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INTRODUCTION

Filamentous micro-organisms are ubiquitous and conspicuous members of activated sludge wastewater treatment plant microbial communities. Excessive growth of filamentous bacteria in activated sludge wastewater treatment plants (WWTPs) can cause serious operational problems. During a scum study on a full-scale plant treating domestic waste (Fig. 1), the dominant filament was a Gram and Neisser negative "*Nostocoida limicola*" morphotype. The Gram-negative "*Nostocoida limicola*" seen in industrial treatment plants belong to the *Alphaproteobacteria*, and these have only rarely been reported in domestic treatment plants. Detection of filamentous bacteria morphotypes involved in scum formation in activated sludge wastewater treatment plants by conventional sludge microscopy is often doomed to fail because of morphological and taxonomical variations. Correct identification of the filamentous bacteria by targeted FISH probes and their association with particular operational features may provide a means of controlling foaming in WWTPs. In this study, we tried to clarify why the *N.limicola*-like organism proliferates.



Figure 1. A) Empuriabrava WWTP carousel reactor and reactor with the clarifier in the center; B) Reactor with the clarifier in the center with a foaming episode

MATERIAL AND METHODS

The sampling period was conducted between September 2009 and May 2010 in Empuriabrava WWTP (Northeastern Spain). The biological reactor volume was 7500 m³ and presented occasionally discharge of septic tanks. During this period there have been two processing systems: biological reactor controlled by ammonium setpoint with oxic and anoxic zones and removal of sludge from the bottom (new line), and two Carousel reactors in which the removal of sludge is done from the upper zone (old line), thus helping to eliminate foam. In this case the system is fully aerobic and controlled by setpoint oxygen.
 Monthly samples of mixed liquor of mixed liquor were taken for microscopic observation. The total extended filamentous microorganism length (TEFL) was calculated using the technique based on the "simplified filament counting technique" described by Jenkins et al. 1993. The abundance of filamentous bacteria by FISH technique in the activated sludge samples was measured according to the subjective scoring method of Eikelboom (2000), where observations are rated on scale from 0 (none) to 5 (extensive growth). The hybridizations were performed at 46 °C for 2 hours except for *Microthrix* that has extended the period of hybridization until 3 h. All the probes (Table 1), labelled at the 5' end with Tamra, were purchased from TibMobiol, Germany. The ELF 97 glucuronide substrate (ELF 97 glucuronide) was used to detect the enzyme β-D-glucuronidase (glucosidase activity) and the ELF 97 phosphate reagent (ELF 97 phosphate) was used to detect acid and alkaline phosphatases.

Table 1. FISH probes used for the identification of filamentous bacteria

Strain	Sequence (5'-3')	Target (Species)	% ^a	Reference
EU18 338 I	GCCTCCCTCCCGTAGAGT	<i>Bacteriophage</i>	20	Amann (1990)
EU18 338 II	GCACCCACCCCTAGGTT	<i>Planctomyces</i>	20	Dalms et al. (1999)
EU18 338 III	GCCTCCACCCGATAGGTT	<i>Verrucomicrobium</i>	20	Dalms et al. (1999)
AL1946	GCCTCCACATCGTT	<i>Alphaproteobacteria</i>	35	Niemi (1997)
ALM426	GCCTCCACATCGTT	<i>Beta-proteobacteria</i>	35	Mans et al. (1992)
BEF126	GCCTCCACATCGTT	<i>Chloroflexi</i>	35	Mans et al. (1992)
GNS8941	AAACCCACCGCTCCGCT	<i>Chloroflexi</i>	35	Glick et al. (2001)
CFX1223	ACATGCTGACATGTTMG	<i>Chloroflexi</i>	35	Snaird et al. (2002)
PP3-1428	TGGCCACCGCTCCGG	Ca. " <i>A. bataviensis</i> "	50	Snaird et al. (2002)
NHL-649	TCCTCCGACATGTTMG	Ca. " <i>A. europaea</i> "	35	Snaird et al. (2002)
MC2-649	CTTCCCGACCTGAGCC	Ca. " <i>A. bataviensis</i> "	35	Snaird et al. (2002)
NHL993	CAAGCCGACCTGAGCC	Ca. " <i>S. italicum</i> "	20	Kragelund et al. (2006)
Helpe1010	GAAGCCGACCTGAGCC	Ca. " <i>S. italicum</i> "	20	Kragelund et al. (2006)
Com9-1033	CACCTCCAGTGGCTCCCGA	Ca. " <i>Com. italica</i> "	35	Kragelund et al. (2006)
Com9-649 ^b	GCCTCCCGCTCCGCT	<i>complanata</i>	50	Kragelund et al. (2006)
DF198	ATCCGAGGGCAATACATCC	Ca. " <i>S.M. bataviensis</i> "	35	Nittami et al. (2009)
CFX179	GCCTCCGCTCCGCT	<i>Chloroflexi</i> var. A ¹²	40	Speirs et al. (2009)
CFX1223	GCCTCCGCTCCGCT	<i>Chloroflexi</i> var. A ¹³	40	Speirs et al. (2009)
CFX1223	GCCTCCGCTCCGCT	<i>Chloroflexi</i> var. B ¹⁴	35	Speirs et al. (2009)
CFX1223	AGCGCTGAGCTTCACTC	<i>Chloroflexi</i> var. B ¹⁵	35	Speirs et al. (2009)
NLMH181	GCCTCCGCTCCGCT	<i>Inosiphon</i> sp.	20	Liu & Seviour (2001)
NLMH729	AGCATCCGACCTCCCT	<i>Inosiphon</i> sp.	20	Liu & Seviour (2001)
NLMH830	GCATCCGACCTCCCT	<i>Inosiphon</i> sp.	20	Liu & Seviour (2001)
NLMH833	CCGACACTACCCACTGT	<i>N. limicola</i> sp.	35	Schäde et al. (2002)
Mpa-all-141	GGTGTGTGACCTTTCGGC	<i>Microthrix</i>	35	Levantani et al. (2006)

^aFA: Formamide percentage

RESULTS AND DISCUSSION

The "*N. limicola*" Gram and Neisser negative filaments did not fluoresce with any of the probes described by Snaird et al. (2002), Kragelund et al. (2006) and Levantani et al. (2004) for the alphaproteobacterial "*N. limicola*" filaments. These filaments fluoresced with the DF 198 probe designed by Nittami et al. (2009) to target alphaproteobacterial *Candidatus Monilibacter bataviensis*-related organisms (see Fig. 2D). The fluorescence microscopy after Nile Blue A staining made it also possible to observe which morphological types of bacteria were able to store PHB. Among the morphological types which were identified with the FISH probes (*Haliscobenobacter hydrossis*, *Chloroflexi* (Fig. 3), Type 0092 variant A, "*Candidatus Alysiosphera europaea*", *Microthrix* (Fig. 4) and *Thiothrix*), only *Monilibacter bataviensis*-related organisms were able to store poly-β-hydroxyalkanoates PHA (see Fig. 2B). The ability to form PHA is an obvious advantage, since storage material can be utilized as energy and/or as a reserve of carbon during periods of unbalanced growth due to unfavourable conditions (Dawes 1991). No exo-enzymatic activity were found for any of the ELF enzymes (β-D-glucuronidase and phosphatase) tested. The lack of ectoenzyme activity in *Monilibacter bataviensis* cluster III has been previously reported by McIlroy et al. (2010) and these authors suggests that these populations are unable to use high-molecular-weight polymeric substrates.
 To understand the ecology of the dominant filamentous bacteria in almost all samples and test their patterns of development, and compounds that support growth, it is necessary to check that conditions prevailing in the plant on the three study periods, since apparently the Carousel system limits their growth and also the circular reactor operated by redox setpoint further limits the development of the *Monilibacter bataviensis* than under ammonium setpoint (Fig. 5). On the one hand, the SS and BOD mean values were similar in the three periods, while the COD increased significantly in the third period. In the case of operational parameters differences were observed for the three periods. The high concentration of MLSS during the first period, along with a very low F/M ratio (0.009 kg BOD/kg MLVSS.d) and sludge age of 77 days produced a highly stressed nutritional environment. Comparing the F/M ratio and sludge age of each period it can be seen that the F/M ratio of the second and third period were higher than the first period, 0.022 and 0.019 kg BOD / kg MLVSS d, respectively. The sludge ages of the second and third period were lower than the first period, 55 and 37 days, respectively. It is clear that the decrease of sludge age allowed a better distribution of nutrients. Comparing period 1 and 2, the growth of *Candidatus Monilibacter bataviensis* organisms can be related with low F/M ratio and high sludge ages. *Monilibacter bataviensis*, is associated with readily short chain fatty acids (acetic and propionic) and these organisms synthesize PHA anaerobically, and then utilize this stored PHA under aerobic conditions (McIlroy et al., 2010). Its development is more competitive in situations of nutritional imbalance and it is obvious that conditions in the third period could limit their growth, along with operational conditions of this period.



Figure 2. A) Gram negative staining of *Monilibacter bataviensis*; B) PHB storage of *Monilibacter bataviensis* by Nile blue A staining; C) *Monilibacter bataviensis* with phase contrast; D) *Monilibacter bataviensis* hybridized with the DF198 probe, 1000x



Figure 3. A) *Microthrix* filaments with phase contrast; B) *Microthrix* hybridized with the mPA-all-1410 probe, 1000x

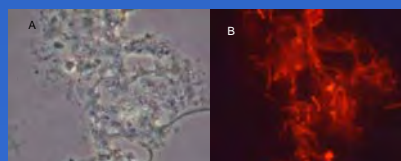


Figure 4. A) *Chloroflexi* filaments with phase contrast; B) *Chloroflexi* hybridized with CFX1223 and GNS8941 mix probes, 1000x



Figure 5. Abundance of the most important filamentous microorganisms in the study period

CONCLUSIONS

The oxygen control system under redox setpoint is more effective than ammonium setpoint to avoid anaerobic conditions of the circular reactor. The control of nutritional conditions in the WWTP, operational measures such as reduction of MLSS, decreased sludge age and increased of the F/M ratio, it seems to help control the growth of *Candidatus Monilibacter bataviensis*-related organisms

References

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