after 1 d)
				+464 (after 2 d)		+393 (after 2 d)

Figure 1

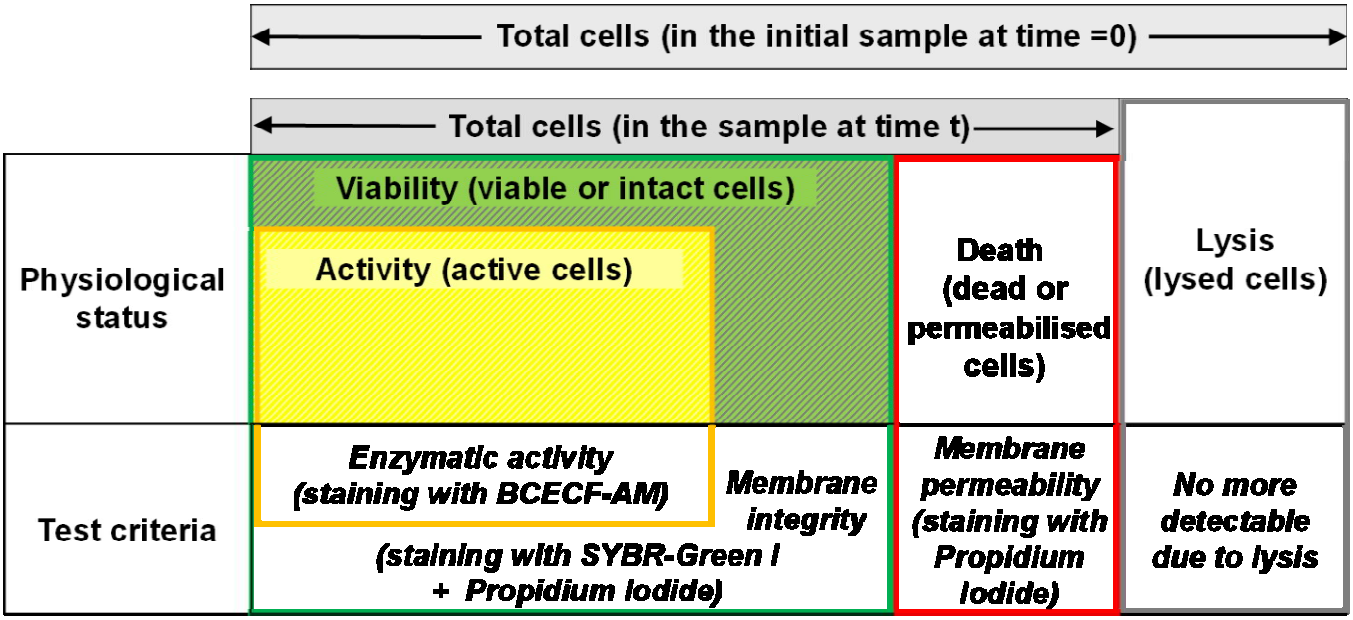

Figure 1. Physiological status of bacterial cells and test criteria for identification. Lysed cells are quantified by difference of total cells at two different times (0, t).

Figure 2

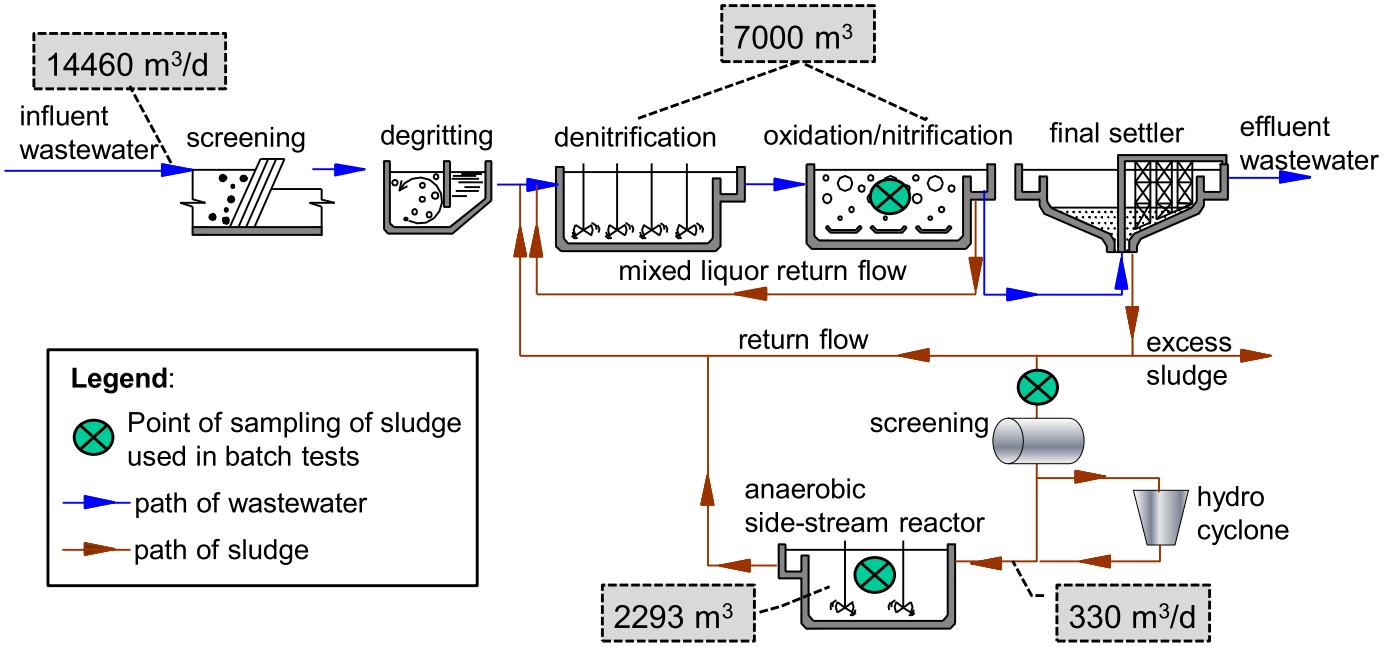

Figure 2. Flow sheet of the WWTP including the anaerobic side stream reactor (ASSR) for sludge reduction. The points of sampling of sludge used in the batch tests are indicated.

Figure 3

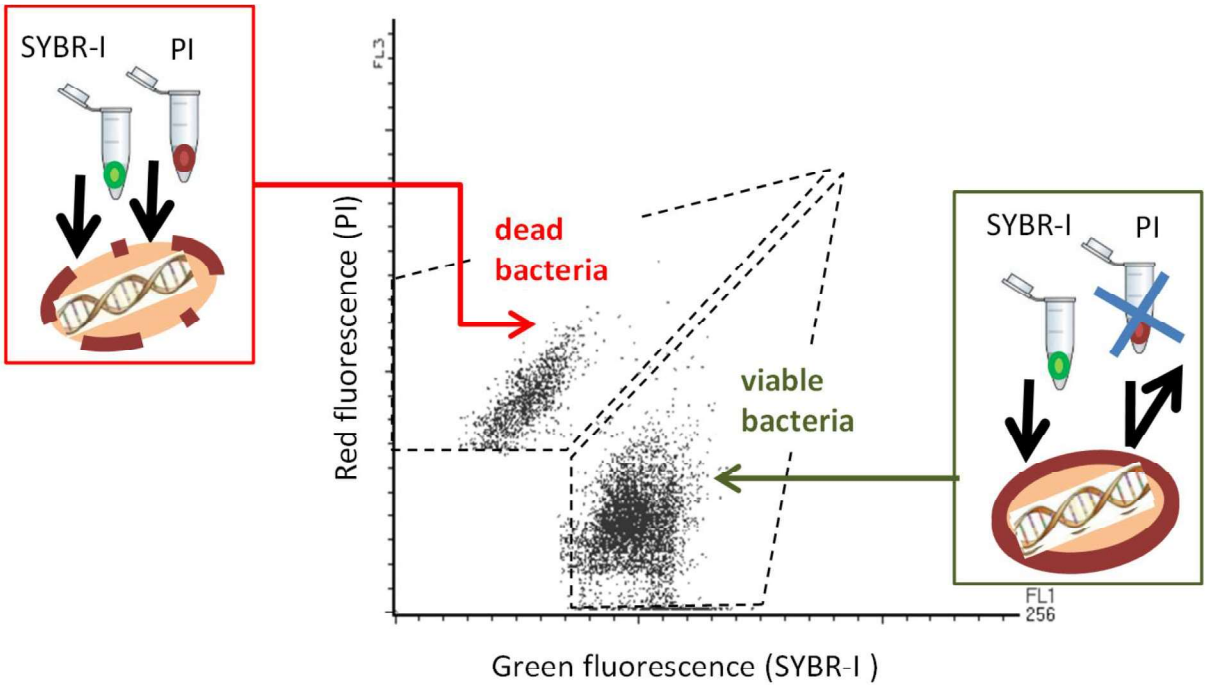

Figure 3. FCM cytogram of viable and dead cells identified by staining with SYBR-I and PI.

Figure 4

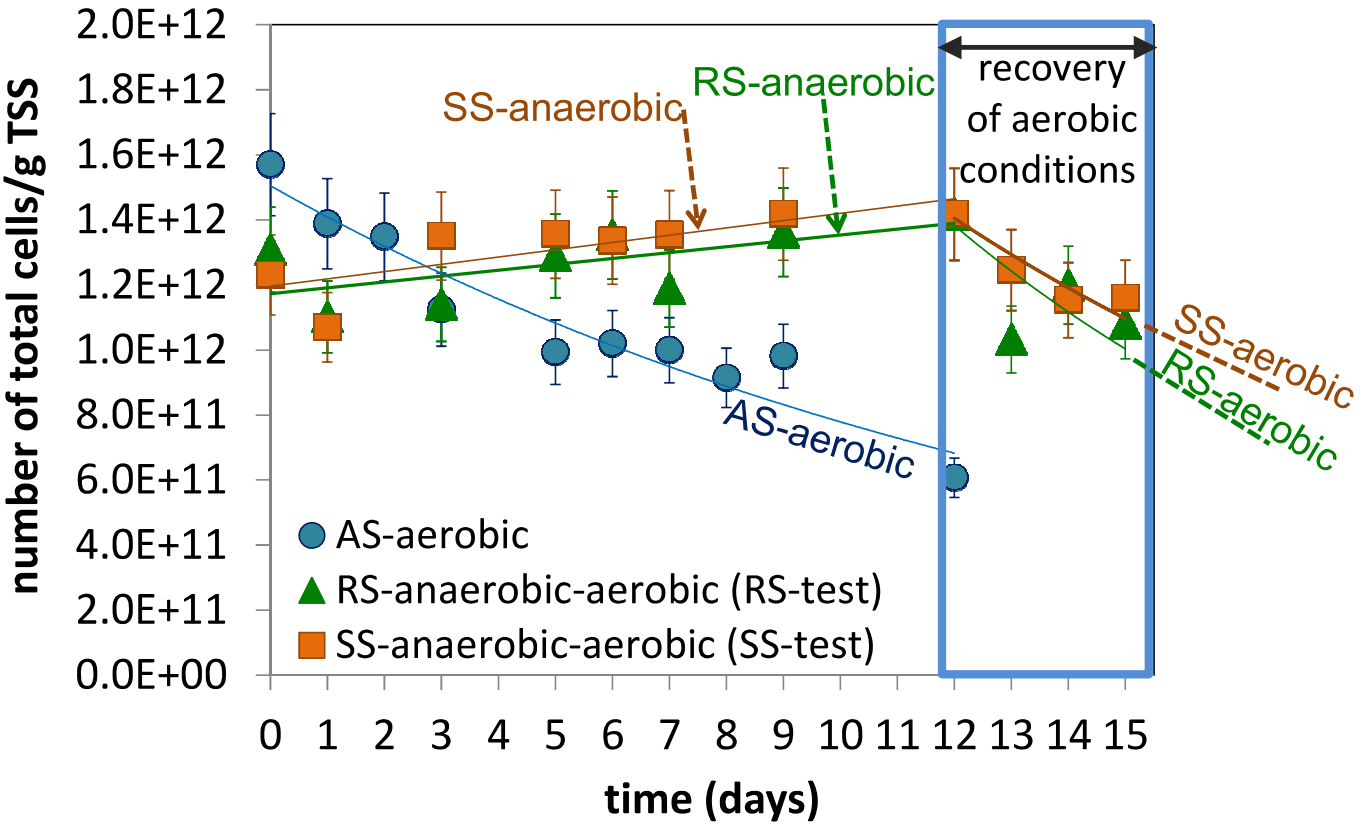

Figure 4. Total bacteria: profiles in the batch tests to compare aerobic conditions (AS-aerobic test) and the transition from anaerobic to aerobic conditions (RS-test and SS-test).

Figure 5

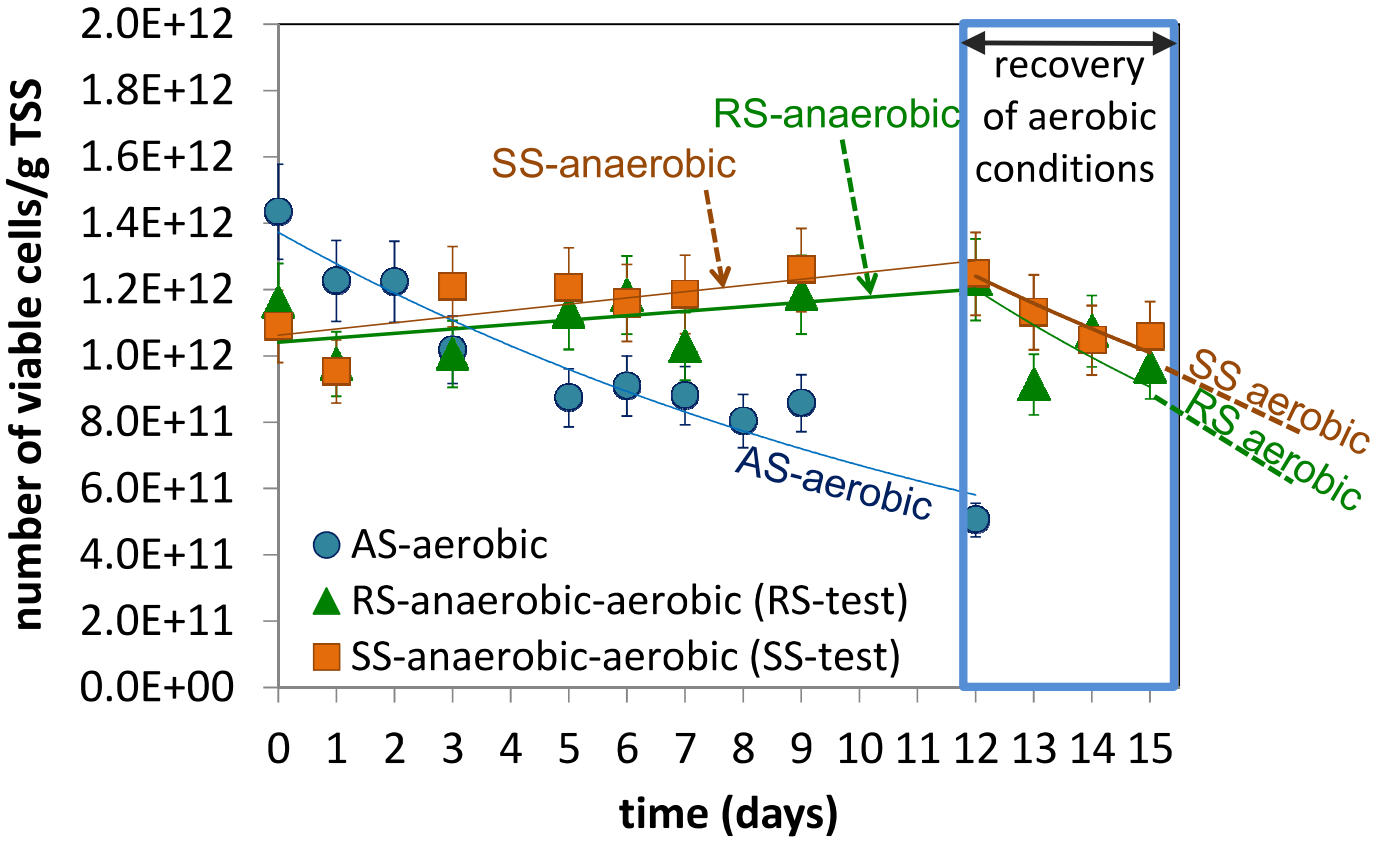

Figure 5. Viable bacteria: profiles in the batch tests to compare aerobic conditions (AS-aerobic test) and the transition from anaerobic to aerobic conditions (RS-test and SS-test).

Figure 6

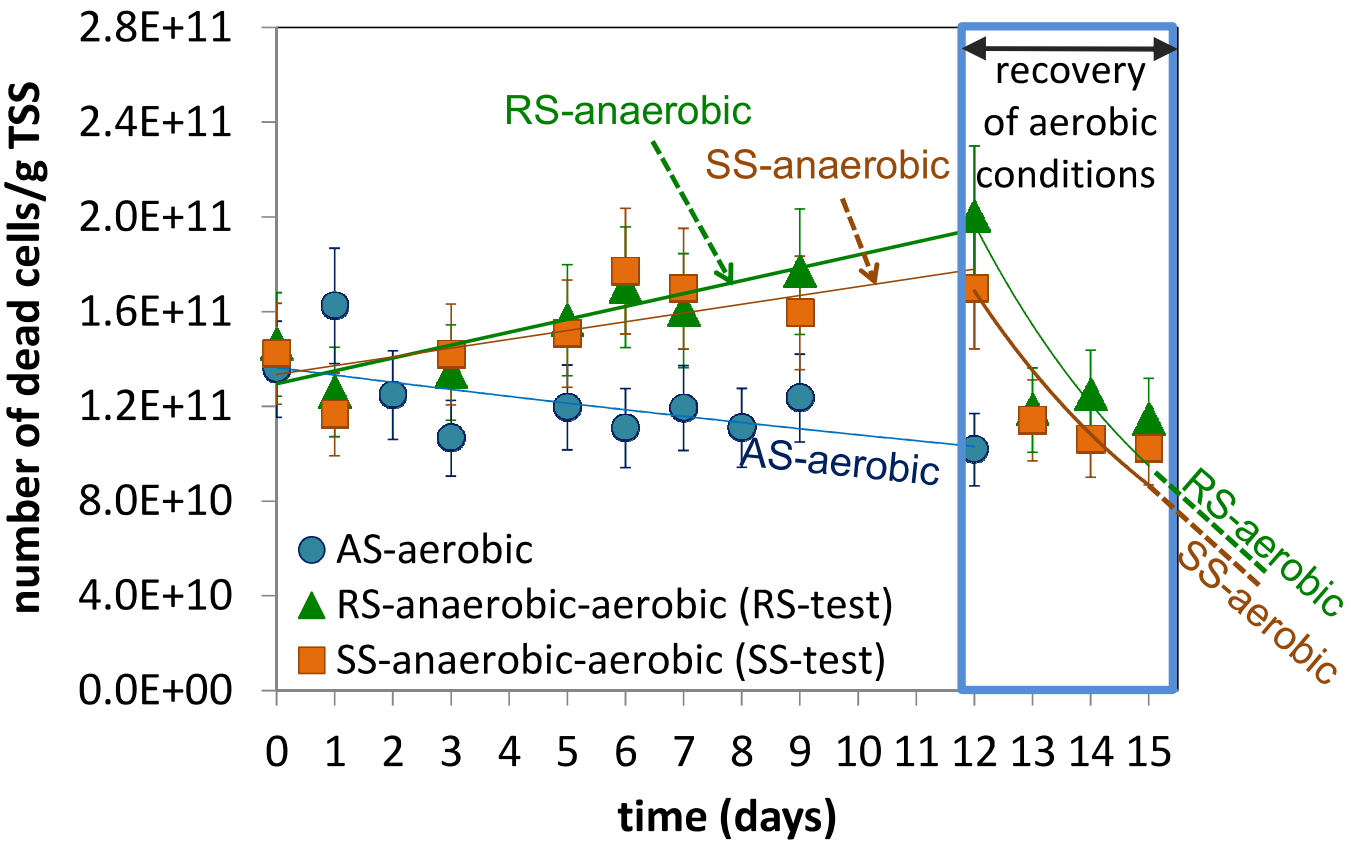

Figure 6. Dead bacteria: profiles in the batch tests to compare aerobic conditions (AS-aerobic test) and the transition from anaerobic to aerobic conditions (RS-test and SS-test).

Figure 7

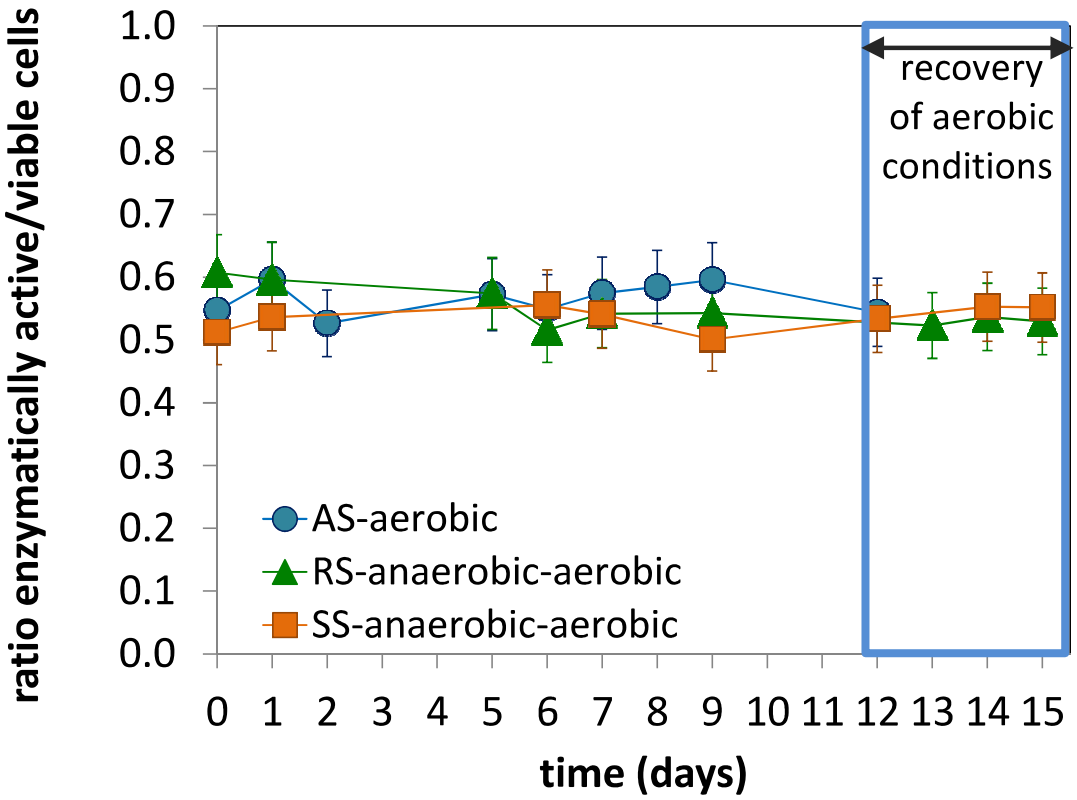

Figure 7. Ratio of enzymatically active bacteria in the batch tests.

Figure 8

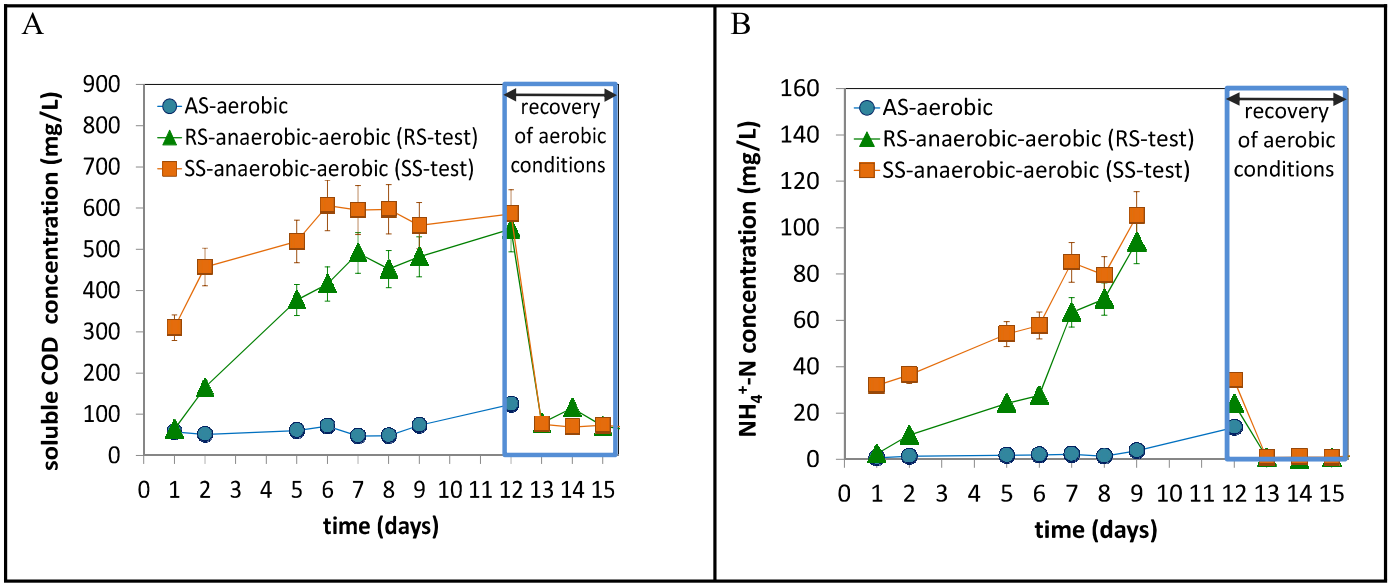

Figure 8. Profiles of soluble COD (A) and NH_4^+-N (B) in the batch tests.