

A comprehensive overview of the Chloroflexota community in wastewater treatment plants worldwide

Francesca Petriglieri^{1*}, Zivile Kondrotaitė¹, Caitlin Singleton¹, Marta Nierychlo¹, Morten K. D. Dueholm¹, Per H. Nielsen^{1*}.

¹ Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, Aalborg, Denmark

* corresponding authors: phn@bio.aau.dk and fp@bio.aau.dk

Abstract Filamentous Chloroflexota are abundant in activated sludge wastewater treatment plants (WWTPs) worldwide and are occasionally associated with poor solid-liquid separation or foaming, but most of the abundant lineages remain undescribed. Here, we present a comprehensive overview of Chloroflexota abundant in WWTPs worldwide, using high-quality metagenome-assembled genomes (MAGs) and 16S rRNA amplicon data from 740 Danish and global WWTPs. Many novel taxa were described, encompassing 4 families, 13 genera and 29 novel species. These were widely distributed across most continents, influenced by factors such as climate zone and WWTP process design. Visualization by fluorescence *in situ* hybridization (FISH) confirmed their high abundances in many WWTPs based on the amplicon data and showed a filamentous morphology for nearly all species. Most formed thin and short trichomes integrated into the floc structure, unlikely to form the typical inter-floc bridging that hinders activated sludge floc settling. Metabolic reconstruction of 53 high-quality MAGs, representing most of the novel genera, offered further insights into their versatile metabolisms and suggested a primary role in carbon removal and involvement in nitrogen and sulfur cycling. The presence of glycogen reserves, detected by FISH-Raman microspectroscopy, seemed widespread across the phylum demonstrating that these bacteria likely utilize glycogen as an energy storage to survive periods with limited resources. This study gives a broad overview of the Chloroflexota community in global activated sludge WWTPs and improves our understanding of their roles in these engineered ecosystems.

Importance Chloroflexota are often abundant members of the biomass in wastewater treatment plants (WWTPs) worldwide, typically with a filamentous morphology, forming the backbones of the activated sludge (AS) floc. However, their overgrowth can often cause operational issues connected to poor settling or foaming, impairing effluent quality and increases operational costs. Despite the importance, few Chloroflexota genera have been characterized so far. Here, we present a comprehensive overview of Chloroflexota abundant in WWTPs worldwide and an in-depth characterization of their morphology, phylogeny, and ecophysiology, obtaining a broad understanding of their ecological role in activated sludge.

Introduction

Microorganisms belonging to the phylum Chloroflexota are frequently observed in the filamentous biomass in activated sludge (AS) wastewater treatment plants (WWTPs). They promote floc-formation by creating the backbone upon which other bacteria can attach (1–3). However, the uncontrolled overgrowth of specific

genera, such as *Candidatus Amarolinea*, can also cause operational issues (4). According to the current taxonomic classification, based on 16S rRNA gene phylogeny (2), Chloroflexota found in WWTPs belong mainly to the classes Anaerolineae and Chloroflexia, with only a few cultured representatives mostly in the family *Anaerolineaceae* (2, 5–10).

Chloroflexota have a versatile facultative anaerobic metabolism, likely providing them with a competitive advantage in treatment plants with nitrogen removal and/or enhanced biological phosphorus removal (EBPR) systems characterized by alternating oxic and anoxic stages (2). The preferred substrates of known Chloroflexota filamentous species are carbohydrates, complex polymers such as cellulose, or amino acids (2). All described genera have the potential for fermentation of different products and some have the genes to carry out dissimilatory nitrate reduction or partial denitrification (11, 12), indicating a potential role in nitrogen removal from wastewater. The isolation of a nitrite-oxidizing bacterium, *Nitrolancea hollandica*, belonging to this phylum further supports their possible involvement in several steps in the nitrogen cycle (13).

Historically, filamentous bacteria in AS were identified using morphological features defined by specific staining methods and light microscopy (14–16), which often resulted in imprecise classification with little phylogenetic resolution (4). The introduction of high-throughput DNA sequencing and bioinformatics tools offered a breakthrough for the profiling of microbial communities. However, incomplete universal reference databases along with a lack of taxonomy for most uncultured lineages, including abundant Chloroflexota in WWTPs has hampered our ability to study these key organisms at lower taxonomic ranks (17). To improve the taxonomic resolution in microbial profiling studies, we introduced the Microbial Database for Activated Sludge (MiDAS), which includes a global, ecosystem-specific 16S rRNA gene reference database for wastewater treatment systems (MiDAS 4) (18). It also serves as a powerful tool for the design of genus- or species-specific fluorescence *in situ* hybridization (FISH) probes, which can subsequently be applied in combination with other techniques (e.g., microautoradiography or Raman microspectroscopy) for physiological characterization (1, 19). Furthermore, the recent retrieval of thousands of high quality (HQ) metagenome-assembled genomes (MAGs) (20, 21), together with the retrieval of several MAGs from Chloroflexota abundant in AS (12, 22–24), will allow us to obtain an improved overview of the phylogeny and role of Chloroflexota in the AS system.

Here, we present a comprehensive overview of the Chloroflexota abundant in WWTPs worldwide, using HQ MAGs and amplicon data from Danish and global WWTPs, in combination with the MiDAS 4 database. In total, we described 4 families, 13 genera and 29 novel species, which appeared to be widely distributed across most continents, and influenced by factors such as climate zone and WWTP process design. The design of specific genus-level FISH probes enabled investigation of their morphology, abundance, and spatial arrangement. Most of the novel Chloroflexota presented a typical filamentous morphology and demonstrated the presence of glycogen reserves, detected by FISH-Raman. Moreover, the annotation of 53 HQ MAGs, recently retrieved from Danish WWTPs (21), provided further insights into Chloroflexota functional potential and their involvement in nutrient cycling. This study represents a first fundamental milestone in the understanding of the ecological role of these microorganisms in the activated sludge microbial community.

Materials and Methods

Sampling and fixation

Sampling of AS was carried out within the Danish MiDAS survey (25) and the global MiDAS project (18). In short, fresh biomass samples from full-scale AS WWTPs were collected and either sent to Aalborg University (Danish MiDAS) or preserved in RNAlater and shipped to Aalborg University with cooling elements (Global MiDAS). Upon arrival, samples were stored at –20°C for sequencing workflows and fixed for FISH with 50% ethanol (final volume) or 4% PFA (final volume), as previously described (26).

Community profiling using 16S rRNA gene amplicon sequencing

DNA extraction, sample preparation, and amplicon sequencing were performed as previously described (18, 25). Briefly, DNA was extracted using a custom plate-based extraction protocol based on the FastDNA spin kit for soil (MP Biomedicals). The protocol is available at www.midasfieldguide.org (aau_wwtp_dna_v.8.0). For Global MiDAS samples, RNA later was removed by centrifugation and resuspension of the sample in 320 μ L PBS. For Danish MiDAS samples, 160 μ L of sample was mixed with 160 μ L of PBS. All samples were transferred to Lysing Matrix E barcoded tubes and bead beating was performed in a FastPrep-96 bead beater (MP Biomedicals) (3 \times 120 s, 1800 rpm, 2 min incubation on ice between beating). Community profiling was performed using 16S rRNA amplicon sequencing. V1-V3 16S rRNA gene regions were amplified using the 27F (AGAGTTTGATCCTGGCTCAG) (27) and 534R (ATTACCGCGGCTGCTGG) (28) primers, and the resulting amplicons were used in all the analyses. The V4 16S rRNA gene region was amplified using the 515F (50-GTGYCAGCMGCCGCGGTAA-30) (29) and 806R (50-GACTACNVGGGTWTCTAAT-30) (30) primers for comparison with the previous dataset. Data was analyzed using R (version 3.5.2) (31), RStudio software (32) and visualized using ampvis2 (version 2.7.5) (33) and ggplot2 (34). The Köppen-Geiger climate zone classification (35) was utilized to categorize the countries participating in the global MiDAS project (18). Details about the climate zones classification and the countries belonging to it can be found in **Table S1**.

Phylogenetic analysis based on the 16S rRNA gene, FISH probe design and evaluation

Phylogenetic analysis of 16S rRNA gene sequences and design of FISH probes for the novel Chloroflexota were performed using the ARB software v.6.0.6 (36). A phylogenetic tree was calculated based on comparative analysis of aligned 16S rRNA gene sequences, retrieved from the MiDAS 4 database (18), using the maximum likelihood method and a 1000 – replicates bootstrap analysis. Coverage and specificity were evaluated and validated in silico with the MathFISH web tool for hybridization efficiencies of target and potentially weak non-target matches (37). When needed, unlabelled competitors and helper probes were designed. All probes were purchased from Biomers (Ulm, Germany), labelled with 6-carboxyfluorescein (6-Fam), indocarbocyanine (Cy3), or indodicarbocyanine (Cy5) fluorochromes.

Fluorescence in situ hybridization (FISH), quantitative FISH (qFISH) and Raman microspectroscopy

FISH was performed as described by Daims et al. (2005) (38). Optimal formamide concentration for each novel FISH probe was determined after performing hybridization at different formamide concentrations in the range 0-70% (with 5% increments). The intensity of at least 50 cells was measured using ImageJ (39) software. Optimal hybridization conditions are described in **Table S2**. EUBmix (40, 41) was used to target all bacteria and NON-EUB (42) was used as a negative control for sequence independent probe binding. Quantitative FISH (qFISH) biovolume fractions of individual genera were calculated as a percentage area of the total biovolume, hybridizing with both EUBmix probes and specific probe. In case of specific probe not overlapping with EUBmix, a mix of EUBmix and CFXmix (43, 44), both labelled in Cy5, were used as a universal probe for total biomass coverage. qFISH analyses, performed using the Daimi image analysis software (45), were based on 30 fields of view taken at 630 \times magnification. Microscopic analysis was performed with Axioskop epifluorescence microscope (Carl Zeiss, Germany) equipped with LEICA DFC7000 T CCD camera or a white light laser confocal microscope (Leica TCS SP8 X). Raman microspectroscopy was applied in combination with FISH to look for the storage polymers polyphosphate (poly-P), glycogen, and polyhydroxyalkanoates (PHA) as previously described (19).

Genome phylogeny, annotation and metabolic reconstruction

A set of 1083 MAGs (NCBI BioProject PRJNA629478, Singleton et al., 2021), meeting the MIMAG HQ draft standards of full-length rRNA genes, completeness >90% and contamination <5% (46), was searched for Chloroflexota members using GTDB-Tk v2.1.0 (RefSeq release 207) ‘de_novo_wf’ pipeline (47). Species representatives, based on 95% average nucleotide identity clustering of the MAGs, and completeness and contamination estimates were determined from Singleton et al., (2021) (**SData 1**). A total of 53 Chloroflexota MAGs were identified. The phylogenetic maximum likelihood tree was created using the concatenated,

trimmed alignment of the 120 single copy gene proteins from the GTDB-Tk de novo workflow, which included our MAGs and representative RefSeq genomes, as well as *Candidatus* (*Ca.*) *Amarolinea aalborgensis*. Three Cyanobacterota genomes (NCBI accession numbers: GCA_000317655, GCA_002813895, GCA_003566215) were used as an outgroup to root the tree. The ~5000 amino acid alignment was used as input for IQ-TREE v2.1.2 (48), which was run using the WAG+G model and 1000x bootstrap iterations using UFBoot ultrafast bootstrap approximation. The tree was visualized in ARB v6.0.3 (36) to set the root using the outgroup Cyanobacterota, and exported for visualization and final aesthetic adjustments in iTOL v6.1.1 (49) and Inkscape v0.92. Pyani v0.2.11 (50) was used to determine average nucleotide identity (ANI).

Genomes were annotated as previously described (51). Briefly, the EnrichM v5.0 ‘annotate’ pipeline (github.com/geronimp/enrichM) was used to annotate the protein sequences of the genomes against the EnrichM v10 database, which included the KEGG (52) orthology (KO) number annotated Uniref100 database. Enrichm ‘classify’ --cutoff 1 was used to determine the presence of 100% complete KEGG modules, such as for transporters and glycolysis (SData2 and 3). Additionally, the MAGs were uploaded to the ‘MicroScope Microbial Genome Annotation & Analysis Platform’ (MAGE) (53) for manual inspection and cross-validation of KO annotations found using EnrichM.

Results and discussion

Phylogenetic evaluation of the Chloroflexota members abundant in WWTPs

The phylogenetic diversity of novel and well-known Chloroflexota abundant in global WWTPs was evaluated using a comparison of genome-based and 16S rRNA gene-based phylogenies to obtain a robust taxonomic assignment and to resolve potential discrepancies between 16S rRNA gene- and genome-based classification methods. The phylogenomic analysis (**Figure 1, Additional File 1**) revealed clustering into different novel families and genera, largely supported by 16S rRNA gene-based classification using the MiDAS 4 reference database (**Figure 2, Additional File 1**).

Of the 53 MAGs analyzed, several belonged to well-known families, such as *Ca.* Promineofilaceae (11 MAGs), *Ca.* Amarolineaceae (4 MAGs), *Caldilineaceae* (2 MAGs), *Roseiflexaceae* (2 MAGs), and *Tepidiformaceae* (10 MAGs), while the remaining represented the proposed new *Candidatus* families Epilineaceae (3 MAGs), Brachytrichaceae (1 MAG), Villigracilaceae (15 MAGs) and Flexifilaceae (5 MAGs). In many cases, the MAGs represented novel genera, as with *Ca.* Epilinea brevis (2 MAGs), *Ca.* Avedoeria danica (1 MAG), *Ca.* Brachytrich odensis (1 MAG), *Ca.* Defluviolinea gracilis (1 MAG) and proxima (1 MAG), *Ca.* Hadersleviella danica (1 MAG), *Ca.* Trichofilum aggregatum (1 MAG), *Ca.* Leptofilum proximum (1 MAG) and gracile (1 MAG), *Ca.* Leptovillus gracilis (1 MAG) and affinis (5 MAGs), *Ca.* Flexicrinis affinis (1 MAG) and proximus (1 MAG), *Ca.* Flexifilum breve (1 MAG) and affine (1 MAG), *Ca.* Fredericiella danica (1 MAG), *Ca.* Ribeiiella danica (1 MAG), *Ca.* Amarobacter glycogenicus (9 MAGs) and *Ca.* Amarobacillus elongatus (1 MAG). A few MAGs clustered together with genomes from known genera and represented new species, such as *Ca.* Promineofilum glycogenicum (1 MAG), *Ca.* Amarolinea dominans (4 MAGs), *Ca.* Caldilinea saccharophila (1 MAG, former *Ca.* Amarithrix (2)) and *Ca.* Kouleothrix ribensis (1 MAG). Interestingly, 13 MAGs corresponded to the well-known genus *Ca.* Villigracilis by comparison with the original 16S rRNA gene sequence used to define the genus (1), including the novel species *Ca.* Villigracilis vicinus (3 MAGs), adiacens (1 MAG), propinquus (1 MAG), affinis (3 MAGs), proximus (3 MAGs) and saccharophilus (2 MAGs). An in-depth analysis of the recently published *Ca.* Villigracilis nielsenii MAG (54) revealed its clustering within the *Ca.* Villigracilaceae family but outside of the *Ca.* Villigracilis genus (**Additional File 1, S Figure 3**), and we therefore propose to rename it *Candidatus* Manresella nielsenii, from the origin of the sludge (**Additional File 2**). A detailed summary of the phylogeny and supporting information for the phylogenetic analysis can be found in **Additional File 1**.

Despite absence of genomic information, 16S rRNA gene-based phylogeny (**Figure 2**), FISH probe design, and experimental analysis (see below) were possible for additional genera abundant in both global and Danish activated sludge samples. Therefore, we propose to rename the genera with placeholder names *midas_g_391*, *midas_g_550*, *midas_g_9648* as *Ca. Amarofilum*, *Ca. Pachofilum*, and *Ca. Tricholinea*, respectively. 16S rRNA gene-based analysis using the MiDAS 4 database showed that *midas_g_169* corresponded to *Ca. Defluviifilum*, as defined by Speirs et al., (2) and we suggest to adopt this name in future studies.

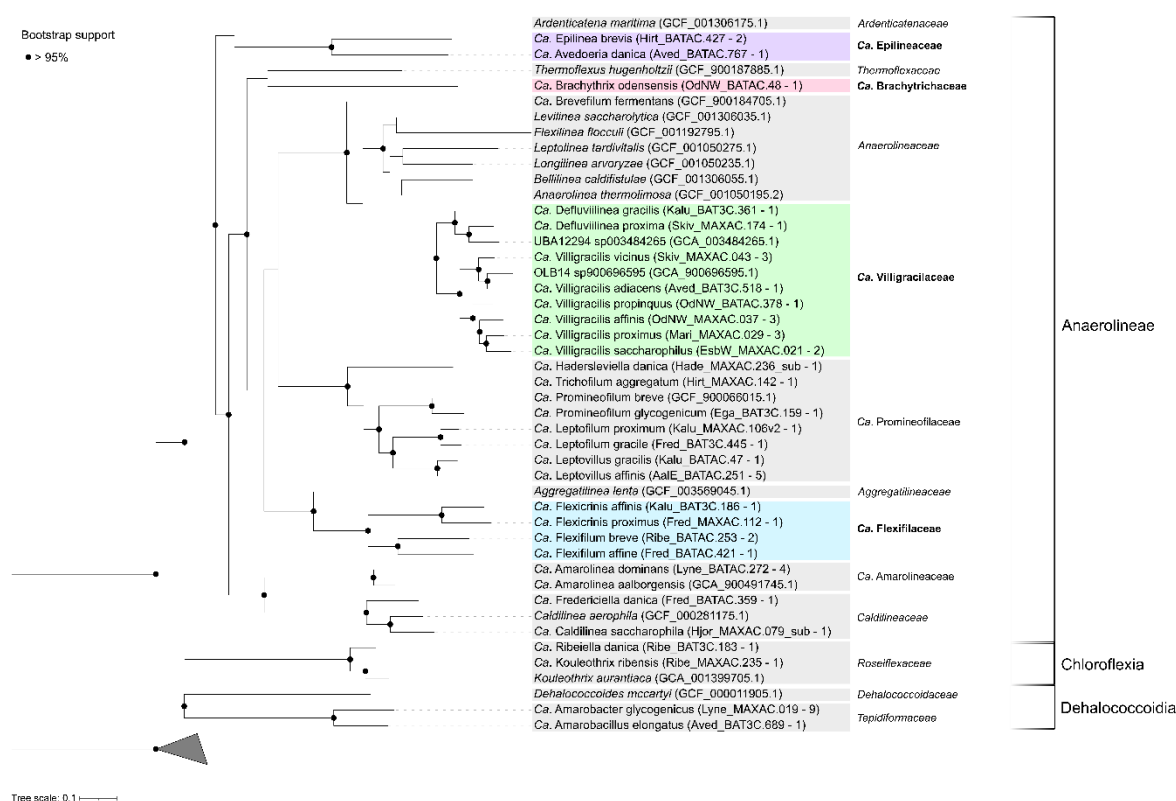


Figure 1. Phylogenetic genome tree of abundant Chloroflexota representatives. The maximum likelihood genome tree was created from the concatenated alignment of 120 single copy marker gene proteins trimmed to 5000 amino acids using the WAG+G model and 1000x UFBoot bootstrap iterations. Bootstrap support > 95% is shown by the solid black circles. Three Cyanobacterota genomes (NCBI accession numbers: GCA_000317655, GCA_002813895, GCA_003566215) were used as an outgroup to root the tree. For NCBI GenBank genome accession numbers see SData File 1. MAGs belonging to novel families are marked with colored boxes, while MAGs clustering with validly published families are marked with grey boxes. MAGs from species representatives are used to construct the tree and indicated between brackets, as well as the number of available MAGs for each lineage. The scale bar represents substitutions per amino acid base.

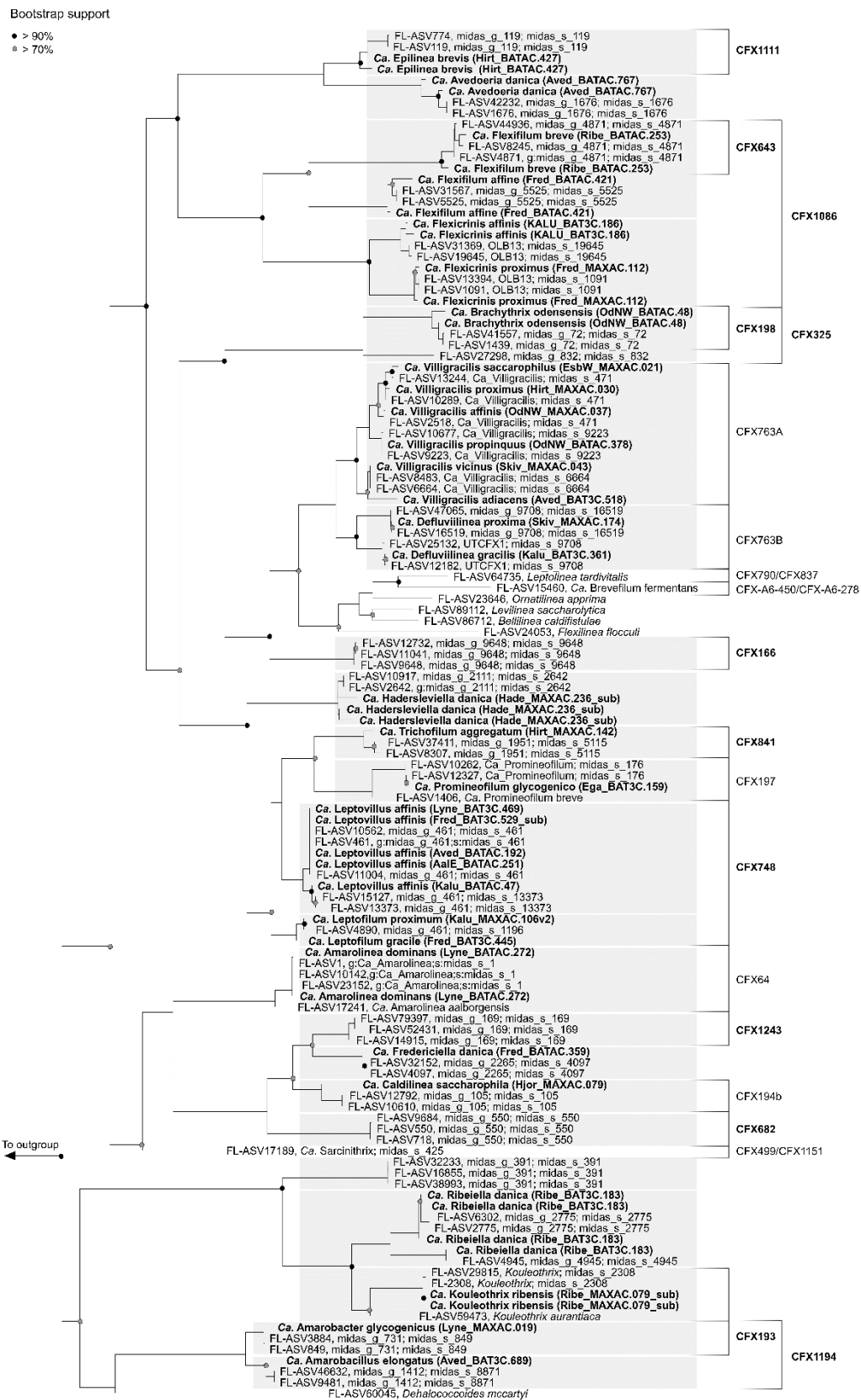


Figure 2. Maximum-likelihood (PhyML) 16S rRNA gene phylogenetic tree of Chloroflexota genera abundant in WWTPs. 16S rRNA gene sequences were retrieved from the global MiDAS 4 database or from the MAGs (bold). 16S rRNA gene sequences belonging to novel species representatives MAGs are indicated in bold blue. Grey boxes are used to indicate the taxonomy of novel species. The alignment used for the tree applied a 20% conservational filter to remove hypervariable positions, giving 1159 aligned positions. Coverage of the existing and designed in this study FISH probes is indicated with black brackets and is based on the MiDAS 4 database (18). Bootstrap values from 1000 re-samplings are indicated for branches with >70% (gray dot) and >90% (black) support. Species of the phylum Cyanobacteria were used as the outgroup. The scale bar represents substitutions per nucleotide base.

Geographical distribution of Chloroflexota in global full-scale WWTPs

We analyzed the occurrence and diversity of both novel and well-known Chloroflexota genera abundant in global AS ecosystems, using data from the global MiDAS survey (18). On a global scale, the phylum Chloroflexota was the fourth most abundant phylum, making up 6.3% of the total reads (**Figure 3a**), similar to previous observations in Denmark (1), Spain (55), and Australia (56). Among the most abundant genera worldwide (**Figure 3b**), several well-known microorganisms appeared to be widespread, such as *Ca. Villigracilis* (1), *Ca. Promineofilum* (11), *Ca. Sarcinithrix* (1), *Ca. Amarolinea* (12), and *Kouleothrix* (57). All other abundant Chloroflexota were mainly undescribed, but potentially important for the process, such as the genus *Ca. Defluviilinea* (former UTCFX1), observed as part of the heterotrophic bacteria in anammox bioreactors (58, 59), or *Ca. Flexifilum*, belonging to the former family A4b, first identified in nitrifying-denitrifying industrial WWTPs (60). Interestingly, few novel genera were only found locally in individual countries, such as the genus *midas_g_75442*, present only in Malaysia (**Figure 3b**).

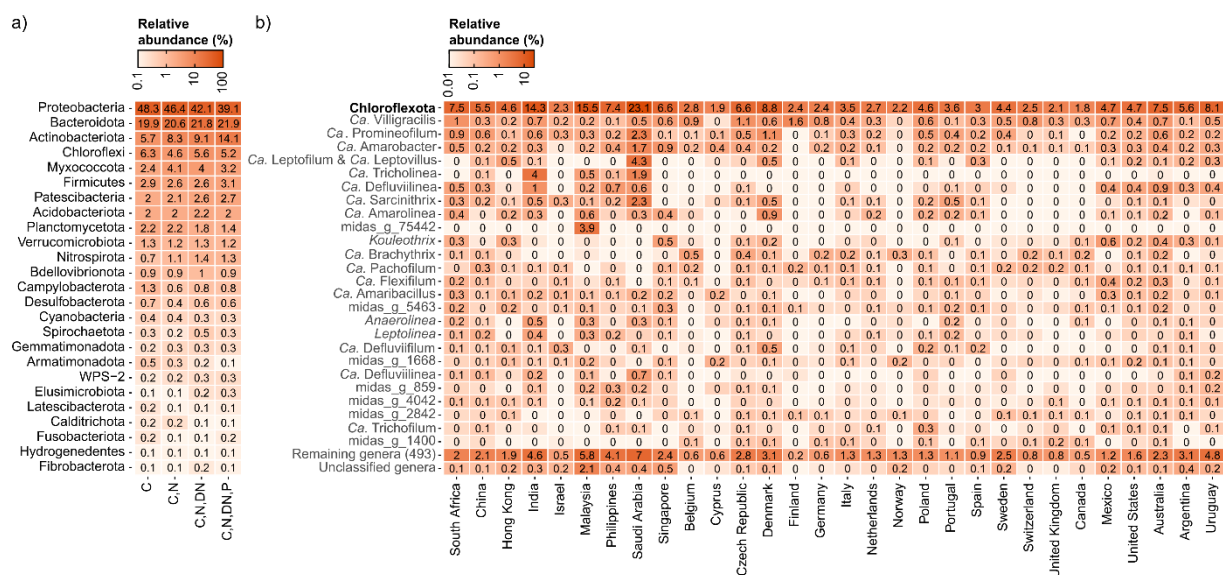


Figure 3. Global average relative read abundance of a) abundant phyla in WWTPs with different process designs and b) abundant Chloroflexota genera in different countries. Results are based on 929 activated sludge samples from 740 WWTPs. C, carbon removal; N, nitrification; DN, denitrification; P, biological P removal.

Ca. Defluviifilum and *Ca. Caldilinea saccharophila* (former *Ca. Amarithrix*), together with the novel *Ca. Amarofilum* and *Ca. Pachofilum*, were commonly found in high abundance in Danish WWTPs, ranging from 0.1 to 1.1% but reaching up to 8% in some samples (**Figure S1**).

Examining global WWTP community composition enabled a deeper insight into factors affecting the occurrence of the different genera. We expected that the previously demonstrated slow-growth and facultative anaerobic metabolism of Chloroflexota species would favor their prevalence in long-sludge age WWTPs and WWTPs with biological N and P removal (2). *Ca. Villigracilis*, *Ca. Promineofilum*, and *Ca. Amarobacter* occurred in higher abundance in WWTPs with a complex process design involving both N and P removal, indicating a potential role in these processes (**Figure 4a**). The same genera also appeared to be influenced by the fraction of industrial wastewater (shown as the COD fraction in the influent), preferring low to medium content (<50%) of industrial wastewater (**Figure 4b**). This observation confirms previous findings, where filamentous Chloroflexota were detected in low abundance by FISH in industrial sludge (61). The differences in the Chloroflexota communities were more accentuated when considering the different climate zones (**Figure 4c**), with highest abundances of all genera observed in dry and temperate climates. Interestingly, some genera seemed to be specific to areas with hot temperatures, such as *Ca. Defluviilinea* and *Ca. Tricholinea*, dominant in the dry and arid climates, or *Ca. Brachythrix*, which seemed predominant in countries with polar climate (**Figure 4c-d**).

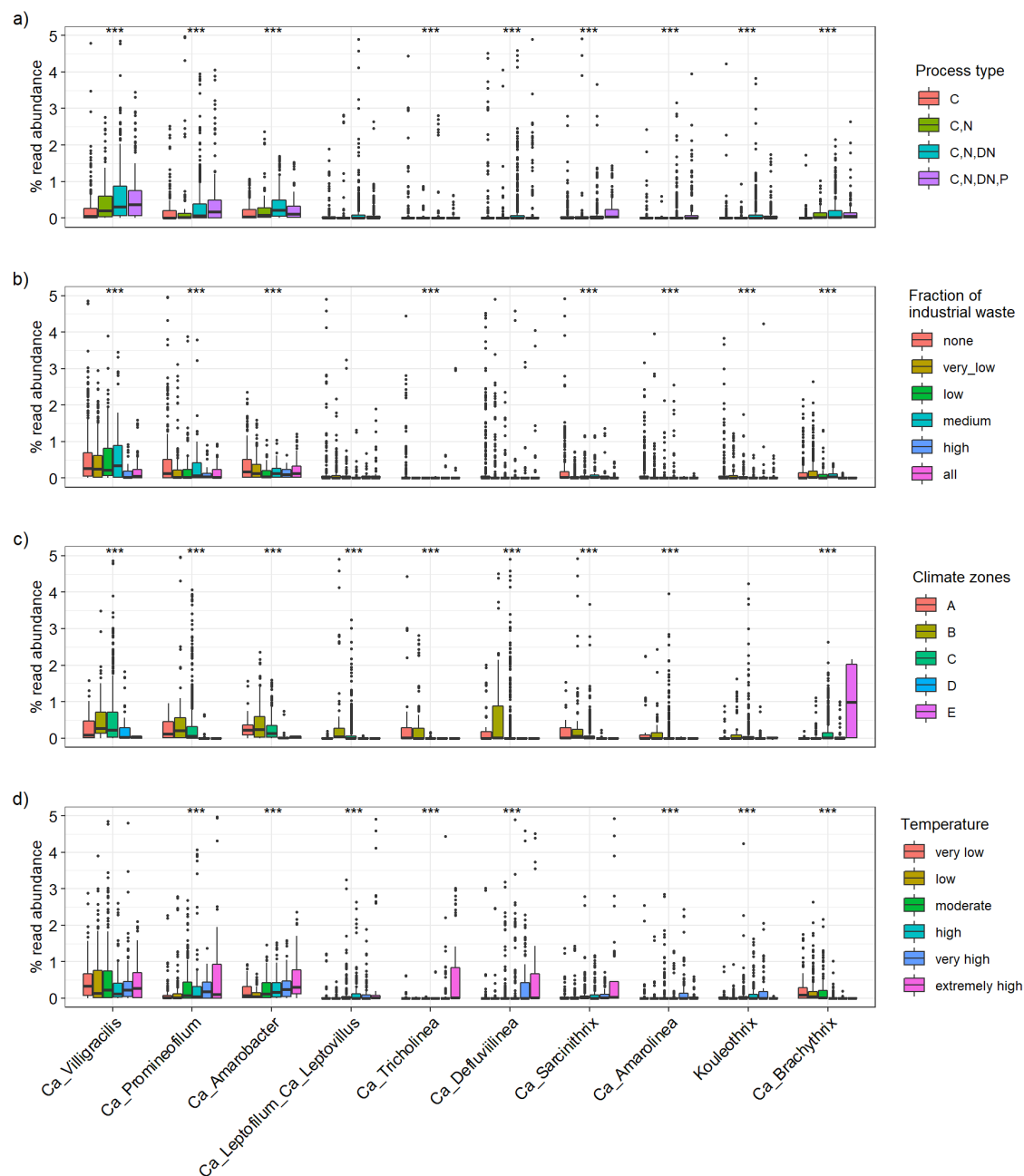


Figure 4. Distribution of selected abundant Chloroflexota genera across the world based on A) process type (C, 113 plants; C, N, 48 plants; C, N, DN, 208 plants, C, N, DN, P, 111 plants), (C, carbon removal; N, nitrification; DN, denitrification; P, biological P removal); B) fractions of industrial wastewater (0% - 169 plants; 0-10% - 105 plants; 11-30% - 67 plants; 31-50% - 41 plants; 51-99% - 20 plants; 100% - 40 plants); C) climate zones (A: tropical/megathermal climates, 29 plants; B: dry (desert and semi-arid) climates, 48 plants; C: temperate/mesothermal climates, 368 plants; D: continental/microthermal climates, 24 plants; E: polar climates, 2 plants), and D) different temperature ranges analyzed in process tanks (1-10.0°C, 43 plants; 10.1-15.0°C, 96 plants; 15.1-20.0°C, 112 plants; 20.1-25°C, 73 plants; 25.1-30.0°C, 48 plants; 30.1-38.0°C, 32 plants). Detailed information about the different climate zones and the countries belonging to each of them is in Table S1. Significant differences within individual groups are indicated by *** (Kruskal-Wallis test, $p < 0.001$). For visualization purposes, samples with abundances higher than 5% are not shown in this figure.

In situ characterization of Chloroflexota abundant in Danish and global WWTPs

We designed new FISH probes to target and characterize the abundant novel Chloroflexota genera *in situ* and re-evaluated the coverage and specificity of existing FISH probes (**Figure 2, Table 1**). *In silico* evaluation of the widely-applied CFXmix (43, 44) using the MiDAS 4 database showed good coverage of the phylum in the AS ecosystem, and it is recommended to be used in combination with EUB mix (40, 41) for better coverage of the Chloroflexota (**Table 1**). All but two of the genera investigated, *Ca. Epilinea* and *Ca. Promineofilum*, did hybridize with the EUB mix. *In silico* evaluation of previously published genus-level FISH targeting *Ca. Amarolinea*, *Ca. Sarcinithrix*, *Ca. Promineofilum*, and *Ca. Villigracilis* probes showed high specificity and hybridized with filaments of variable length and thickness (1, 3, 11, 61, 62) (**Figure 2, Table 1**). The majority of the novel Chloroflexota appeared to have the same conventional morphology (**Figure 5**), with filaments of different length and thickness often found in bundles inside the flocs, or sometimes creating inter-floc bridges (**Figure 5; Table 1**). Epiphytic bacteria were found on protruding filaments belonging to *Ca. Flexifilum* and other bacteria of the *Flexifilaceae* family, *Ca. Trichofilum*, *Ca. Defluviifilum*, *Ca. Amarofilum* and *Ca. Pachofilum* (**Figure 5**). Interestingly, the short filaments of *Ca. Epilinea* were found sometimes to be themselves attached to other Chloroflexota filaments (**Figure 5**). The surface adhesion mechanism of these microorganisms is still unclear, but the presence of pili has been previously reported for several isolates (63–65) and these appendages could mediate the adhesion process (66). The two genera from the order Dehalococcoidia, *Ca. Amarobacter* and *Ca. Amarobacillus*, were small rods.

The FISH probes designed for the novel Chloroflexota genera were applied to Danish and, when possible, global activated sludge samples for FISH-based quantification (**Table S3**). Amplicon sequencing relative read abundances were in most cases very similar, while a few genera (*Ca. Defluviifilum* and *Ca. Leptofilum*) had slightly lower and some slightly higher (*Ca. Amaribacillus*, *Ca. Amaribacter* and *Ca. Epilinea*) read abundances compared to qFISH results. These differences are likely due to variations in cell size, extraction efficiency, and 16S rRNA gene copy number variation (67). The 16S rRNA gene copy numbers in the MAGs analyzed in this study varied from 1 to 5 (**SData 1**), which could lead to overestimation of some genera when using amplicon-based quantification (67). Primers are also known to introduce bias in amplicon sequencing (67). Therefore, the composition of the Chloroflexota community was compared using two commonly applied primer sets (the V1-V3 and V4 regions of the 16S rRNA gene sequences) (**Figure S2**). The overall relative abundance of the phylum Chloroflexota was comparable, but significant differences appeared in the relative abundances of specific genera (**Figure S2**). The V4-dataset showed lower relative abundances of some genera such as *Ca. Promineofilum* and *Ca. Leptofilum*, and almost complete disappearance of *Ca. Villigracilis* and *Ca. Defluviilinea*, which were likely not targeted by the V4 primer-set. These findings and the similarity of the abundances calculated by qFISH and V1-V3 amplicon sequencing confirmed that the V1-V3 primer set is more suited to encompass the diversity of the phylum Chloroflexota in AS systems. This is of particular importance if the potential effect on settling properties of these filamentous bacteria is evaluated using amplicon sequencing data.

To investigate the *in situ* physiology of the novel Chloroflexota genera, we performed Raman microspectroscopy in combination with the new FISH probes. This approach allows the detection of general cellular components, such as nucleic acids, membrane lipids, or proteins (68), as well as storage polymers important for the physiology of microorganisms involved in nitrogen or phosphorus removal in activated sludge (19, 51). Most of the Chloroflexota genera showed the presence of peaks characterizing common biological components, such as phenylalanine, nucleic acids, lipids, as well as a peak for glycogen, which most likely serves as a storage compound to survive periods with low energy sources (**Figure S3**). The presence of glycogen, which appears to be a conserved feature of the phylum, confirms the potential for glycogen accumulation proposed by our metabolic predictions (see below) and studies of other known Chloroflexota (11, 12, 63). Although granules of PHAs and poly-P have been identified previously in Chloroflexota isolates (63), these storage polymers were not detected *in situ*.

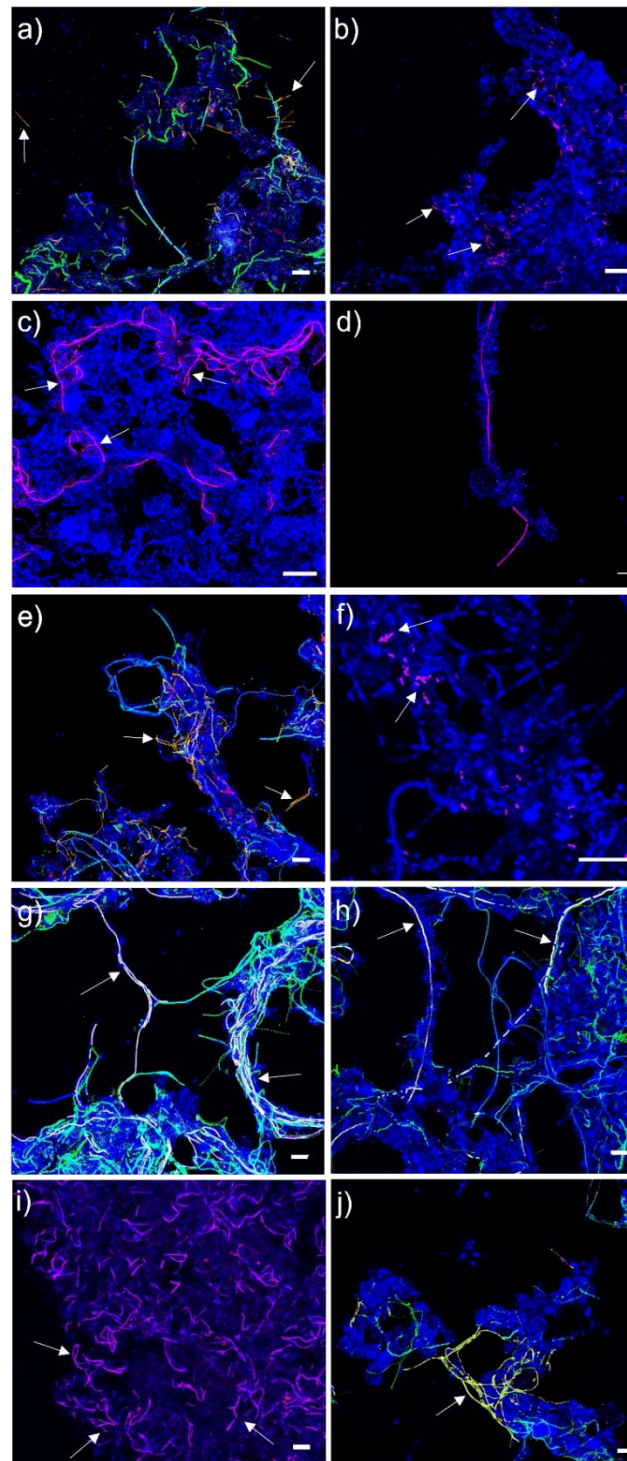


Figure 5. FISH micrographs of novel Chloroflexota genera in full-scale activated sludge. All bacteria were targeted with EUBmix (blue), in some micrographs CFXmix (green) was also applied to target most bacteria belonging to the Chloroflexota phylum. Specific probe targets: a) *Ca. Epilinea* (yellow); b) *Ca. Brachythrix* (magenta); c) *Ca. Trichofilum* (magenta); d) *Ca. Flexifilum* (magenta); e) - *Ca. Leptofilum* and *Ca. Leptovillus* (yellow); f) *Ca. Amarobacter* (magenta); g) *Ca. Amarofilum* (white); h) *Ca. Pachofilum* (white); i) *Ca. Tricholinea* (magenta); j) *Ca. Defluviifilum* (yellow). Scale bar is 20 μm .

Metabolic potential of the abundant *Chloroflexota* genera

Functional analysis of the 53 *Chloroflexota* MAGs revealed similar metabolisms to previously published models (11, 12). They exhibited a very versatile metabolism, revealing heterotrophic lifestyles with possible involvement in degradation of complex organic compounds and utilization of a wide selection of different sugars and amino acids as carbon sources (**Figure 6, SData 2 and 3**). Manual inspection of the MAGs confirmed that all the MAGs encoded pathways for full central carbon processing, through glycolysis, pentose phosphate pathway, and TCA cycle (**Figure 6, SData 2 and 3**).

Aerobic uptake of different substrates is a shared trait exhibited by members of this phylum (1, 61). This widespread trait was confirmed in our MAGs, as ABC transporters for glucose/mannose (*gts*), fructose (*frc*), ribose (*rbs*), xylose (*xyl*), and glycerol 3-phosphate (*ugp* and *malk*) were predicted across all the MAGs (**SData 2 and 3**). Additionally, genes encoding a putative simple sugar ABC transporter and a multiple sugar transport system (*ggu*) were widely distributed across the MAGs (**SData 2 and 3**). Genes encoding for degradation of different sugars, such as galactose (*gal*), lactose (*lacZ*), sucrose (*malZ*), fructose (*scrK* or *fruK*) or xylose (*xyl*) were also present across all MAGs (**Figure 6, SData 2 and 3**). Additionally, the use of lactate as a carbon source in some of the MAGs was indicated by the presence of genes encoding for lactate permease (*lctP*) and lactate utilization (*lldG*, *lldF*, *lldE*, *ldhA*, *dld*) (**Figure 6, SData 2 and 3**). Several MAGs (17/53) also indicated the potential degradation of cellulose to glucose, with genes encoding endoglucanase and beta-glucosidase (*blgX* and/or *blgB*) (**Figure 6, SData 2 and 3**).

The presence of genes encoding for the transport of peptides (*dpp*), oligopeptides (*opp*), branched-chain amino acids (*liv*), and a putative polar amino acids transport system indicated that amino acids could be another important energy source (**SData 2 and 3**). Nearly all the MAGs encoded the alanine dehydrogenase (*ald*) for the oxidation of alanine to pyruvate under anoxic conditions. Furthermore, genes encoding the degradation of different amino acids were widespread in all the MAGs and included glutamate dehydrogenase (*gdhA*), arginase (*arg*), tryptophan (*tnaA*), asparagine (*ansA*), aromatic amino acids (*paaABCDE* and *paaK*), histidine (*hutHUI*, *ficD*, and *fold*), and L-serine (*sdaA*). However, previous experimental studies did not show amino acid uptake under oxic or anoxic conditions in *Ca. Villigracilis*, *Ca. Amarolinea*, or *Ca. Promineofilum* (1, 11, 61), therefore further experimental validation is needed.

The acetate transporter gene (*actP*) was encoded only in two MAGs, belonging to the genera *Kouleothrix* and *Ca. Brachythrix*. The uptake of acetate and short-chain fatty acids has also not been experimentally confirmed in some of the abundant genera (1, 61, 69). Nonetheless, the presence of the gene *acs* was widespread in the MAGs, suggesting the potential use of acetate or fatty acids as carbon sources by these organisms, maybe deriving from internal pools (**Figure 6, SData 2 and 3**). The glyoxylate cycle was complete in part of the genomes (15/53), but only one MAG, belonging to *Ca. Brachythrix*, encoded the full potential for aerobic pyruvate oxidation to acetyl-CoA (*aceE* and *aceF*) (**Figure 6, SData 2 and 3**). Additionally, genes encoding for biosynthesis (*fadD*) or beta-oxidation of fatty acids (*acd*, *fadJ*, and *fadA*) were also widespread in the genera and identified in nearly all MAGs (**Figure 6, SData 2 and 3**). Interestingly, oxidation of long-chain fatty acids appeared to be one of the preferred energy sources for the bacteria belonging to the two non-filamentous genera in the order Dehalococcoidia, *Ca. Amarobacter* and *Ca. Amarobacillus*. They had a high copy number of genes involved in this pathway (*fadD*, *acd*, *paaF*, *fadJ*, *fadA*), comparable to those found in the well-known lipid user in AS systems *Ca. Microthrix* (70), indicating their potential specialization as lipid consumers (**Figure 6, SData 2 and 3**). Acetate use as carbon source is common in the Dehalococcoidia isolates (71, 72) and beta-oxidation has also been proposed as a potential metabolic route to obtain carbon and reducing equivalents by Dehalococcoidia found in marine sediments (73). Further analysis using transcriptomics and/or proteomics, could help to clarify the activity and expression level of these enzymes.

Anaerobic sugar uptake has previously been demonstrated *in situ* in several *Chloroflexota* genera and the potential for fermentation of substrates to acetoin or lactate was indicated in the genomes of *Ca. Amarolinea*

and *Ca. Promineofilum* (1, 11, 12, 61). The potential for fermentation was encoded by most of the MAGs (*ALDH*, *adh* and *adhE*), with ethanol as a possible by-product (**Figure 6, SData 2 and 3**). Part of the MAGs (15/53) encoded genes for formate dehydrogenase (*fdh* and/or *fdoGHI*), potentially used to reduce formate produced during anaerobic fermentation, as suggested for *Ca. Promineofilum breve* (11).

Alternative electron acceptors under anaerobic conditions included nitrate, with potential dissimilatory nitrate reduction to nitrite (*narGHI*) identified in the four MAGs associated with the genus *Ca. Amarolinea*, as previously reported for *Ca. Amarolinea aalborgensis* (12) and in the two MAGs representing the new genus *Ca. Defluviilinea* (**Figure 6, SData 2 and 3**). Genes for nitrite reduction to nitric oxide (*nirK*) were present in 10/53 MAGs, while no MAGs encoded potential for nitric oxide reduction to nitrous oxide (*norBC*). 25 MAGs encoded genes (*nosZ*) for potential reduction of the latter to gaseous nitrogen (**Figure 6, SData 2 and 3**). The potential for dissimilatory nitrite reduction to ammonia (*nrfAH*) was widespread across the MAGs, similar to previous findings for *Ca. Amarolinea aalborgensis* (12) or *Ca. Promineofilum breve* (11), while no potential for nitrification was detected (**Figure 6, SData 2 and 3**). The potential for sulfate reduction to H₂S through the assimilatory pathway (*sat*, *cysH* and *sir*) was also widespread across the MAGs (**Figure 6, SData 2 and 3**). Some Chloroflexota bacteria are described to be involved in the biogeochemical sulfur cycling (74–76).

Many bacteria that live under alternating oxic-anoxic conditions produce storage compounds, such as poly-P, glycogen, and PHA, which can be used in dynamic systems when environmental carbon or energy reserves are scarce. Examples are polyphosphate-accumulating organisms, involved in biological P removal in activated sludge (77). Genes indicating the potential for polyphosphate accumulation, such as the phosphate transporters (*pit*, *pstSCAB*) and the polyphosphate kinase (*ppk*) were widespread across the MAGs (**Figure 6, SData 2 and 3**). However, this storage compound was not detected in any Chloroflexota genus *in situ*. Only MAGs belonging to *Ca. Epilinea* and *Ca. Avedoeria* encoded the full potential for PHA accumulation (*phaABC*) (**Figure 6, SData 2 and 3**), but this intracellular polymer was not detected experimentally. Previous genome studies indicated the presence of glycogen as a storage compound in *Ca. Amarolinea aalborgensis* and *Ca. Promineofilum breve* (11, 12). The potential for glycogen biosynthesis was confirmed by the identification of the genes involved in the pathway (*glgABC*, *galU*, *glgP*, and *glgY*), as well as for its degradation (*malQ*) (**Figure 6, SData 2 and 3**), and experimentally validated in most of the genera.

The potential for carbon fixation through the enzyme RuBisCO and Calvin-Benson-Bassham cycle was also present in several MAGs (9/53), as previously observed for *Kouleothrix* and other Chloroflexota genera (2) (**SData 2 and 3**). Interestingly, the potential for bacteriochlorophyll biosynthesis and photorespiration were encoded by *Ca. Ribeiiella danica* and *Ca. Kouleothrix ribensis*, similar to the isolates of the class Chloroflexia *Roseiflexus castenholzii* and *Chloroflexus aurantiacus* (**SData 2 and 3**) (23). As for the latter, *Ca. Ribeiiella* and *Kouleothrix* present a fused form of the genes encoding for type II photosystem reaction centers (*pufLM*), recognized by manual inspection of the genomes (23). However, it is unclear if these pathways have a role in the activated sludge environment, where light is not easily accessible and the Calvin–Benson–Bassham pathway would be an energetically expensive alternative to the available organic carbon. Further analysis of the activity and expression level of these enzymes could help to clarify their role *in situ*.

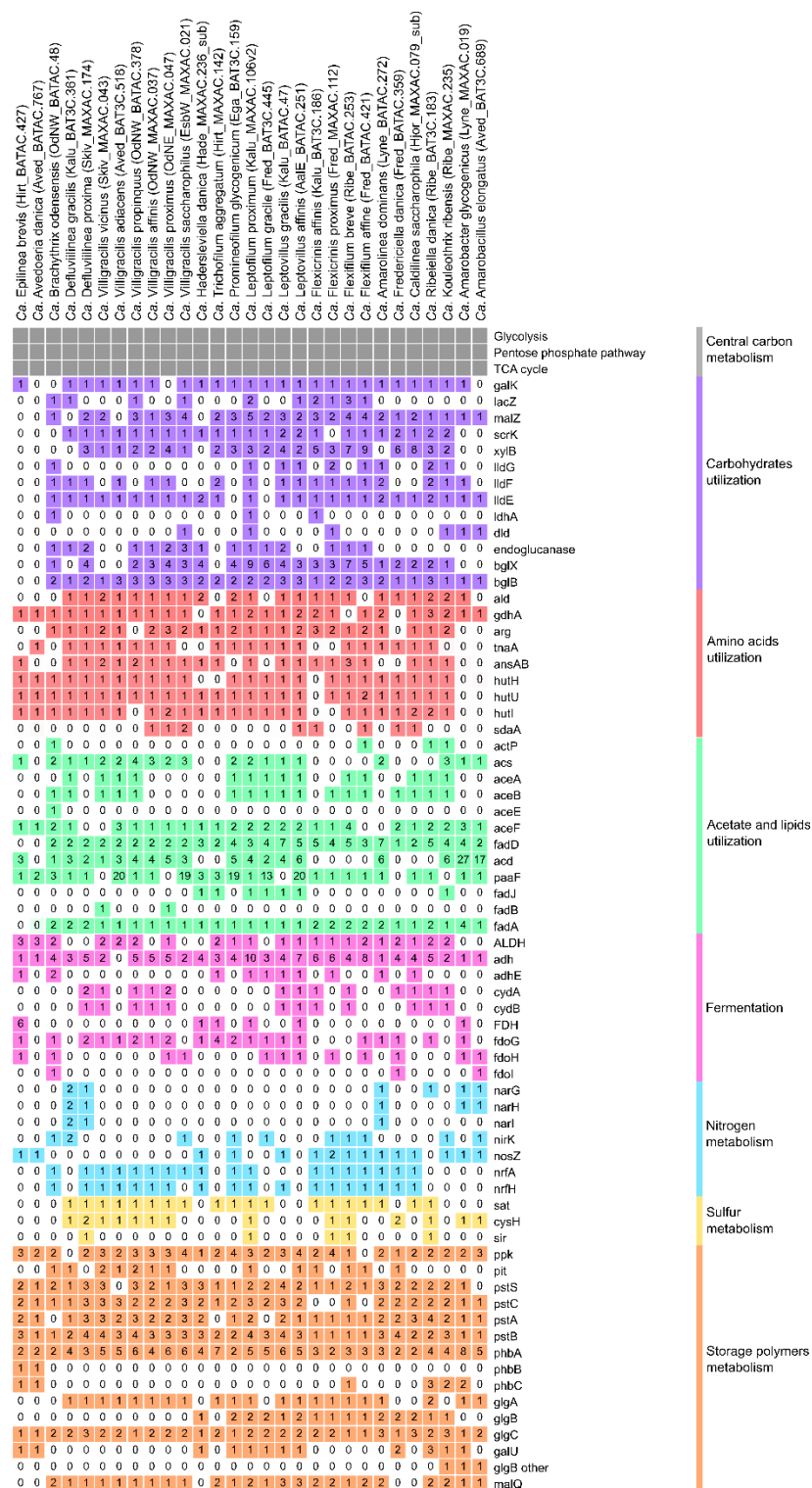


Figure 6. Basic functional potential of the Chloroflexota MAGs. For the full list of gene names and associated KO numbers see Data S2-3. The MAGs and genomes are ordered as in the genome tree in Figure 1. For simplicity, only MAGs from species representatives are shown. Numbers indicate gene copy number.

Table 1. Summary table of morphology and ecophysiology of known Chloroflexota genera.

Genus	Morphology (Length × width (μm))	Eikelboom type (14, 16)	FISH probe	Metabolic potential				Reference
				Physiology	Carbon sources	Electron Acceptors	Intracellular storage polymers*	
<i>Ca. Epilinea</i>	Filamentous (4-57 × 0.4-0.7)	Unknown	CFX1111, CFXmix	Heterotroph, Facultative anaerobe	Amino acids	O ₂ ; N ₂ O	-	This study
<i>Ca. Avedoeria</i>	NA	Unknown	NA	Heterotroph, Facultative anaerobe	Amino acids	O ₂ ; N ₂ O	NA	This study
<i>Ca. Brachythrix</i>	Filamentous (5-18 × 0.5-0.9)	Unknown	CFX198, CFXmix, EUBmix	Heterotroph, Facultative anaerobe	Carbohydrates, amino acids, fatty acids	O ₂ ; NO ₂ ⁻	Glycogen	This study
<i>Ca. Villigracilis</i>	Filamentous (12-50 × 0.3-0.4)	0803	CFX763A, CFXmix, EUBmix	Heterotroph, * Facultative anaerobe*	Carbohydrates*, amino acids*, fatty acids	O ₂ *	Glycogen	(1)
<i>Ca. Defluviilinea</i>	Filamentous (5-30 × 0.2-0.4)	0803	CFX763B, CFXmix, EUBmix	Heterotroph *, Facultative anaerobe*	Carbohydrates*, amino acids*, fatty acids	O ₂ *	Glycogen	(1)
<i>Ca. Hadersleviella</i>	Filamentous (30–50)	0803	CFX841, CFXmix, EUBmix	Heterotroph, Facultative anaerobe	Carbohydrates, amino acids, fatty acids	O ₂ ; N ₂ O	NA	(2, 78) This study
<i>Ca. Trichofilum</i>	Filamentous (60- 200 × 0.6-0.8)	0092	CFX841_2, CFXmix, EUBmix	Heterotroph, Facultative anaerobe	Carbohydrates, amino acids, fatty acids	O ₂	Glycogen	This study
<i>Ca. Promineofilum</i>	Filamentous (20-140 × 0.8)	0092	CFX197, CFXmix	Heterotroph *, Facultative anaerobe*	Carbohydrates*, fatty acids, amino acids	O ₂ *; NO ₂ ⁻ ; N ₂ O	Glycogen	(11)
<i>Ca. Leptofilum</i>	Filamentous (10-70 × 0.7-0.9)	Unknown	CFX748, CFXmix, EUBmix	Heterotroph, Facultative anaerobe	Carbohydrates, amino acids, acetate	O ₂	Glycogen	This study
<i>Ca. Leptovillus</i>	Filamentous (10-70 × 0.7-0.9)	Unknown	CFX748, CFXmix, EUBmix	Heterotroph, Facultative anaerobe	Carbohydrates, amino acids, fatty acids, acetate	O ₂ ; N ₂ O	Glycogen	This study

<i>Ca. Flexicrinis</i>	Filamentous (40-110 × 0.7-1.1)	Unknown	CFX1086, CFXmix, EUBmix	Heterotroph, Facultative anaerobe	Carbohydrates, amino acids, fatty acids	O ₂	Glycogen	This study
<i>Ca. Flexifilum</i>	Filamentous (>100 × 0.8-1.1)	Unknown	CFX643, CFXmix, EUBmix	Heterotroph, Facultative anaerobe	Carbohydrates, amino acids, fatty acids	O ₂	Glycogen	This study
<i>Ca. Amarolinea</i>	Filamentous (20-140 × 2.2)	0092	CFX64, CFXmix, EUBmix	Heterotroph*, Facultative anaerobe*	Carbohydrates*, amino acids, fatty acids	O ₂ *; NO ₃ ⁻ *; N ₂ O	Glycogen	(1, 79)
<i>Ca. Fredericiella</i>	NA	Unknown	NA	Heterotroph, Facultative anaerobe	Carbohydrates, amino acids	O ₂ ; N ₂ O	NA	This study
<i>Caldilinea</i>	Filamentous (70-200 × 0.8)	0675	CFX194b, CFXmix, EUBmix	Heterotroph*, Facultative anaerobe*	Carbohydrates, amino acids, fatty acids	O ₂ *; N ₂ O	Glycogen	(2)
<i>Ca. Ribiella</i>	NA	Unknown	NA	Heterotroph, Photothroph, Facultative anaerobe	Carbohydrates, amino acids	O ₂	Glycogen	This study
<i>Kouleothrix</i>	Filamentous (>200 × 0.5-0.7)	1851	CHL1851, CFXmix, EUBmix	Heterotroph, *Photothroph*, Facultative anaerobe*	Carbohydrates*, amino acids, acetate*, fatty acids	O ₂ *; NO ₂ ⁻ ; N ₂ O	NA	(57)
<i>Ca. Amarobacter</i>	Rod-shaped (1-2 × 0.3-0.5)	NA	CFX193, CFXmix, EUBmix	Heterotroph, Facultative anaerobe	Fatty acids, carbohydrates, amino acids	O ₂ ; NO ₃ ⁻	Glycogen	This study
<i>Ca. Amarobacillus</i>	Rod-shaped (1-2 × 0.3-0.5)	NA	CFX1194, CFXmix, EUBmix	Heterotroph, Facultative anaerobe	Fatty acids, carbohydrates, amino acids	O ₂ ; NO ₃ ⁻	Glycogen	This study
<i>Ca. Sarcinithrix</i>	Filamentous (<200 × 0.6-0.8)	0914	CFX499/CFX1151, CFXmix, EUBmix	Heterotroph*, Facultative anaerobe*	Carbohydrates*	O ₂ *; NO ₃ ⁻ *	Glycogen	(1)
<i>Ca. Amarofilum</i>	Filamentous (80-100 × 1-2)	Unknown	CFX122, CFXmix, EUBmix	NA	NA	NA	Glycogen	This study

<i>Ca. Pachofilum</i>	Filamentous ($>150 \times 1.2-1.8$)	Unknown	CFX682, CFXmix, EUBmix	NA	NA	NA	Glycogen	This study
<i>Ca. Tricholinea</i>	Filamentous ($10-60 \times 0.5-0.9$)	Unknown	CFX166, CFXmix, EUBmix	NA	NA	NA	Glycogen	This study
<i>Ca. Defluviifilum</i>	Filamentous ($20-250 \times 0.8-1.3$)	0675	CFX1243, CFXmix, EUBmix	NA	NA	NA	Glycogen	This study
midas_g_1668 (former <i>Ca.</i> <i>Catenibacter</i>)	Filamentous ($40-160 \times 1-1.5$)	0041	CFX86a, CFXmix, EUBmix	NA	NA	NA	NA	(2)
midas_g_344 (former <i>Ca.</i> <i>Catenibacter</i>)	Filamentous ($40-160 \times 1-1.5$)	0041	CFX86b, CFXmix, EUBmix	NA	NA	NA	NA	(2)
<i>Ca. Trichobacter</i> (midas_g_13117)	Filamentous (30–50)	0803	CFX998, CFXmix, EUBmix	NA	NA	NA	NA	(2, 78)

Metabolic annotation was determined by metabolic annotation (no star) or experimentally validated (*); NA= not applicable; - = negative.

Ecological significance of Chloroflexota in activated sludge

This study provides a broad overview of the Chloroflexota abundant in AS WWTPs and contributes to recognizing their role in these systems. The phylum Chloroflexota encompasses a variety of different metabolisms, ranging from haloalkane-reducers (73, 80, 81), to anoxygenic photosynthetic microorganisms (23, 64, 82, 83), sponge holobionts (84–86), and extremophiles (87), all with roles in carbon, sulfur, and nitrogen cycling. It is therefore unsurprising that members of the Chloroflexota are present in the activated sludge ecosystem worldwide, although most lineages remain undescribed.

Our comprehensive approach, which includes the utilization of genome- and 16S rRNA gene-resolved phylogeny, allowed the identification of four novel families, 14 novel genera and 29 species, most of which are widely distributed across the continents and are seemingly influenced by factors such as climate zones, temperature, and WWTP process design. However, the variation in presence and abundance between countries and even between plants in the same country may also be largely influenced by immigration of microbial populations with the influent and seasonality (88, 89).

Generally, filamentous bacteria act as the backbone of activated sludge flocs, to which floc-forming bacteria attach and grow, typically as microcolonies. However, this beneficial role can change if the filaments are proliferating when the environmental conditions promote their growth, resulting in bulking and poor sludge-water separation (90). The Chloroflexota has often been associated with bulking episodes, consequently the morphological characterization of the novel genera is essential (2, 4). Most of the novel genera are, however, characterized by thin and short trichomes, some were short and attached to other filaments, so they did not form filamentous bridging which prevents the flocs from clustering as is known from *Ca. Amarolinea* and a few others, stressing that most are likely good for the floc formation. All filamentous members of Chloroflexota belonged to the orders Anaerolineae and Chloroflexia while the members from Dehalococcoidia, *Ca. Amarobacter* and *Ca. Amarobacillus* were rod shaped, showing that not all AS Chloroflexota are filamentous.

Confirming previous findings, Chloroflexota abundant in AS likely have a heterotrophic and facultative anaerobic lifestyle, which may explain their higher abundance in plants with more complex designs with oxic and anoxic conditions (1, 2, 11, 91). The different genera have versatile metabolisms, but with a seemingly important role in carbon cycling, with the potential ability to use various sugars and amino acids, but in some cases also lipids and acetate. They are assumed to have high hydrolytic activity, and the production of exoenzymes for polysaccharide degradation has been previously shown *in situ* and suggests their importance in degrading the exopolymeric matrix, rather than soluble substrates (91, 92). Fermentation seems to be widespread across all the genera and likely sustains these organisms during anoxic conditions, as shown for some of the related isolates (7, 8, 10, 93). Despite being fermentative and facultative anaerobic microorganisms, Chloroflexota abundant in AS systems generally appear to die-off when the biomass is fed to anaerobic digesters, likely due to the substantial differences in environmental conditions, such as higher temperatures or salinity, while different Chloroflexota filaments seem to be predominant in the anaerobic digester environment (94–96). Their involvement in nitrogen, sulfur, and phosphorus cycling seems to be more limited. However, these organisms still have an important role in nutrient cycling if considered as active members of the bacterial consortia typical of the activated sludge floc, producing essential substrates that can be used by other microorganisms. Our study provides the foundation for future in-depth characterization of their physiology and relationships with other microorganisms. Following this, we believe gene expression and regulation studies are the next important steps forward in understanding the ecology of these process-critical bacteria.

Formal taxonomic proposal

Etymologies and protologues for the novel proposed species are provided in Additional File1.

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