***Candidatus Monilibacter* spp., common bulking filaments in activated
sludge, are members of Cluster III *Defluviicoccus***

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Running title: Bulking filaments in activated sludge

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Abstract

Two alphaproteobacterial Neisser negative ‘Nostocoida limicola’ morphotypes differing slightly in their trichome diameter and filament regularity were dominant populations in the Bendigo, Victoria, Australia activated sludge community removing phosphorus (P). Neither responded to the FISH probes available for any of the other alphaproteobacterial ‘N.limicola’ morphotypes. Instead both fluoresced with the DF988 FISH probe designed originally to target alphaproteobacterial cluster II *Defluviicoccus* tetrad forming organisms. A 16S rRNA based clone library from this biomass revealed that the alphaproteobacterial clones grouped closely with *Candidatus* ‘Monilibacter batavus’ and *Defluviicoccus* clones in a cluster separate to the existing cluster I and II *Defluviicoccus*. When a FISH probe was designed against these, it only hybridized to the thinner and less abundant ‘N.limicola’ morphotype. Micromanipulation-RT-PCR was used to selectively recover the main ‘N.limicola’ morphotype and a FISH probe designed against the 16S rRNA clones generated from it showed only this filament fluoresced. From FISH based surveys, both ‘N.limicola’ variants occurred frequently in phosphorus removal activated sludge systems in Australia treating domestic waste. The data suggest that they represent two new strains of *Candidatus* ‘Monilibacter’, which on this evidence are filamentous members of the genus *Defluviicoccus*, a potential competitor for the polyphosphate accumulating organisms in these communities.

Keywords: activated sludge; *Alphaproteobacteria*; bulking; *Candidatus* ‘Monilibacter batavus’; *Defluviicoccus*; FISH; ‘Nostocoida limicola’

Introduction

Bulking and foaming of activated sludge caused by excessive proliferation of filamentous bacteria is still a major operational problem in many wastewater treatment plants (WWTPs) [13, 16, 24]. So far, more than 30 filament morphotypes have been recognized by their microscopic characteristics in plants treating domestic wastes, and further diversity seen in industrial plant biomass samples [10, 13, 24]. It is now clear that a single filament morphotype may contain several phylogenetically unrelated bacteria. Examples are the three groups of 'Nostocoida limicola' I, II and III, which differ in their cell diameters [11]. After 16S rRNA sequencing, each was shown to belong to a different bacterial phylum [5, 20, 21].

The phylogenetic diversity among members of the N. limicola II morphotype is more extensive, containing as it does members of the *Actinobacteria* [5], '*Chloroflexi*' [31] and the *Alphaproteobacteria* [19]. The *Alphaproteobacteria* 'N.limicola' morphotype previously reported as predominant in industrial plants [15, 16, 19, 35, 36], and rarely in those treating domestic wastes [32] contains six distantly related phylogenetic clusters [15, 19, 36]. These include five uncultured species, '*Candidatus Combothrix italica*', '*Candidatus Alysiosphaera europaea*', '*Candidatus Monilibacter batavus*', '*Candidatus Alysiumicrobium bavaricum*' and '*Candidatus Sphaeronema italicum*' [19]. FISH probes have been designed for all of these and applied to samples from industrial plants [35], and in combination with microautoradiography (FISH/MAR), their ecophysiological attributes described [15].

During a study on a full scale bulking EBPR plant treating domestic waste the dominant bulking filament was a Neisser negative alphaproteobacterial 'N. limicola' morphotype.

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3 These filaments fluoresced with the DF988 FISH probe designed to target the
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5 alphaproteobacterial Cluster II *Defluviicoccus vanus* related tetrad forming organisms or
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7 TFO [27]. Furthermore, they did not fluoresce with any of the probes described by Snaidr
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9 *et al.*, [35] for their alphaproteobacterial 'N. limicola' filaments. Therefore this study
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11 focussed on identifying this filament, and with the FISH probes designed against it, to
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13 determine its distribution in full scale treatment plants.
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19 **Materials and Methods**

20 **DNA extraction.**

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22 Two activated sludge samples were taken from the Bendigo, Victoria, Australia
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24 enhanced biological phosphorus removal (EBPR) plant treating domestic waste, in
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26 February and October 2007. This plant operates as a modified University of Cape Town
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28 (MUCT) configuration. Samples were immediately fixed in ethanol and stored at -20 °C.
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31 The DNA was extracted using three different methods to attempt to minimise possible
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33 extraction biases associated with each: 1) the sodium trichloroacetate based method of
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35 McIlroy *et al.*, [25] , 2) the phenol chloroform based method of McVeigh *et al.* [26], and 3)
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37 the FastDNA SPIN Kit (Qbiogene, Melbourne, Australia). The DNA extracts were stored
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39 at -20 °C.
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48 **PCR amplification.**

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50 Five PCR reactions were performed with each DNA extract using a GeneAmp PCR
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52 System 9700 thermal cycler (ABI, CA, USA), to minimize PCR associated biases. 16S
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54 rRNA genes were amplified with primers U27f (5'-GAGTTTGATCMTGGCTCAG-3')

and U1492r (5'-GGYTACCTTGTTACGACTT-3') [17], under the following conditions, 1 cycle 10 min 95 °C, 30 cycles (30 sec 94 °C, 30 sec 50 °C, 2 min 72 °C) and 1 cycle 10 min 72 °C. Each PCR reaction mixture (50 µl) contained 1 µl of template DNA, 200 nM of each primer, 200µM of each deoxyribonucleoside triphosphate, 1x PCR buffer, 2.5 mM of MgCl₂, and 0.025 U/µL of AmpliTaq Gold (ABI). The amplified products for each PCR reaction were combined and electrophoresed on 1.5 % agarose gels. A band corresponding to approximately 1500 bp was excised with a clean razor and purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) according to manufacturers' instructions.

Clone library construction.

The purified PCR products were cloned into the pGEM-T Easy Vector System (Promega), and presence of correctly sized inserts was confirmed by colony PCR. Plasmids from each clone were extracted with the Wizard *Plus* SV Minipreps DNA Purification System (Promega) as per manufacturer's instructions. For all the extracted plasmids, 16S rRNA gene sequences of about 500 bp were determined at the Australian Genome Research Facility (AGRF, Brisbane, Australia). For the clones with partial sequences of interest only (see Results for selection criteria), the complete insert sequences were then determined at AGRF using internal 16S rRNA primers [17], and assembled with Geneious software v 3.6.2 (Biomatters, Auckland, New Zealand).

Cell sorting-RT-PCR.

The RT-PCR method described by Levantesi *et al.*, [19] was used to recover and sequence the ‘Nostocoida limicola’-like filamentous bacteria from a fresh biomass sample from the Bendigo biomass. More than 30 of the target filament morphotypes were micromanipulated with a Skerman [34] micromanipulator. These filaments were suspended in 10 µl of sterilized milliQ water and subjected to RT-PCR with the Invitrogen SuperScript™ III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, USA), as described by Levantesi *et al.*, [19]. Preparation of the 16S rRNA gene clone library was then performed as described above.

Phylogenetic tree construction and FISH probe design.

Possible chimeric sequences were assessed using Bellerophon v3 [8], Mallard [3], and Pintail [2] software, and all putative chimaeras were eliminated from subsequent analyses. The remainder were added to the ARB database [22] and aligned. A maximum likelihood phylogenetic tree was constructed from these and selected related sequences (Fig. 2). Oligonucleotide probes were designed using the probe design/match tools in ARB. In order to evaluate the formamide concentration required for optimum stringency, all newly designed probes were assessed against PFA-fixed activated sludge collected from Bendigo with hybridization buffer containing 15-50% formamide in 5% incremental steps. Their assessed optimal formamide concentrations, sequences and target sites are given in Table 1. All probes were purchased from Proligo (Melbourne, Victoria), and were tagged with either Fluos, Cy3 or Cy5 fluorochromes.

FISH analysis.

Activated sludge samples were taken from aerobic tanks of 12 full-scale (EBPR) plants in eastern Australia (details given in Table 3). Samples were fixed in 4% paraformaldehyde for 3 h and then stored at -20 °C according to Daims *et al.*, [7]. FISH analyses on fixed samples were performed following the method of Snaird *et al.* (1997). Formamide concentrations used for existing FISH probes (Table 1), which were labelled with either Cy3, Cy5 or Fluos (5(6)-carboxyfluorescein-N-hydroxy-succinimide ester) fluorochromes and purchased from Prologo (Sydney, NSW) were those described in the original publications. For probes designed in this study, their optimal hybridization conditions were obtained as described above. Samples mounted in vectashield® Vector Laboratories, Burlingame, CA) were either examined with a Nikon Eclipse 800 epifluorescence microscope, or with a Leica TCS SP2 confocal scanning laser microscope. Appropriate controls were incorporated into all the analyses

Staining

To detect polyphosphate and PHA in cells, 4',6-diamidino-2-phenylindol (DAPI) [14] and Nile blue A [30] respectively were used on both fresh and fixed samples.

Nucleotide sequence accession numbers.

The nucleotide sequence data for clones from the alphaproteobacterial 'N.limicola' morphotype reported in this paper were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB445107– AB445110.

Results

FISH Survey of Bendigo Biomass

When samples of biomass from the Bendigo EBPR plant were being surveyed for presence of the alphaproteobacterial *Defluviicoccus* TFO cluster II members with the DF988 probe originally designed to target these [27], two Neisser negative ‘Nostocoida limicola’ II filament morphotypes, differing slightly in their trichome diameters and regularity of cell shape, also fluoresced with it (Fig. 1a,b) and also the ALF968 probe. However, neither fluoresced with the DF1020 FISH probe designed to target the same *Defluviicoccus* populations [27], nor the DF218 and DF618 FISH probes for members of the cluster I *Defluviicoccus* of Wong *et al.*, [39] or any of the probes targeting the alphaproteobacterial ‘Nostocoida limicola’ filament morphotypes described by Snaidr *et al.*, [35], including the MC2-649 probe targeting *Candidatus* ‘Monilibacter batavus’ (data not shown).

16S rRNA sequence analyses of the Bendigo biomass

A clone library of 75 partial sequences (first 500bp) of bacterial 16S rRNA genes from the Bendigo biomass contained nine alphaproteobacterial sequences. These fell into two OTUs (data not presented).

One representative clone from each OTU (A40 and B29) was then selected for complete sequencing, and the resulting phylogenetic tree is given in Fig. 2. Both sequences were most closely related (98.8% similarity) to an uncultured bacterial clone mle1-13 (AF280850) recovered from an industrial activated sludge treating pharmaceutical waste [18] and designated as a member of ‘cluster’ III *Defluviicoccus* by Wong & Liu [38].

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3 These sequences also clustered with clones MC2 (AY428763), *Candidatus* 'Monilibacter
4 batavus' (AY590701) and MU073 (AM157605) (Fig. 2).

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7 Sequences of A40 and B29 clones were then screened for the presence of the target
8 sequences for the DF988 probe and absence of the MC2-649 probe target sequence, a
9 choice based on the earlier FISH data. The 16S rRNA from both had three mismatches
10 with the DF988 probe, as shown in Table 2, but these were all terminal of the 22 base
11 oligonucleotide probe. This meant that total complementarity existed with the other 18
12 nucleotides, and would explain why these filaments fluoresced with it. Thus, a competitor
13 probe [23] was designed (DF988c) with a perfect match with the A40 and B29 target sites,
14 and as shown in Figs 1c,d, applying this successfully eliminated any fluorescent signal
15 from the filament with the DF988 probe. Thus, it is recommended that the DF988c probe
16 be used in combination with the DF988 probe. The MC2-649 probe has a single central
17 mismatch with the 16S rRNA sequences of the A40 and B29 clones (Table 2), but the
18 recommended hybridisation stringency [19] was clearly sufficient to ensure these
19 'N.limicola' filament morphotypes did not fluoresce with it.
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41 **Probe design.**

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43 Thus, two new probes (DF198 and DF1013) were designed against clones in the
44 cluster. Probe DF198 targeted the 16S rRNA sequences of all the members of the cluster
45 III *Defluviicoccus* including clones A40 and B29, while probe DF1013 targeted only the
46 A40 and B29 clone sequences (Fig. 2). Two helper probes [12] , DF997H and DF1032H
47 were designed for use with the DF1013 probe as it targeted an area thought to be relatively
48 inaccessible to probes [4]. Of these helpers DF997H was required to increase the
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3 fluorescence signal strength of the DF1013 probe as assessed subjectively by eye, although
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5 the addition of the DF1032H did not noticeably improve it. A competitor probe, DF1013c,
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7 was also designed to reduce the possibility of the probe binding to the mle1-13
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9 (AF280850) sequence, which has a single mismatch with it. Clone sequences encompassed
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11 by each probe are shown in Fig. 2.
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15 FISH analysis with the DF198 probe and Bendigo sample showed that both
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17 morphological variants of this 'N.limicola' filament fluoresced with it (Fig. e-h). On the
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19 other hand, only the thinner and less regular 'N.limicola' filaments present in much smaller
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21 numbers fluoresced with the DF1013 probe (Fig. e-f). These results suggest that while the
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23 dominant 'N.limicola' in Bendigo samples is a member of the same cluster, it is
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25 phylogenetically distinct from the A40 and B29 clones. A corresponding sequence was not
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27 recovered in the clone library, suggesting either DNA extraction problems, despite efforts
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29 to avoid this commonly encountered problem, or PCR associated biases [37]. Hence its
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31 identity was sought by a different approach.
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38 **Retrieval of 16S rRNA sequences by micromanipulation and RT-PCR**

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42 The micromanipulation cell sorting-RT-PCR method was applied to recover dominant
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44 'N.limicola' filaments from a Bendigo biomass sample, and another clone library was
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46 generated. Partial sequences (about 500bp) of 13 clones revealed that six (designated C17,
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48 C23, C38, C41, C43, and C49) had the DF988 probe target sequence, all with the same
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50 terminal three nucleotide mismatches. Five of these (C17, C23, C38, C41 and C49) had
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52 two mismatches with the DF1013 probe target site (Table 2) while C43 was 99.6% similar
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54 to clone A40 obtained earlier. These features were consistent with the FISH data for the
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dominant 'N. limicola' morphotype. As four of the five clones (C17, C38, C41 and C49) had identical partial sequences, only clones C17 and C23 were fully sequenced. Both fell into the same cluster as clones A40 and B29 (Fig. 2) (sharing 98.6-98.9% sequence similarity). They share the same marker sequences with the A40 and B29 clones at the target sites for the DF988, MC2-649 and DF198 FISH probes. Thus, a new probe, DF1004 that targeted only the C17 and C23 sequences was designed. Because of possible accessibility problems with the target site, helper probes DF987H and DF1021H were also designed. Of these, DF987H improved the fluorescence signal of the DF1004 probe but application of the DF1021H probe seemed unnecessary. A competitor probe, DF1004c, was also designed to reduce any likelihood of the DF1004 probe binding to the *Candidatus* 'M. batavus' clone (AY590701), which contains just a single mismatch with it (Table 2).

When this DF1004 FISH probe was applied together with the helpers and competitor to a sample from the Bendigo plant, only the dominant 'N. limicola' filaments hybridized with it (Fig. 1g,h), suggesting that the C17 and C23 clones contained the sequences from this filament.

Distribution of these filamentous *Defluviicoccus* in full scale EBPR plants

These DF198, DF1004 and DF1013 FISH probes were applied to biomass samples taken from other activated sludge samples from EBPR plants in eastern states of Australia (Table 3). The two 'N. limicola' filament morphotype variants fluoresced with either DF1004 or DF1013 probes, as in the Bendigo plant sample, although their numbers varied, ranging from some in a few plants to very common and excessive in others, based on the subjective criteria of Jenkins *et al.* [13]. In all cases, the morphotype dominant in the Bendigo plant

was more abundant in all samples examined. These data would suggest that filaments detected with these new probes are common members of the microbial communities in full-scale EBPR plants treating domestic waste in eastern Australia.

Production of polyP and PHA

None of the alphaproteobacterial FISH probed 'N. limicola' morphotypes in any of the biomass samples contained polyP after DAPI staining. A few (approx. <20%) in each sample stained for PHA with Nile blue A.

Discussion

This paper shows that two variants of a Neisser negative alphaproteobacterial 'N.limicola' morphotype dominating the community of EBPR activated sludge communities are members of the cluster III *Defluviicoccus* proposed by Wong *et al.*, [38]. It extends the known biodiversity of this functionally important group of bacteria in activated sludge communities removing P [29, 33] , and is the first report of a filamentous member of this group, whose members have been described previously as TFO or single celled organisms. Each of the two morphological variants was phylogenetically distinct, based on their 16S rRNA sequence (98.6-98.9 percent similar) but whether these represent separate species is not known.

Clones possessing or lacking marker sequences elucidated from preliminary FISH analyses could be identified in the generated clone libraries (Fig 2) and selected for further examination. By this approach FISH probes could be designed confidently to target both morphotype variants, and those described here embrace all currently known members of

the cluster III *Defluviicoccus*. FISH based surveys with them showed the alphaproteobacterial 'N.limicola' filaments were common and often dominant members of communities in most full scale EBPR plants examined in eastern Australia, and were probably responsible for bulking in some of these (e.g. Bendigo, Victoria; Nambour and Coolum, Queensland). Their possible impact on P removal in the plants was not pursued. These alphaproteobacterial clones are all phylogenetically very closely related (98.1 to 98.8% 16S rRNA shared similarity) to the 'N.limicola' filament morphotype *Candidatus* 'M. batavus' [19] which, from the data presented here, must now also be considered a member of the genus *Defluviicoccus*. However, they appear to differ in their distribution, with *Candidatus* 'M. batavus' not reported widely in EBPR communities, but seemingly found mainly in industrial wastewater treatment plants [19]. *Candidatus* 'M. batavus' also stains Neisser positively and Gram variably [15], staining reactions different to the 'N. limicola' filaments described here. Furthermore, the MC2-649 FISH probe designed to target *Candidatus* 'M. batavus' [19], did not impart fluorescence to either of the 'N. limicola' variants seen in our study, as the sequence of each possessed a single mismatch with its target site (Table 2). However, this MC2-649 probe was designed from a partial sequence from clone MC2 (581 bp) [35] and has low specificity. It also targets a number of other alphaproteobacterial sequences and members of the *Acidobacteria* and *Candidatus* division SBR1093. Furthermore, many deposited 16S rRNA sequences in the Greengenes database [9] contain a single, often terminal, mismatch with it. Members of the genus *Defluviicoccus* are thought to be responsible for EBPR failure by out-competing the polyphosphate accumulating organisms (PAO) for substrates in the anaerobic zone of these plants, and using their stored PHA to synthesise glycogen and not

polyphosphate under aerobic conditions [29, 33]. Consequently, their phenotype is described as that of a glycogen accumulating organism or GAO. So the question is whether the *Defluviicoccus*-related alphaproteobacterial 'N. limicola' filament morphotypes described here, which were seen so frequently in EBPR systems possess the same *Defluviicoccus*-like GAO phenotype [29, 33]. In particular, whether they assimilate substrates and synthesise PHA anaerobically, and subsequently fail to synthesise polyP aerobically, producing glycogen instead, needs to be determined.

FISH/microautoradiography (FISH/MAR) data from Kragelund *et al.*, [15] with filaments in non-EBPR processes suggest that MC2-649 FISH probed *Candidatus* 'M. batavus' assimilated only acetate, propionate and pyruvate aerobically, and only very weak acetate uptake was detected anoxically. However, PHA synthesis occurred and filaments stained positively with the Neisser stain, although whether this latter reaction reflects polyP presence is not clear.

No polyP granules could be detected in any of the FISH probed filaments in EBPR biomass samples stained with DAPI in our study, and PHA storage appeared to be a highly variable property with only a few staining positively with Nile blue A. Clearly more studies are required to see whether these filaments including *Candidatus* 'M. batavus' are found in EBPR plants globally, and to resolve their ecophysiology by FISH/MAR and their possible impact on EBPR community functioning. The availability of the FISH probes and the additional phylogenetic information on their biodiversity described here should assist in this task.

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Fig. 1 FISH micrographs for the activated sludge biomass from Bendigo wastewater treatment plant. a) Phase contrast b) corresponding FISH image using probe DF988 (CY3: Red); c) Phase contrast d) negative FISH signal for DF988 (CY3: Red) in the presence of unlabelled competitor probe DF988c; e,f) FISH overlays with probes EUBmix (Fluos: Green), probe DF1013 (CY3: Red), probe DF198 (CY5: Blue). g) Phase contrast image h) FISH overlay with probes EUBmix (Fluos: Green), probe DF1004 (CY3: Red), probe DF198 (CY5: Blue).

Fig. 2. Maximum likelihood tree of the complete *Deffluviicoccus* –related sequences (all sequences were at least 1250 bp long). Sequences from this study are represented in bold. Parsimony bootstrap values were calculated as a percentage of 1000 analysis and are only indicated for values ≥ 75 %. ○ Indicates a bootstrap value of ≥ 75 % and ● indicates a bootstrap value of ≥ 95 %. The scale bar corresponds to 0.1 substitutions per nucleotide position. * Indicates partial sequences added using the ‘quick add’ function in ARB. Probe coverage is indicated by brackets, where broken lines indicate the absence of sequence information at the probe site. The outlier used was *Rhodocyclus tenuis*.

Table 1 FISH probes used in this study; their specificities, sequences, target sites and hybridization formamide concentrations.

Probe name	Specificity	Sequence (5'-3')	Target site ^a	FA(%) ^b	References
EUB338 ^c	Most bacteria	GCTGCCTCCCGTAGGAGT	338-355	0-60	[1]
EUB338-II ^c	<i>Planctomycetales</i>	GCAGCCACCCGTAGGTGT	338-355	0-60	[6]
EUB338-III ^c	<i>Verrucomicrobiales</i>	GCTGCCACCCGTAGGTGT	338-355	0-60	[6]
ALF968	Most <i>Alphaproteobacteria</i>	GGTAAGGTTCTGCGCGTT	968-985	35	[28]
DF988	<i>Defluviicoccus vanus</i> -related organisms, cluster 2	GATACGACGCCCATGTCAAGGG	988-1009	35	[27]
MC2-649	<i>Candidatus Monilibacter batavus</i>	CTCTCCCGGACTCGAGCC	649-666	35	[35]
DF198	<i>Candidatus Monilibacter batavus</i> -related organisms	ATCCCAGGGCAACATAGTCT	198-217	35	This study
DF1004	C17 & C23	TAAGTTTCCTCAAGCCGC	1004-1021	35	This study
DF1013	A40 & B29	GAAGTGAAGGCTCGAGTTTC	1013-1032	35-50	This study
DF988c	A40 & B29	GCCGCGACGCCCATGTCAAGGG	988-1009		This study
DF1004c	Competitor for DF1004	TAACTTTCCTCAAGCCGC	1004-1021		This study
DF1013c	Competitor for DF1013	GAAGTGAAGGCTTGAGTTTC	1013-1032		This study
H966	Helper for DF988	CTGGTAAGGTTCTGCGCGTTGC	966-987		[27]
DF997H	Helper for DF1013	CCCAAGCCGCGACGCCCA	997-1014		This study
DF1032H	Helper for DF1013	CCTGTGTGGCGTCCAGCC	1032-1049		This study
DF987H	Helper for DF1004	GACGCCCATGTCAAGGGC	987-1004		This study
DF1021H	Helper for DF1004	CCAGCCGAAGTGAAGGCT	1021-1038		This study

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- 1 ^a *Escherichia coli* rRNA numbering (Brosius *et al.*, 1981); ^b Formamide concentration (FA) in the hybridization buffer (v/v); ^c
- 2 Equimolar concentrations of EUB338, EUB338-II and EUB338-III were used in a mixture called EUB mix.
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Table 2 Mismatches in target sites between FISH probes and clone sequences retrieved in this study.

MC2-649 Probe	C	T	C	T	C	C	C	G	G	A	C	T	C	G	A	G	C	C
MC2-649 Target	G	A	G	A	G	G	G	C	C	U	G	A	G	C	U	C	G	G
clones A40, B29, C17 and C23	G	A	G	A	G	G	G	C	C	U	G	A	G	U	U	C	G	G

DF988 Probe	G	A	T	A	C	G	A	C	G	C	C	C	A	T	G	T	C	A	A	G	G	G
DF988 Target	C	U	A	U	G	C	U	G	C	G	G	G	U	A	C	A	G	U	U	C	C	C
clones A40, B29, C17 and C23	C	G	G	C	G	C	U	G	C	G	G	G	U	A	C	A	G	U	U	C	C	C

DF1013 Probe	G	A	A	C	T	G	A	A	G	G	C	T	C	G	A	G	T	T	T	C
DF1013 Target	C	U	U	G	A	C	U	U	C	C	G	A	G	C	U	C	A	A	A	G
clones C17 and C23	C	U	U	G	A	C	U	U	C	C	G	A	A	U	U	C	A	A	A	G

Table 3 Summary of the 12 activated sludge samples and its FISH analysis with the new probes developed in this study.

Plant Location	System configuration	FISH with DF198 ^a	FISH with DF1004 ^a	FISH with DF1013 ^a
Bendigo, Victoria	MUCT ^b	+++	+++	++
Castlemaine, Victoria	MUCT ^b - intermittent decant	-	-	-
Coolum, Queensland	OD ^c with anaerobic zone	++	++	++
Dalby, Queensland		++	++	+
Kyneton, Victoria	MUCT ^b Lutzig- Ettinger	-	-	-
Logan, Queensland	5 stage Bardenpho	+	+	+
Maroochy, Queensland	5 stage Bardenpho	+	+	+
Merrimac, Queensland	MUCT	+	+	+
Morpeth, NSW	Johannesburg version II	+	+	+
Nambour, Queensland	4-stage Westbank	+++	+++	++
Thorneside, Queensland	Bardenpho 3stage	+	+	+
Watella, Queensland	MUCT variation	+	+	+

+++ = excessive to abundant ; ++ = very common to common; + = some to few; - = none according to Jenkins et al. (2004)

MUCT = Modified University of Cape Town configuration ; OD = oxidation ditch.

Figure
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