PHA storage from several substrates by different morphological types in an anoxic/aerobic SBR

D. Dionisi*, C. Levantesi**, V. Renzi*, V. Tandoi** and M. Majone*

* Department of Chemistry, University of Rome “La Sapienza”, p.le A. Moro 5, 00185 Rome, Italy
** Water Research Institute, National Research Council, via Reno 1, 00198 Rome, Italy

Abstract
An activated sludge was cultivated on a mixture of several soluble substrates (acetate, ethanol, glucose, glutamic acid, peptone, Tween 80, starch, yeast extract) in an anoxic/aerobic SBR. Highly dynamic conditions in the SBR (feast-famine regime) caused fast removal of most COD in the anoxic phase (in particular acetate, ethanol, glutamic acid and glucose were totally removed) and relevant contribution of storage. In spite of that, filament abundance was always high, as is typical of bulking sludges. Filaments which developed in the reactor were characterized on a morphological basis and on the basis of their ability to grow and to store polyhydroxyalkanoates (PHAs). Three main filaments prevailed in the biocenosis, whose relative abundance was varying with time: Nostocoida limicola II, (two different morphological types), Haliscomenobacter hydrossis and an unidentified one. It was found that maximum growth rate was higher for floc-formers than for filaments on each of the tested substrates. Epifluorescence showed that storage ability was more widespread among floc-formers than in the filaments. Only one type of Nostocoida limicola II was able to store PHAs. The obtained data show that aerobic growth on the little residual fraction of COD from the anoxic phase was enough to support high abundance of filamentous microorganisms.

Keywords
Bulking; denitrification; Haliscomenobacter hydrossis; Nostocoida limicola II; PHAs; storage

Introduction
In several plant configurations, the biomass grows under transient (unsteady) conditions, even though the overall process can be considered under steady-state conditions. Transient conditions are typical for configurations where a concentration gradient of the substrate is produced (like plug-flow configuration of the aeration tanks) or, to a greater extent, where biomass experiences alternately (vs. time or flow direction) high and low substrate concentrations, as in sequencing batch reactors, contact-stabilization processes or processes with a selector for bulking control. More importantly, transient conditions are also present in nutrient removal processes where the biomass experiences alternating anaerobic, anoxic or aerobic environments and a metabolic stress is added to the kinetic one (Daigger and Grady, 1982). It is widely accepted that under these dynamic conditions growth becomes unbalanced, i.e. substrate can be removed without a corresponding regular increase in every cellular component (with no or little synthesis of active biomass): in particular intracellular storage of the substrate (i.e. substrate uptake and related synthesis of internal polymers) can become important (Majone et al., 1999). Therefore, storage can play a dominant role in substrate uptake in nitrogen removal processes based on pre-denitrification, particularly when an external source of readily biodegradable COD (RBCOD) is added to increase the denitrification rate (Isaacs et al., 1994; Hasselbad and Hallin, 1996).

Storage can be a key-factor in microbial competition and therefore in bulking control. As a matter of fact, highly dynamic processes are reported to be effective in avoiding proliferation of filaments, because floc-formers are reported to have in general higher storage capacity than filaments (Grau et al., 1982). Under anoxic conditions a metabolic pressure is added to the kinetic one, because most filamentous microorganisms are reported not to be
able to denitrify. In spite of highly dynamic conditions and of the metabolic pressure, nitrogen removal processes often suffer for bulking problems (Wanner, 1993).

Till now literature evidence of storage is mainly focused on acetate or glucose, under aerobic conditions, and each substrate being the only carbon source. On the contrary, wastewater is a complex mixture of several substrates (Henze et al., 1994) and in many plants the carbon source is firstly taken up by biomass under anoxic conditions. Therefore the aim of the present paper is to study the behaviour of activated sludge in a nitrogen removal SBR in the presence of a mixture of several substrates (low and high molecular weight), with main reference to the role of poly-hydroxyalkanoate (PHA) storage and its effect on population dynamics.

Methods

SBR operation

The SBR was operated with 4 cycle per day (6 h/cycle, first 2 h 30 min anoxic, remaining 3 h 30 min aerobic, feed 1 min starting 30 min after the beginning of the cycle, in order to assure anoxic conditions during the feed, Figure 1). The mixed liquor was withdrawn just before the new feed, without any settling phase; so hydraulic retention time and sludge age were the same (6 d) and well controlled independently from settling properties of the sludge (Table 1). The particular arrangement was also chosen in order to study population dynamics in the sludge as resulting only from biological competition for the substrate between flocformers and filaments, with no additional effect due to different settling properties. Due to high HRT, feed COD was quite high (3,800 mg/L), in order to apply the chosen organic load rate (OLR 633 mgCOD/Ld, Table 1). The carbon sources in the feed (Table 2) were several soluble compounds representing different classes of organic substances in a range of molecular-weights (MW): volatile fatty acids (acetate), sugars (glucose and starch), alcohols (ethanol), proteins (glutamic acids, peptone and yeast extract) and long chain fatty acids (Tween 80). Thiourea and nitrate were added in the feed to inhibit nitrification and to provide nitrate for denitrification, respectively. Mineral medium composition is reported in Majone et al. (2001). During the last period of SBR operation the feed pattern was changed: only 1 cycle per day was made (the first 8 hours were anoxic, the remaining period was aerobic). All the other operating parameters remained unchanged.

During operation, total suspended solids (TSS), volatile suspended solids (VSS), sludge volume index (SVI) were measured three times per week by sampling the mixed liquor at the end of the aerobic phase. Once a week soluble COD, acetate, glutamic acid, ethanol and glucose were determined, by sampling the mixed liquor at the end of both anoxic and aerobic phase. The analytical procedure was reported elsewhere (Majone et al., 2001).

Microbial identification

Once a week the SBR biomass was characterized by microscopic observation (bright field, phase contrast and epifluorescency), after Gram, Neisser and Nile Blue staining. Filament morphological identification and filament abundance were done according to Eikelboom and van Buijsen (1983) and Jenkins et al. (1993). FISH analysis was performed according to Amann et al. (1995), using the probe ALF1b (Manz et al., 1992) for detecting the α-subclass of Proteobacteria and the eubacterial probe EUB338 (Amann et al., 1990). The ALF1b and EUB338, labelled respectively at the 5’ end with CY3 and fluorescein, were purchased from MWG Biotech, Germany. A Gram post-staining was performed according to Jenkins et al. (1993).

Batch tests

In order to investigate the role of each substrate as the single carbon source, two types of
batch tests were performed. In order to evaluate the relative ability of the filamentous and
floccforming biomass to grow under excess substrate condition, the first type of batch test
(low biomass inoculum, approximately 1 mgVSS/l) was performed with each substrate,
under anoxic (nitrate) and aerobic conditions. During this type of test, the mixed liquor was
sampled and slides were taken at the end of the test for microscope observation. In order to
investigate the role of storage, the second type of batch tests (high sludge inoculum,
approximately 250 mgVSS/l) was performed with each of the four low-MW well defined
substrates (acetate, ethanol, glutamic acid and glucose), in the presence of either nitrate,
nitrite or oxygen. Storage was quantified during aerobic or anoxic (nitrate) tests, where the
mixed liquor was regularly sampled for substrate, PHA, carbohydrates and ammonia
analysis, according to the methodology reported elsewhere (Majone et al., 2001).
Moreover, in order to investigate the ability of individual morphotypes to store PHAs the
mixed liquor was sampled and slides were taken at the beginning and at the end of the test
for epifluorescence microscope observation.
Results and discussion

SBR operation

The SBR was operated for a long time under a typical feast-famine regime (feed in 1 minute over a 6 hour cycle). In particular, the four low-MW substrates that were analytically determined (acetate, ethanol, glucose and glutamic acid) were completely removed during the first part of the anoxic phase. Also for other substrates, most removal was in the anoxic phase (Table 1). It is also noteworthy that in spite of high removal (93%), the residual COD was still quite high (247 mgCOD/l at the end of the anoxic phase); this residual COD was apparently “non biodegradable” under the chosen OLR and sludge age.

During SBR operation, SVI was usually high, even though highly variable (Figure 2). Three filamentous morphotypes were prevailing in the biomass, whose relative abundance was varying with time (Figure 3): *Nostocoida limicola II* (two different morphological types), *Haliscomenobacter hydrossis* and an unidentified filament that did not correspond to any of the Eikelboom types. Morphological characterization of the main observed morphotypes is reported in Table 3. One type of *Nostocoida limicola II* (named type A) was Gram variable and Neisser positive, with filament length usually > 200 µm, while type B was both Gram and Neisser negative, with filament length usually < 100 µm. The unidentified filamentous bacterium was straight or smoothly curved, of variable length (100–300 µm), diameter 1 µm, composed of cells of variable shape (square, rectangular, barrel), Gram and Neisser negative and gave negative response to the S test. Besides filamentous organisms, many tetrads forming Gram positive bacteria were detected in the sludge, morphologically differing from the G bacteria isolated and characterized by Blackall *et al.* (1997); in fact Fish analysis showed that tetrad-forming bacteria did not belong to the α subclass. A slight improvement was observed in the last period of the SBR run when the feed pattern was modified to 1 cycle/day: the SVI profile and the abundances of the various filaments showed a slight and slow tendency to decrease after the change of operating conditions.

Storage and growth in batch tests

Growth during substrate removal was investigated starting from a very small inoculum (batch tests at 1 mgVSS/l) under aerobic and anoxic (nitrate) conditions with each of the eight substrates of the SBR feed as single carbon source. Once a significant amount of biomass had been formed, filaments or tetrad-forming bacteria were not detectable with any substrate tested. These data show that floc-forming bacteria have a higher maximum growth rate than filamentous bacteria with each one of the substrates in the feed, as usually reported in the literature (Wanner, 1993). Therefore, the growth of filaments was probably
supported by aerobic utilization of residual slowly biodegradable substrate: indeed, even though all substrates were soluble, 28 mgCOD/l apparently behaved as slowly biodegradable, so remaining available in the aerobic phase at low concentrations.

PHA formation during substrate removal was also investigated (batch tests at about 250 mgVSS/l) for the four low-MW well defined substrates (acetate, ethanol, glucose and glutamic acid, each one as the only carbon source). Storage phenomena (PHA or glycogen) exerted a significant role (both under anoxic and aerobic tests) for acetate, ethanol and glucose (Table 4). No significant PHA or glycogen storage was observed from glutamic acid, even though an intense fluorescence after Nile Blue staining was developed in the sludge. The presence of fluorescence could indicate storage products of lipidic nature other than PHA, as already hypothesized for pure cultures grown on acetate (Fixter et al., 1986; Tandoi et al., 1998). On the other hand, storage of glutamic acid in form of poly-aminoacid has been reported under anaerobic conditions (Satoh et al., 1998) and would be coherent with combined carbon and nitrogen balance obtained in the present study (Majone et al., 2001). However, further research is clearly needed to ascertain whether glutamic acid can be actually stored and eventually in which form.

As reported in Figure 4 for a typical anoxic batch test with ethanol, the fluorescence microscopy after Nile Blue staining made it also possible to observe which morphological types of bacteria where able to store PHB. In general, fluorescence was present in the largest amount inside the flocs under all conditions (including in the presence of nitrite as the only electron acceptor). Among the five morphological types which were individuated (Table 5), only Nostocoida limicola II (type B) was able to store PHA (from acetate and ethanol) or other compounds of lipidic nature fluorescent under Nile Blue staining (from glutamic acid). The evidence of storage by Nostocoida limicola II (type B) was found in the presence of oxygen or nitrate while it was not in the presence of nitrite. Specific FISH analysis directed to the identification of the Nostocoida limicola II morphotype B has been...
This filamentous bacterium, differently from *Nostocoida limicola II* recently isolated from conventional wastewater treatment plants (*Actinomycetes* subphylum, Blackall *et al.*, 2000), belongs to the α-subclass of Proteobacteria. The results of FISH analysis are reported in Figure 5 (a, b).

<table>
<thead>
<tr>
<th>Storage contribution to substrate removal and solid formation</th>
<th>Acetate</th>
<th>Ethanol</th>
<th>Glucose</th>
<th>Glutamic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>storage on removed substrate ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• PHA</td>
<td>0.30</td>
<td>0.34</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>• Carbohydrate</td>
<td>0</td>
<td>0</td>
<td>0.73</td>
<td>0</td>
</tr>
<tr>
<td>storage on solid formation ratio</td>
<td>0.46</td>
<td>0.48</td>
<td>0.98</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 4** Bright field and fluorescence photo of a Nile Blue stained slide taken during an anoxic batch test with ethanol (three out of the five described morphological types are visible)

**Table 5** Fluorescence for the different morphological types

<table>
<thead>
<tr>
<th>Morphological Type</th>
<th>Acetate aerobic</th>
<th>Acetate anoxic with nitrate</th>
<th>Ethanol anoxic with nitrate</th>
<th>Glutamic acid aerobic</th>
<th>Glutamic acid anoxic with nitrate</th>
<th>Glucose every tested condition</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nostocoida limicola II</em> type A</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Nostocoida limicola II</em> type B</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Haliscomenobacter hydrossis</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unidentified filament</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tetrads forming</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Floc formers</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Done. This filamentous bacterium, differently from *Nostocoida limicola II* recently isolated from conventional wastewater treatment plants (*Actinomycetes subphylum*, Blackall *et al.*, 2000), belongs to the α-subclass of Proteobacteria. The results of FISH analysis are reported in Figure 5 (a, b).
Conclusions
The presented data show that filamentous microorganisms can survive also in highly dynamic systems. Even though the system exerted a strong pressure, both kinetic and metabolic (the removed COD was mainly consumed in the anoxic phase), three different filaments (two types of Nostocoida limicola II, Haliscomenobacter hydrossis, and an unidentified one) grew at significant abundance (at typical values of bulking sludge). Neither storage response nor fast growth were probably key factors in filament survival under the studied conditions. As a matter of fact, while storage exerted an important role in substrate removal and storage ability was widespread among flocformers, only one of the described filamentous organisms was able to store PHA (α Nostocoida limicola II, also in anoxic conditions). None of the filaments could grow faster than floc-formers with any of the different substrates. The little amount of COD that was degraded aerobically was apparently enough to allow filament growth in the described system. In particular, the occurrence of Haliscomenobacter hydrossis, which is usually considered an “oxic zone grower”, was confirming such evidence. When the feed pattern was modified from 4 to 1 cycle per day, the SVI and abundance of main filamentous morphotypes showed a slight and slow tendency to decrease. However, such an evidence has to be confirmed by more experimental work.

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References


