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# The microbiome of wastewater treatment plants across the world

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**Abstract:** Microbial composition in wastewater treatment plants across the world was investigated based on samples collected at 740 wastewater treatment plants from 31 countries. In order to improve identification of microorganisms from wastewater treatment plant ecosystem, we have created an improved full-length 16S rRNA gene reference database and taxonomy that allows species-level identification of all microbes using amplicon sequencing. The taxonomy provides a common vocabulary and will facilitate sharing of knowledge across the scientific field. Here we present the first results giving a complete overview of the species present, their global distribution, and for the known functional groups, such as nitrifiers and bacteria involved in biological P-removal, also the diversity across treatment plant type, country, and continent.

**Keywords:** Microbial diversity; full-length 16S rRNA gene; global survey

Biological wastewater treatment and resource recovery rely on a multitude of microbes with a lot of process-critical species to carry out key processes. Microbial community management ensures well-working plants, but this requires reliable identification of the microbes present and establishment of a link to their functions. Recently, a survey of the microorganisms in wastewater treatment plants across the world provided an overall knowledge about the diversity and distribution of the microbial communities present therein (Wu et al., 2019). However, many microbes are still poorly described, and the existing taxonomic reference databases are incomplete and cannot resolve the diversity to more than family or genus level, not to the species level, as is needed to link identity to function.

The gold standard for microbial community analyses is 16S rRNA amplicon sequencing, where a small piece of the 16S rRNA gene of all organisms in the sample is PCR amplified, sequenced, and taxonomically classified by comparison to a full-length 16S rRNA gene reference database. This provides an overview of the microbial taxa present and their relative abundance. However, reliable classification at the lower taxonomic ranks, such as genus or species, requires that the reference database includes sequences with high sequence similarity to the amplicons and that the sequences in the reference database are classified at all taxonomic ranks. None of these criteria are met in the large public databases available (e.g., SILVA, GreenGenes, RDP).

In order to create a comprehensive, global 16S rRNA gene reference database for microbes in wastewater treatment systems, we have sequenced more than 5,200,000 full-length 16S rRNA genes from samples collected at 740 WWTP from 31 countries using our recently developed method (Karst et al., 2018). To provide a robust taxonomic classification for all reference sequences, we have introduced AutoTax (Dueholm et al., 2019), a simple, yet efficient strategy to create ecosystem-specific full-taxonomy for all taxonomic ranks, including for the first time species-level classification. Danish wastewater treatment plants have been investigated, and the overall microbial composition is well described in these plants (Nierychlo et al., 2019, MiDAS 3). Here we present the first results of the global survey of microbes in

different plant and process types: with C-removal, with C-removal and nitrification, with N-removal, and plants with N and P removal, using the new reference database with approx. 90,000 unique full-length 16S rRNA gene sequences (MiDAS 4).

Amplicon sequencing was applied to investigate identity and abundance of the microbial communities in all WWTPs. By applying the MiDAS 4 taxonomy and near-perfect full-length reference sequences, the microbial community was characterized at sub-genus to sub-species-level. In total, we found 9,469 genera (of which 86% were novel genera) and 31,426 species (of which 95% were novel species). The overall community structure showed some dependence of country and continent (Figure 1.1). Also, using multivariate statistics, the plants showed a tendency that communities from warm climates clustered together as did those from cold climates. The process type of the plants also showed clear differences in community structure. Figure 1.2 shows an example of plants with C-removal only and plants enhanced biological P removal.

Different functional groups were investigated in greater details, at genus or species level. The nitrifying communities were less diverse than expected (Figure 1.3). Among the ammonium oxidizers (AOB), only the genus *Nitrosomonas* was abundant. Among the nitrite oxidizers (NOB), two genera were abundant: *Nitrospira* and *Nitrotoga*.

The diversity of known PAO was also smaller than expected in plants with enhanced biological P-removal (EBPR) (Figure 1.4). Three genera were abundant: *Ca. Accumulibacter*, *Dechloromonas* and *Tetrasphaera*. No specific correlations to country or process parameters were found. When the species were studied in greater detail, each genus comprised of 5-10 different species across the plants investigated. Many of these were present across the world, indicating substantial similarity among the EBPR organisms worldwide.

The diversity and distribution of other known bacterial groups were also investigated in details, such as filamentous bacteria, denitrifiers, and glycogen accumulating organisms (GAO). Moreover, we found many abundant species where we have no prior knowledge about their function. They made up the majority of the genera present, thus showing a huge effort is needed to reveal the function of these.

The results presented in this study provide the first comprehensive list of microbes at the species level present across the world. The application of the new MiDAS 4 taxonomy shows the benefit of using MiDAS species-level taxonomy, which reveals a huge undescribed diversity and also introduces placeholder names so that all novel species can be uniquely identified. This makes it for the first time possible to compare high-resolution microbial data across studies and forms an important resource for all future studies of wastewater microbiology. Furthermore, all available knowledge about the microbes can be found at our MiDAS webpage <http://www.midasfieldguide.org>.

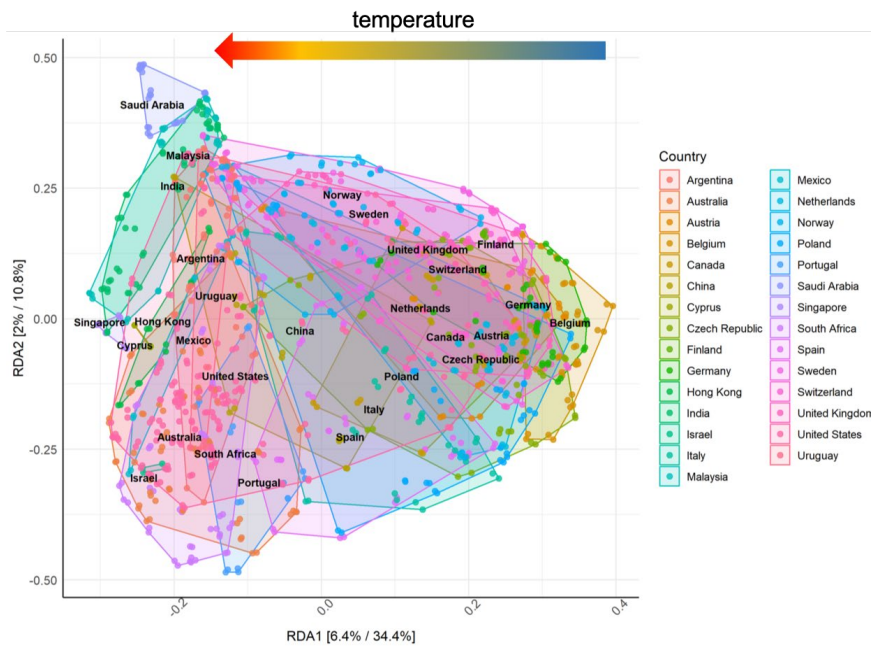


Figure 1.1 Overall community composition in 667 WWTPs worldwide

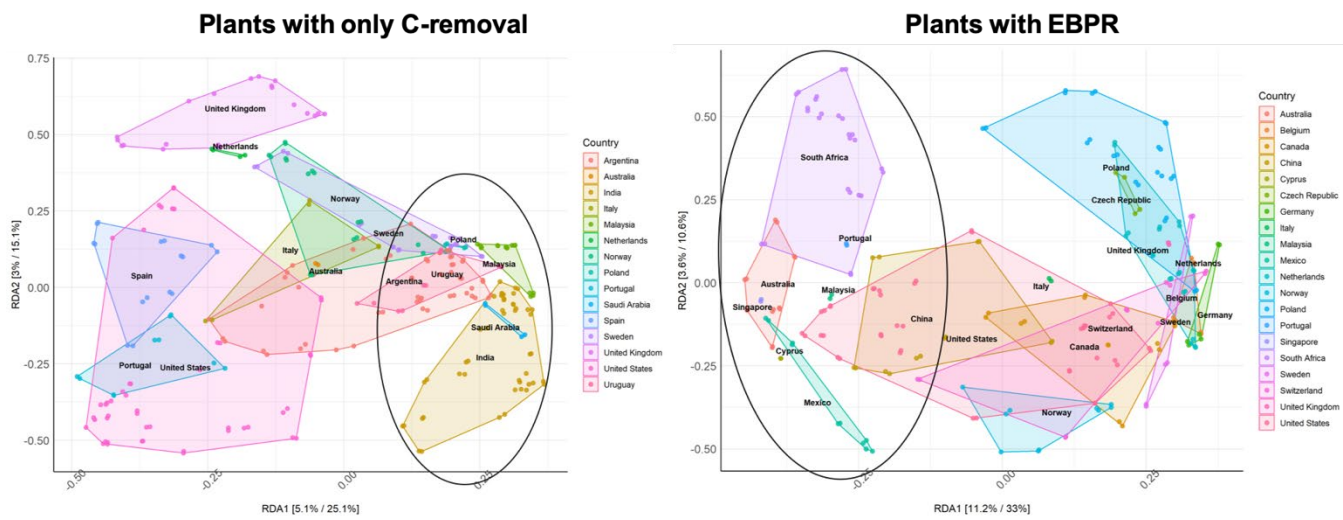


Figure 1.2 Overall community composition in plants with C-removal only and in plants with biological N and P removal. Circles indicate countries with a warm climate.

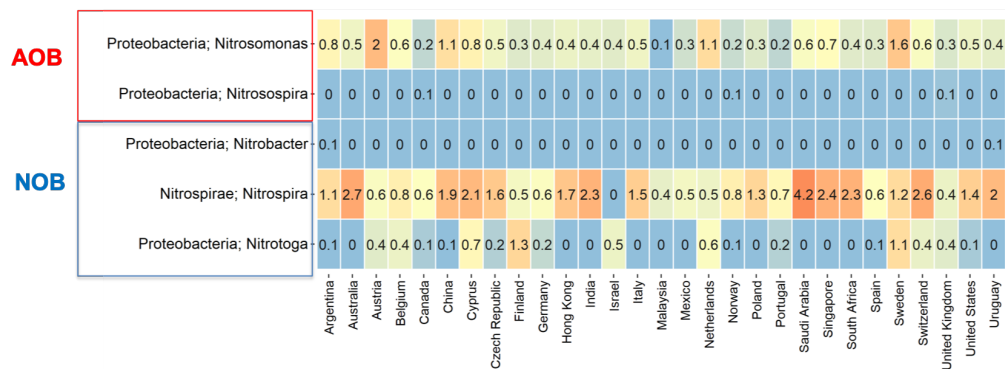


Figure 1.3 Presence of nitrifiers in WWTPs around the world

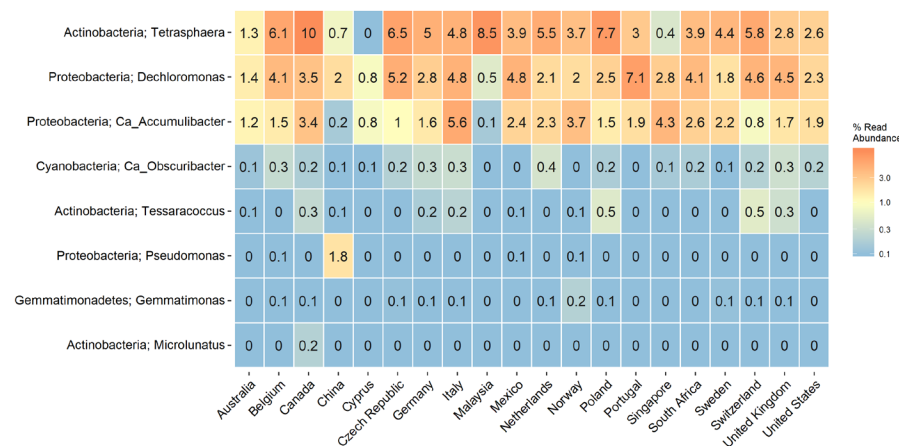


Figure 1.4 Presence of putative PAOs in EBPR plants around the world

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