Control of *Microthrix parvicella* by aluminium salts addition

N. Durban, L. Juzan, J. Krier and S. Gillot

ABSTRACT

Aluminium and iron chloride were added to a biological nutrient removal pilot plant (1,500 population equivalent) treating urban wastewater to investigate the control of *Microthrix parvicella* bulking and foaming by metallic salts. Monitoring plant performance over two 6-month periods showed a slight impact on the removal efficiencies. Addition of metallic salts (Me; aluminium or aluminium + iron) at a concentration of 41 mmol Me(kg MLSS·d) (MLSS: mixed liquor suspended solids) over 70 days allowed a stabilization of the diluted sludge volume index (DSVI), whereas higher dosages (94 mmol Me(kg MLSS·d) over 35 days or 137 mmol Me(kg MLSS·d) over 14 days induced a significant improvement of the settling conditions. Microscopic observations showed a compaction of biological aggregates with an embedding of filamentous bacteria into the flocs that is not specific to *M. parvicella* as bacteria from phylum Chloroflexi are embedded too. The quantitative polymerase chain reaction targeting *M. parvicella* further indicated a possible growth limitation in addition to the flocculation impact at the high dosages of metallic salts investigated. DSVI appeared to be correlated with the relative abundance of *M. parvicella*.

Key words | aluminium salts, bulking, filamentous bacteria, wastewater

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INTRODUCTION

Filamentous bulking causes several operational problems in municipal activated sludge wastewater treatment plants (WWTPs), especially those operated at low sludge loads (Jenkins *et al.* 2004). The excessive growth of filamentous bacteria interferes with settling and compaction of biological aggregates (Jenkins *et al.* 2004). *Microthrix parvicella (M. parvicella)* has been recognized to be the most dominant filamentous bacteria for a long time (Jenkins *et al.* 2004) and still is (Mielczarek *et al.* 2012). Bulking due to *M. parvicella* proliferation occurs more often during wintertime, when mixed liquor temperature is below 15 °C (Knoop & Kunst 1998; Miana *et al.* 2002). As *M. parvicella* is a strong hydrophobic filament, it can also cause foaming when in excess. This bacterium from the Actinobacteria class is a microaerophilic organism which preferentially feeds on long-chain fatty acids (Andreasen & Nielsen 2000; Rossetti *et al.* 2005). Highly versatile, *M. parvicella* shows different metabolic strategies to adapt to a wide range of operating conditions (low nutrient concentration, anaerobic, anoxic and aerobic conditions) (Rossetti *et al.* 2005).

Several strategies have been proposed in order to control the proliferation of *M. parvicella* in WWTPs, such as the installation of tank selectors (Jenkins *et al.* 2004) and/or the application of adequate operating parameters such as a relatively low sludge retention time or a dissolved oxygen (DO) concentration above 2 mg/L (Jenkins *et al.* 2004; Xie *et al.* 2007). However, their effectiveness is not always guaranteed (Noutsopoulos *et al.* 2010) and bulking or foaming crises still require rapid curative treatment. In such conditions, the addition of aluminium salts in the range of 1.5 to 4.5 g Al(kg MLSS·d) (corresponding to 55 to 167 mmol Me(kg MLSS·d) (MLSS: mixed liquor suspended solids) has been proposed to control the proliferation of *M. parvicella* in low-loaded WWTPs. This dosage seems to specifically target *M. parvicella* and to be harmless to the rest of the biomass (Roels *et al.* 2002).

Experiments reported in the literature highlight an improvement in the settling properties of activated sludge and a decrease in floating sludge after the addition of aluminium salts, associated with a reduction of *M. parvicella* abundance. The authors generally observed an increase in the size of biological aggregates together with the integration of *M. parvicella* into floc material (Nielsen *et al.* 2005; Paris *et al.* 2005). The main mechanisms proposed to

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explain the impact of aluminium dosage on *M. parvicella* include a reduction of its activity and of the availability of its preferential substrate, induced by the co-precipitation of aluminium with lipophilic substances (Paris *et al.* 2005), an increase in substrate diffusion (Nielsen *et al.* 2005), a temporary inhibition of surface-associated exoenzymes activity (Nielsen *et al.* 2005) and/or the compaction of *M. parvicella*'s polymeric layer (Hamit-Eminovski *et al.* 2010).

The objectives of this study were to analyse the impact of the addition of metallic salts on process performance and on activated sludge microbiology. Until now, the impact of metallic salts on *M. parvicella* bulking was either investigated on real WWTPs or on pilot plants principally focusing on the appearance/disappearance of foam and sludge settling properties (Roels *et al.* 2002; Paris *et al.* 2005) or studied using diverse methods on a laboratory scale (Nielsen *et al.* 2005; Hamit-Eminovski *et al.* 2010; Knaf *et al.* 2013). The main purpose of this study was to couple the pilot plant monitoring approaches with quantification and identification of bacteria by microscopic and microbiological analyses.

To this aim, aluminium chloride solutions were added to an industrial-scale pilot plant experiencing seasonal bulking due to *M. parvicella*. Two solutions containing only aluminium chloride ($AlCl_3$) or aluminium chloride and iron chloride ($FeCl_3$) were tested with different modes of addition (gradual or rapid). The process performances of the pilot as well as the settling properties of the activated sludge were monitored over two 6-month periods. Process results were compared to microscopic observations of the floc morphology and the localization of filamentous bacteria (FISH: fluorescence *in situ* hybridization), and with quantification data of *M. parvicella* and of total bacteria (qPCR: quantitative polymerase chain reaction).

METHODS

Activated sludge plant and analyses

The tests were carried out on a biological nutrient removal industrial-scale pilot plant located at the largest WWTP of the Parisian area (Seine Aval). The pilot plant has been operated by the Greater Paris Interdepartmental Sanitation Authority (Siaap) since 2005. It consists of an activated sludge system designed to treat the wastewater of 1,500 population equivalent. The pilot plant is continuously fed with pretreated influent from the Seine Aval WWTP, with an average flow rate of 313 m^3/d. Figure 1 depicts the pilot plant's process.

Daily samples (or twice a week for floating sludge extracted from the surface of the secondary clarifier using scum removal equipment) were taken at several points of the pilot plant using different sampling plans (Figure 1). Analyses of MLSS, mixed liquor volatile suspended solids (MLVSS), chemical and biochemical oxygen demand (COD and BOD_5), ammonia (NH_4-N) and total Kjeldahl nitrogen (TKN), total phosphorus (P_t), orthophosphates (PO_4-P) and pH were performed using European standard methods. Aluminium and iron concentrations were measured (NF EN ISO 11885) at least once a week. Temperature was continuously monitored in C2 and in the influent (SensoLyt® 700 IQ, YSI, Yellow Springs, USA), and the DO concentration was continuously monitored in each aerated tank (FDO® 700 IQ, YSI, Yellow Springs, USA). The settling properties of the activated sludge (from C3) were characterized daily through the measurement of the diluted sludge volume index (DSVI).

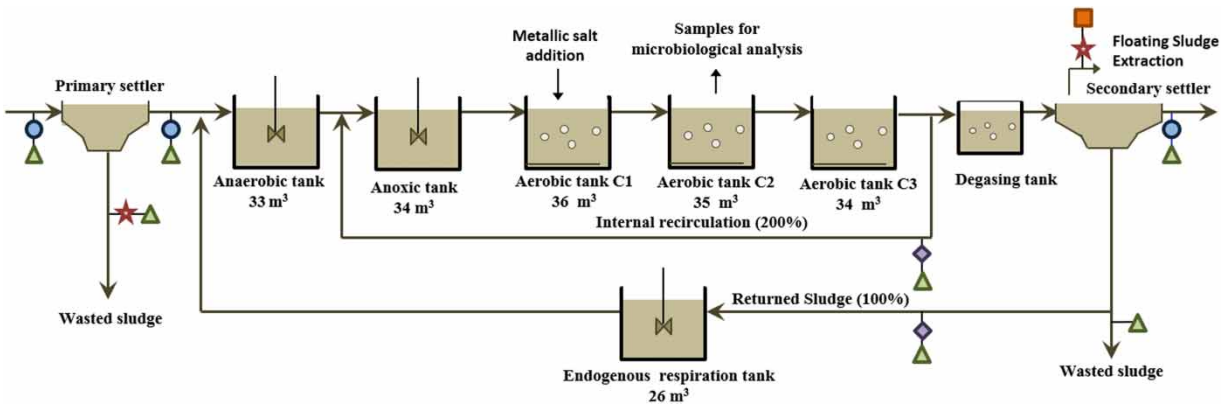

Figure 1 | Scheme of the pilot plant: circle, 24 h flow proportional samples; diamond, daily grab samples; star, two samples a week; triangle, flowmeters; square, floating sludge extraction monitoring.

Activated sludge samples were collected at least once a week in C2 for microbiological analysis. They were cooled to 4 °C and prepared for DNA extraction and microscopic observation (FISH, Gram and Neisser staining) within 12 h. For DNA extraction, 50 mL of mixed liquor was centrifuged for 15 min at 5,800 *g* at 4 °C and the pellet was stored at −20 °C. Each DNA extraction was performed in triplicate with the MoBio PowerSoil® DNA isolation kit following the manufacturer's instructions. DNA concentration and quality were measured by spectrophotometry (Biowave II, Biochrom Ltd, Cambridge, UK).

Aluminium dosage and operating conditions

Two aluminium chloride solutions were tested, the first containing only $AlCl_3$ (30.5% (w/v) – series 1) and the second also containing a small fraction of $FeCl_3$ (24% (w/v) of $AlCl_3$ and 8% (w/v) of $FeCl_3$ – series 2, called Al-Fe-Cl_3 in the following). Those chemicals usually form the basis of commercial solutions proposed to control bulking.

For the first series, two dosages were applied (Table 1). The objective of the first trial (Trial 1) was to stabilize the DSVI value below 150 mL/g. A dosage of 41 mmol Al(kg MLSS·d) was therefore applied for 70 days. This dose is below the range recommended in the literature for *M. parvicella* bulking control. As the DSVI gradually deteriorated after the end of the salt addition, a second trial (Trial 2) was run after a transition phase of 24 days without any dosage. The goal was to rapidly decrease the DSVI value; a higher dose of aluminium salts was therefore applied, 137 mmol Al(kg MLSS·d) for 14 days.

During the second series, two doses were also applied in a gradual manner: a first dose of 16 mmol Me(kg MLSS·d) (Me = aluminium, or aluminium + iron) was applied before and after a higher dosage (94 mmol Me/(kg MLSS·d) for 35 days). Main operating conditions are reported in Table 1.

The pilot was operated at a low food to micro-organisms ratio (F/M < 0.13 kg BOD_5/kg MLVSS/d) during both periods. Ranges of operating parameters are inherent to the pilot operation and correspond to load variations (series 1) and/or to MLSS variations induced by the effect of the salt addition on foam. Operating conditions are, however, comparable between the two series of measurements.

Filament localization and biological aggregate morphology

Identification of filamentous bacteria was based on their Gram and Neisser staining characteristics and on their morphological characteristics as described in Jenkins *et al.* (2004). The filamentous bacteria abundance (FA) was estimated using the subjective 0 to 6 scale proposed by Jenkins *et al.* (2004), referring to a given number of filamentous bacteria per floc. Light microscopy observations were made with an A2 Imager microscope and Zen lite 2011 software (Zeiss).

These observations were completed by FISH analyses in order to identify and localize specific filamentous bacteria in the activated sludge samples. The fluorescently labelled oligonucleotide probes used (MWG Eurofins Operon) targeted the total bacteria with EUBmix (Daims *et al.* 1999), *M. parvicella* with MPAmix (Erhart *et al.* 1997) and

Table 1 | Operating conditions

	Series 1 – $AlCl_3$		Series 2 – Al-Fe-Cl_3	
F/M ratio (kg BOD_5/kg MLVSS/d)	0.06–0.13		0.09–0.11	
Sludge age (d)	11–17		12–17	
[MLSS] C3 (g/L)	1.3–3.5		1.7–2.8	
[DO] in aerated tanks (mg/L)	C1: 2.1–2.3 C2: 1.7–3.9 C3 4.6–6.8		C1: 2.3–2.7 C2: 2.8–3.3 C3: 7.0–8.2	
Temperature in C2 (°C)	13.1–22.9		12.1–24.3	
DSVI (mL/g)	79–191		88–220	
Metallic salts (Me) dosage (mmol Me(kg MLSS·d)): Series 1: $AlCl_3$ (30.5%); Series 2: $AlCl_3$ (24%), $FeCl_3$ (8%)	Initial state (days 0–18) Trial 1 (days 19–88) Intermediate state (days 89–112) Trial 2 (days 113–126) Final state (days 127–180)	0 41 0 137 0	Initial state (days 0–44) Phase I (days 45–65) Phase II (days 66–100) Phase III (days 101–125) Final state (days 126–180)	0 16 94 16 0

phylum Chloroflexi with CFXmix (Gich *et al.* 2001; Bjornsson *et al.* 2002). Paraformaldehyde-fixed samples were prepared following the protocol proposed by Amann (1995) with an additional permeabilization step as recommended for *M. parvicella*'s detection (Kragelund *et al.* 2007). All samples were observed with a confocal laser scanning microscope (Axiovert 200 M LSM 510 META, Zeiss) and Zen 2007 Light Edition software.

Abundance of *M. parvicella* and total bacteria

Quantification of *M. parvicella* and total bacteria was performed by qPCR (CFX 96 Real Time System, Biorad) with primers Mp0828S/Mp1018A and 341F/534R targeting 16S rRNA genes described respectively in Kaetzke *et al.* (2005) and Muyzer *et al.* (1993). Standard curves were obtained using 10-fold dilution series of a linearized plasmid solution (Hou *et al.* 2010). The pEX-A (2,450 bp) plasmid vector containing the targeted 16S rRNA gene sequence was used for total bacteria (194 bp) and *M. parvicella* (210 bp) quantification (Eurofins MWG Operon). The reaction mixture (25 μL) contained 2 μL of DNA template (5 ng/μL of DNA extract or diluted plasmid solution), 12.5 μL of iQ SYBR Green Supermix 1X (Biorad) and 0.4 μM and 0.5 μM of primers for *M. parvicella* and total bacteria quantification, respectively.

RESULTS AND DISCUSSION

Removal efficiency

In both series, metallic salt addition led to a decrease in the total suspended solids (TSS) concentration of the effluent (from 15 to 5 mg/L in series 1 and 20 to 8 mg/L in series 2) that slightly enhanced TSS, COD and TKN removal efficiencies (by a few per cent; see Supplementary material, Figure S1, available in the online version of this paper).

Concerning phosphorus removal, the addition of metallic salts should a priori have led to an increase in the removal efficiency by precipitation. However, as shown in Figure 2 for series 2 and in Supplementary material, Figure S2, for series 1 (available online), the initial removal efficiency obtained through biological P-removal was rather high (87% on average) and the increments were therefore low (about 7%). When the addition was stopped, a decrease

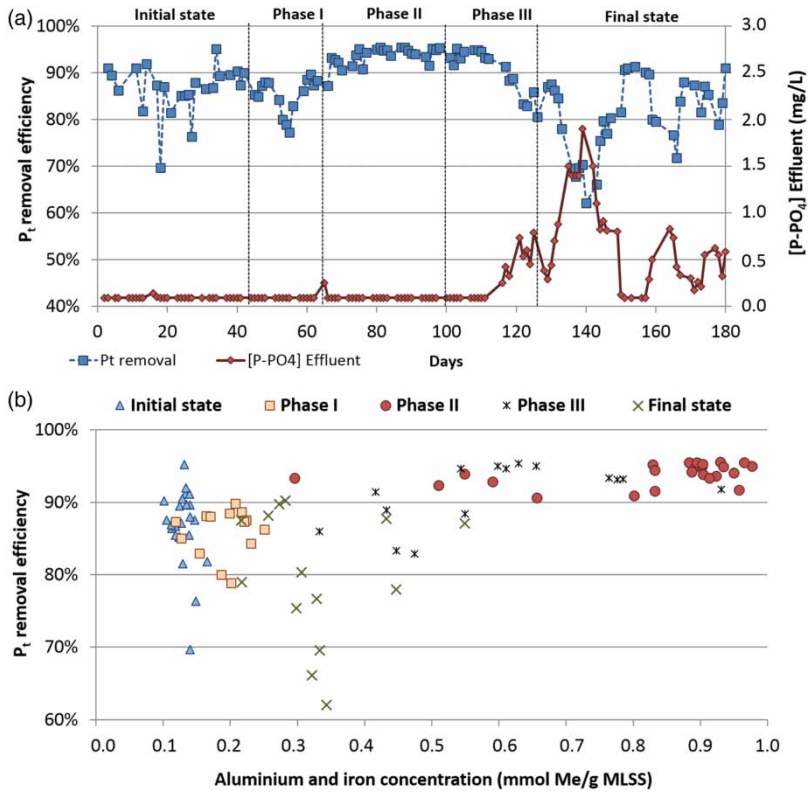

Figure 2 | P-removal efficiency versus time (a) and as a function of the metallic salt concentration (b) for series 2.

in the P-removal efficiency was observed, in relation to an increase in the concentration of orthophosphates in the effluent (Figure 2 and Supplementary material, Figure S2) to a level significantly higher than prior to the dosage. Polyphosphate-accumulating organisms (PAO) may have been washed out, certainly due to the competition phenomenon between biological and chemical P-removal.

Impact of metallic salt addition on settling properties and filamentous bacteria

DSVI and sludge temperature values monitored during the experiments are reported in Figure 3. The variation of the DSVI is expressed as the slope of DSVI values and is also reported as a function of the dose applied (Figure 4).

At the initial state, observed bulking and foaming were due to the proliferation of *M. parvicella*. Four other filamentous bacteria were identified, in top down order: Type 0041/0675 and Type 0092, both belonging to phylum Chloroflexi, *Thiothrix* sp. and *Nostocoida limicola*. Figure 5 shows the localization of *M. parvicella* and bacteria from phylum Chloroflexi before and during Trial 2.

At the smallest dosages (series 1, Trial 1 and series 2, Phase I), DSVI was maintained constant and foam disappeared within 10–15 days despite a rather high filamentous abundance (FA; between 4 and 5 for both series, data not shown). The potential reduction of the sludge hydrophobicity may explain the gradual foam disappearance, such as described by Paris *et al.* (2005). At these dosages, corresponding to a concentration between 0.2 and 0.5 mmol Me/g MLSS in the mixed liquor, *M. parvicella* was still observed free in the water phase.

In order to effectively reduce DSVI values, a higher dosage is required. This is the case during Trial 2 (series 1) and Phase II (series 2), where the concentration in the

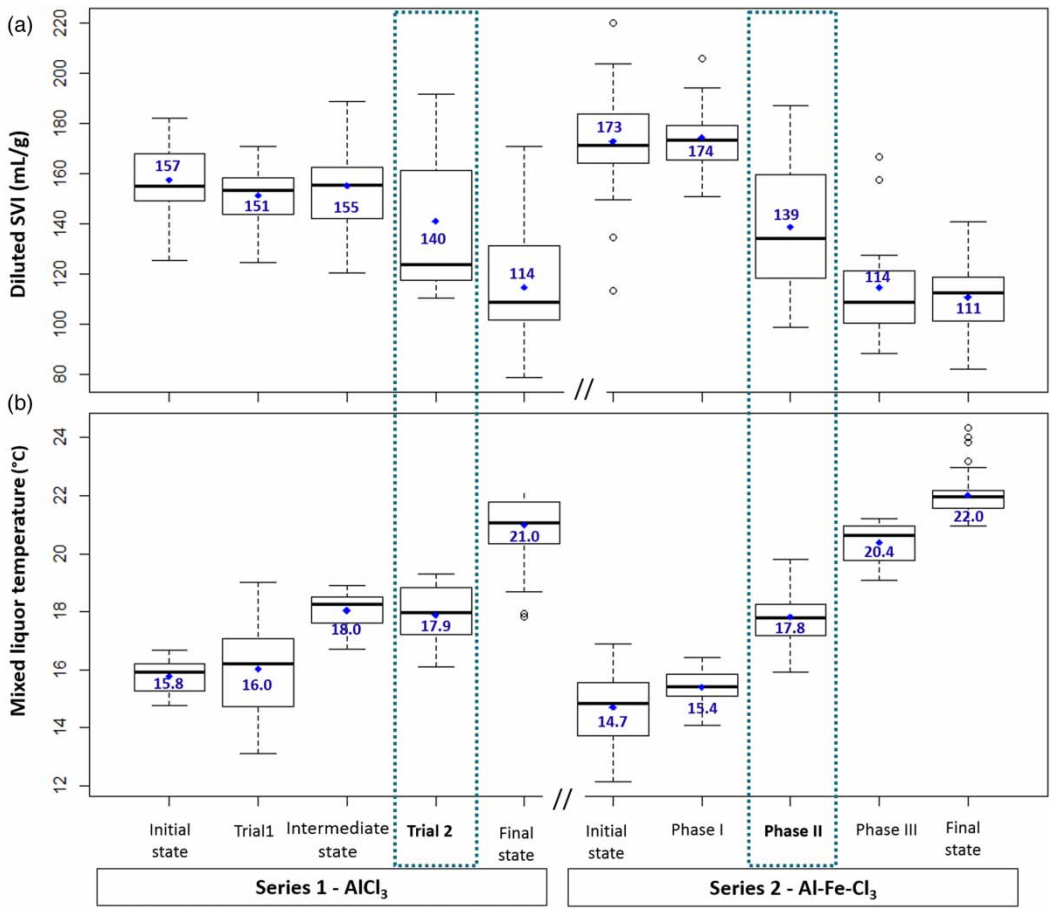

Figure 3 | Box plot representation of DSVI (a) and temperature in C2 (b) during the different phases of the experiments. Circles correspond to the higher dosage in each series. The average value for each period is indicated by the diamond and the median by the thick line. Ends of the whiskers represent the lowest/highest data still within 1.5 interquartile range of the lower/upper quartile.

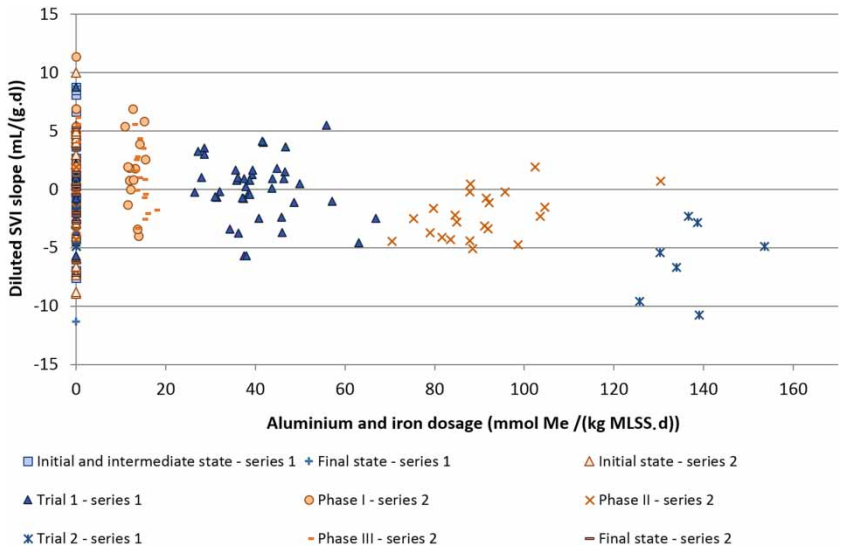

Figure 4 | Variation of DSVI as a function of the applied dose of metallic salts.

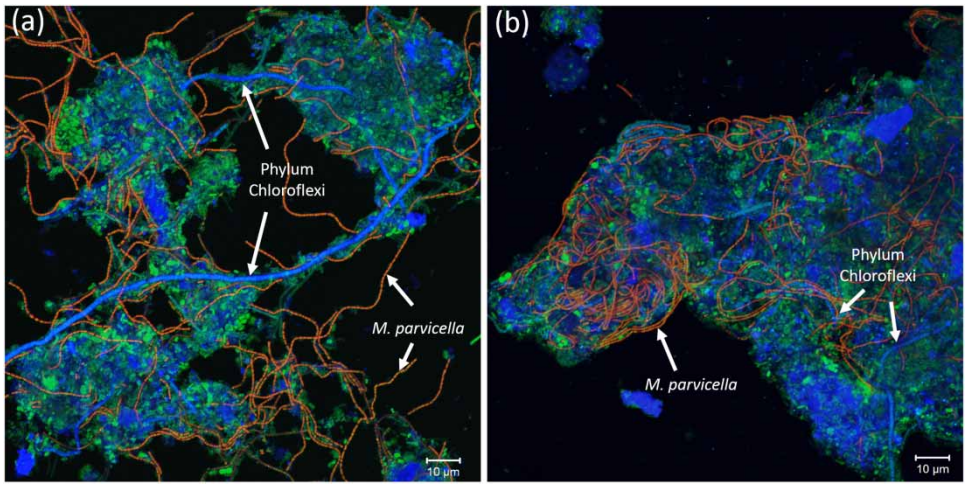

Figure 5 | FISH microscopic observations of activated sludge (Trial 2, series 1) – *M. parvicella*: MPAmix; phylum Chloroflexi: CFXmix; total bacteria: EUBmix. (a) One day before Trial 2; (b) 7th day of Trial 2.

mixed liquor reached values between 0.5 and 1.1 mmol Me/kg MLSS. Metallic salt addition led to foam disappearance within a few days. Filamentous bacteria were no longer free in the water phase (Figure 5) and biological aggregates were of larger size and more compact. DSVI values significantly decreased as well as the overall filamentous abundance (FA from 5 to 3).

While the integration of *M. parvicella* into biological aggregates during metallic salt addition has already been reported (Nielsen *et al.* 2005; Paris *et al.* 2005), results presented in Figure 5 show that filamentous Chloroflexi are also attracted into biological aggregates. Type 0041/0675 and Type 0092, both Gram-negative and respectively sheathed and non-sheathed bacteria, are not known to be particularly hydrophobic (Jenkins *et al.* 2004). Other physicochemical properties may explain the observed embedding of these filaments into flocs, such as chemical interactions between surface polysaccharides of bacteria and cations (Higgins & Novak 1997).

After the dosages in both series, the overall FA increased, with reappearance of free filaments in the water phase and poorly compacted flocs. Despite unfavourable temperatures for *M. parvicella* growth (Figure 3), settling characteristics progressively deteriorated.

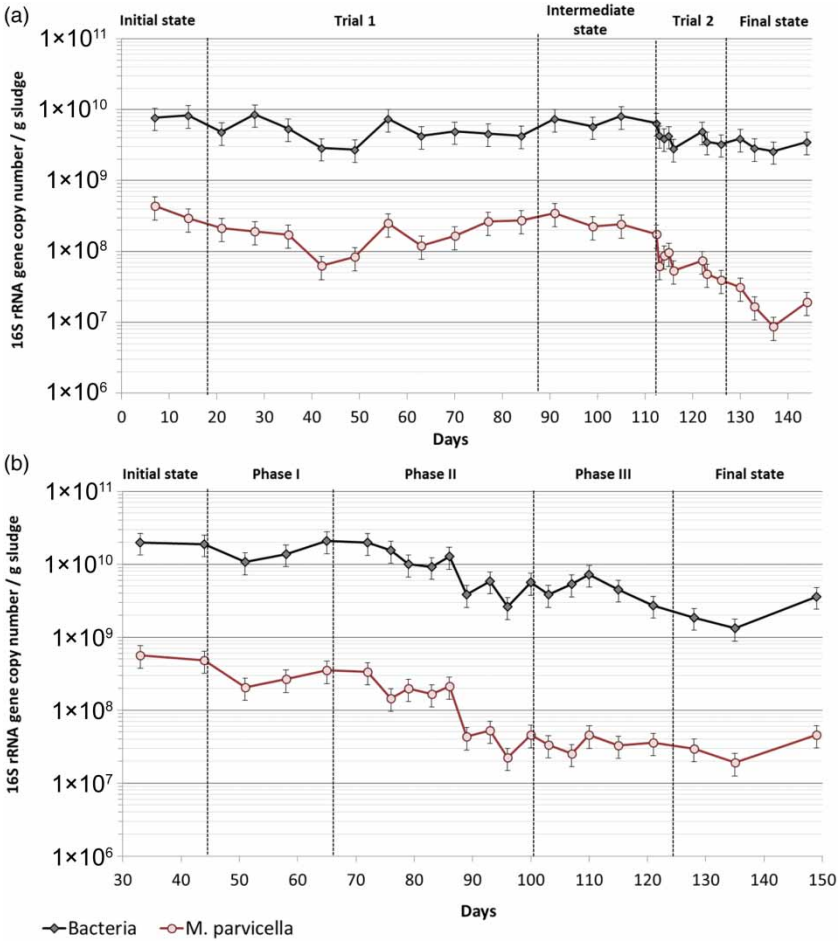

Figure 6 | 16S rRNA gene copy number of total bacteria and *M. parvicella* during series 1 (a) and series 2 (b).

Quantification of *Microthrix parvicella*

Results of qPCR absolute quantification of *M. parvicella* and total bacteria are displayed in Figure 6.

For both series, the copy number of total bacteria comprised between 7.8×10^9 and 2.0×10^{10} copies/g. Those values are similar to literature ones determined in activated sludge samples (Dionisi *et al.* 2003). *M. parvicella* concentration was between 2.9×10^8 and 5.7×10^8 copies/g. The relative abundance of the copy number was around 3.5% of the total bacteria, in accordance with published data for a WWTP encountering foaming (Kaetzke *et al.* 2005).

The smaller dosages applied had no significant impact on *M. parvicella* concentration (in number of cc/g). Maintaining constant DSVI values during those experiments was therefore mainly due to a coagulation effect of salts on the biological aggregates. During the intermediate state of series 1, the slight decrease in *M. parvicella* abundance can be attributed to a temperature rise from 16 to 20 °C (Zhang *et al.* 2010). Increasing the amount of added salts amplifies this decrease (series 1, Trial 2).

The total bacteria were also impacted by a sudden increase in the aluminium dose (series 1, Trial 2). Increasing

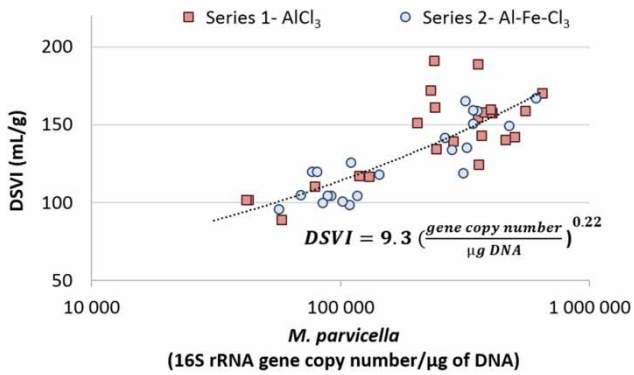

Figure 7 | DSVI as a function of the number of gene copies of *M. parvicella*.

progressively, the dosage seems to be less detrimental to the biomasses (Phase II, series 2). However, in the later experiments, the duration of the test may have impacted the number of copies/g, suggesting a growth limitation in addition to the coagulation effect.

As shown in Figure 7, the sludge volume index is correlated with the copy number of *M. parvicella* 16S rRNA gene.

Given this correlation, measuring *M. parvicella* abundance by qPCR on WWTPs showing seasonal bulking would be interesting in order to develop an early warning method to detect bulking and foaming. Quantification of filamentous bacteria could also help in modelling DSVI and thereafter settling characteristics of activated sludge.

CONCLUSIONS

Measurements performed to investigate the impact of aluminium salts at different dosages to control bulking and foaming caused by *M. parvicella* in activated sludge show that:

- the metallic salts induced no negative impact on the plant removal efficiencies, except a decrease of P-removal when additions were stopped, suggesting a PAO washout during the dosage;
- improvement of the settling conditions is certainly due to flocculation mechanisms; at a concentration comprising between 0.2 and 0.5 mmol Me/g MLSS, *M. parvicella* growth was not affected;
- at a salt concentration higher than 0.7 mmol Me/g MLSS, filamentous bacteria were embedded into the aggregates and qPCR analyses suggest a growth limitation in addition to the coagulation effect.

According to these results, aluminium salts should be added progressively and over a few weeks in order to efficiently control *M. parvicella* bulking without impacting the remaining biomass.

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