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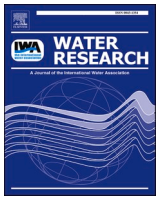

Full-scale activated sludge transplantation reveals a highly resilient community structure

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ABSTRACT

Well-functioning and stable microbial communities are critical for the operation of activated sludge (AS) wastewater treatment plants (WWTPs). Bioaugmentation represents a potentially useful approach to recover deteriorated systems or to support specific AS processes, but its application in full-scale WWTPs is generally problematic. We conducted a massive transplantation (in one day) exchanging AS from a donor to a recipient full-scale WWTP with similar process type (biological removal of nitrogen and phosphorus) and performance, but with differences in microbial community structure. The treatment performance in the recipient plant was not compromised and the effluent quality remained stable. The AS community structure of the recipient plant was initially very similar to the donor AS, but it almost completely restored the pre-transplantation structure approximately 40 days after transplantation, corresponding to 3 times the solid retention time. Most of the unique species of donor AS added to recipient AS disappeared quickly, although some disappeared more slowly the following months, indicating some survival and potentially a time limited function in the recipient plant. Moreover, the addition in higher abundance of most species already present in the recipient AS (e.g., the polyphosphate accumulating organisms) or the reduction of the abundance of unwanted bacteria (e.g., filamentous bacteria) in the recipient plant was not successful. Moreover, we observed similar abundance patterns after transplantation for species belonging to different functional guilds, so we did not observe an increase of the functional redundancy. Investigations of the microbial community structure in influent wastewater revealed that for some species the abundance trends in the recipient plant were closely correlated to their abundance in the influent. We showed that a very resilient microbial community was responsible for the outcome of the transplantation of AS at full-scale WWTP, potentially as a consequence of mass-immigration from influent wastewater. The overall results imply that massive transplantation of AS across different WWTPs is not a promising strategy to permanently solve operational problems. However, by choosing a compatible AS donor, short term mitigation of serious operational problems may be possible.

1. Introduction

Successful operation of wastewater treatment plants (WWTPs) depends on optimal and well-functioning microbial communities. Microorganisms belonging to various functional guilds (e.g., nitrifiers, denitrifiers, polyphosphate accumulating bacteria) play an active role in removal of carbon, nutrients, and micropollutants. The stability of these communities relies on a balance between several different driving forces, such as process design, influent wastewater composition (including microbial immigration), operational parameters (e.g., oxygen level and solids retention time, SRT), and environmental conditions (e.g., temperature) (Chen et al., 2017; Dottorini et al., 2021; Griffin and

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Wells, 2017; Isazadeh et al., 2016; Ofiţeru et al., 2010). Many WWTPs are reported to have stable operation, whereas others frequently experience operational problems, which can lead to deterioration or collapse of the treatment process. These problems include poor solid-liquid separation due to filamentous microorganisms, poor nitrification, or challenges with biological removal of phosphorus (Johnston et al., 2019; Kristensen et al., 2020; Nierychlo et al., 2020a; Wágner et al., 2015).

Bioaugmentation has the potential to address some of these issues by adding selected microorganisms (pure cultures or mixed consortia) to the process tanks in order to improve operation or restore a collapsed activated sludge process (Dueholm et al., 2015; Nguyen et al., 2018). In this case, bioaugmentation by mixed cultures has been successfully applied to improve the nitrification process (Stenström and la Cour Jansen, 2016), for the start-up of full-scale activated sludge processes (Guo et al., 2010), and for the removal of pollutants from industrial wastewaters in lab-scale (Nzila et al., 2016). Applications of bioaugmentation are also well known for other ecosystems, such as sediments treated for the recovery from organic pollutants (e.g., Payne et al., 2019), or such as the human gut to introduce well-functioning microbial communities into patients with an unhealthy community. In the first case, a successful treatment can be characterised by the establishment of the exogenous strain in the local community, despite the cell abundance of the augmented microbes decreased after bioaugmentation (Xu et al., 2022). In the second case, faecal transplantation of the human gut can be effective in treating *Clostridium difficile* infections and other gastrointestinal problems (Gupta et al., 2016). To our knowledge, a full-scale activated sludge transplantation has never been performed to evaluate whether it can be used for establishing a more "healthy" community in a WWTP facing operational problems.

Bioaugmentation acts as an artificial colonization or invasion event, and depending on its extent, it may represent a great disturbance for the local community. The local microbial community composition may remain unaffected (resistant), be temporarily affected but restore its original profile after some time (resilient), or be permanently affected even if maintaining the original process performance (redundant) (Allison and Martiny, 2008). Such patterns have been reported in literature for different ecosystems. Shade et al. (2012) showed that a lake microbial community affected by a major disturbance was able to recover without any permanent effect. In contrast, Vuono et al. (2016) found significant differences in the community composition and diversity in an activated sludge system pre and post-disturbance event.

A successful bioaugmentation, or transplantation in WWTPs, implies a partial or total change of composition of the receiving local microbial community maintaining process performance, therefore resembling the case of functional redundancy. Even if bioaugmentation might be immediately beneficial for the local community, the persistence over time of the bioaugmented bacteria is often problematic. Lab-scale studies report that the establishment of invading communities depends on, for example, inoculum density (or invaders abundance), predation (due to protozoan grazing and bacteriophages infection), invaders turnover, niche availability, competition, and higher diversity of invaders compared to local communities (Kinnunen et al., 2016; Nzila et al., 2016). The role of bacterial mass-immigration from influent wastewater in WWTPs is rarely taken into account in relation to bioaugmentation. Vuono et al. (2016) reported that during disturbance of an activated sludge system, colonization of immigrating bacteria from the influent was successful in some cases, while several activated sludge taxa, not present in influent wastewater, were definitively washed out from the system. In a recent study, we reported that, in addition to biotic and abiotic parameters, mass-immigration can have a high impact on full-scale AS community assembly (Dottorini et al., 2021). Thus, given the open-system character of full-scale WWTPs, mass-immigration may affect the outcome of bioaugmentation.

To evaluate the impact of disturbance on the microbial community structure of a full-scale WWTP, we performed a massive bioaugmentation in the form of activated sludge transplantation in a full-scale WWTP, which, to our knowledge, has not been reported before. We identified two plants in Denmark and we exchanged 75% of activated sludge biomass from one plant (donor) to the other plant (recipient). The selected plants had the same process design (biological nitrogen and phosphorus removal), the same overall operation, but some differences in community structure. First, we evaluated how such massive disturbance affected the performance and the overall microbial community structure of the recipient plant. Secondly, we evaluated the outcome of transplantation (successful or unsuccessful) considering activated sludge community resilience and functional redundancy. The successful outcome of transplantation included the possibility to retain in the recipient plant new species introduced from the donor plant (unique species), and the possibility to boost or mitigate shared species present in both plants, e.g., to increase functional redundancy within important functional guilds or remove unwanted filamentous bacteria. Lastly, we evaluated the influence of bacterial mass-immigration from influent wastewater for the transplantation outcome. These findings provided guidance about the possibility to carry-out a full-scale transplantation to solve operational problems in an AS system and practical insights about the resilience of activated sludge microbial community.

2. Methods

2.1. Characteristics of recipient, donor WWTP and transplantation process

The two full-scale WWTPs involved in transplantation were both located in Odense, Denmark; the recipient plant (36,000 PE) was Odense Nordøst and the donor plant (410,000 PE) was Ejby Mølle. Both plants were operated with Biodenipho nitrogen removal and enhanced biological phosphorus removal (EBPR). However, they differed in the presence of the pre-clarifier (only present at the donor WWTP) and for the received fraction of industrial wastewater (55% of COD in donor and 20% in recipient plant), but the characteristics of the influent wastewater (IWW) of the two WWTPs were very similar (Table S1 - SI).

The sludge transplantation was carried out within a few hours in one day by trucks (20th April 2017, 7:00-22:30). Activated sludge of the recipient plant was emptied as much as was practically possible (approx. 75% of the biomass) and it was filled up with activated sludge from the donor plant (Fig. S1 - SI). Thus, the new sludge composition in the recipient WWTP consisted of approx. 25% original recipient activated sludge and 75% of donor activated sludge.

2.2. Chemical analysis and calculations

Process parameters were collected from both WWTPs from 31/12/2015 to 08/11/2017, by means of online sensor data and laboratory measurements. Chemical analyses of biomass samples were performed at the laboratory of the WWTPs. Nanocolor standard test kits from Macherey-Nagel (Düren, Germany) were used to measure COD (Chemical Oxygen Demand), total nitrogen (N), total phosphorus (P) in the influent wastewater and effluent water. Measurements from influent and effluent were used to calculate COD removal, N removal and P removal. The spectrophotometer used for analysis was Nanocolor UV/VIS II from Macherey-Nagel (Düren, Germany). The online sensors used for the analysis of phosphate (PO_4^{3-}), ammonium (NH_4^+), nitrate (NO_3^-) and total suspended solids (TSS) were analyzed by P700IQ Analyzer, AmmoLyt® Plus 700 IQ, NitraLyt® Plus 700 IQ and Visolid 700 IQ from WTW (Xylem Analytics, Weilheim, Germany), respectively. Diluted sludge volume index (DSVI) was measured according to the standard methods (APHA et al., 2012).

2.3. Bacterial community analysis and identification

Biomass samples of activated sludge (AS) and influent wastewater (IWW) from both full-scale WWTP were collected regularly over one

year (Fig. S2 - SI, Table S2 - SI) for a total of 135 samples. AS samples were collected from 01/12/2016 to 09/11/2017 as grab-samples from the process tank. After collection, AS samples were stored in 2 ml tubes. IWW samples were collected from 03/04/2017 to 26/07/2017 as sub-samples of 24-h flow proportional samples approximately on the same day as activated sludge samples of the same plant. After collection, IWW samples were stored in 50 ml tubes. Both AS and IWW samples were kept frozen at -20° C until analysis. DNA extraction and 16S rRNA amplicon sequencing was performed for each collected AS and IWW samples.

Detailed protocols for the DNA extraction from activated sludge (version: aau_wwtp_dna_v7.1) and wastewater influent (version: aau_ww_dna_v1.0) can be found on https://www.midasfieldguide.org/guide/protocols. Briefly, prior to DNA extraction, 0.5 ml of activated sludge and 15 ml of influent wastewater samples were homogenized using Heidolph RZR 2020 (Heidolph Instruments, Germany) with glass/Teflon tissue grinder. 15 ml of influent wastewater samples were filtered through 0.2 μm polycarbonate membrane to immobilize bacteria for DNA extraction. DNA extraction from both activated sludge and influent wastewater samples was performed using FastDNA Spin kit for soil (MP Biomedicals, USA). FastPrep 24 (MP Biomedicals, USA) was used for bead beating of the samples for 4 times 40 s at 6 m/s. The extracted DNA was stored at -20° C until library preparation.

Bacterial V1-V3 16S rRNA gene amplicon sequencing was performed for both the activated sludge and influent wastewater samples, using the protocol (aau_b16S_v1.2) available on https://www.midasfieldguide.org/guide/protocols. Briefly, the extracted DNA quality was evaluated using Nanodrop1000 (Thermo Fisher Scientific, USA), and with Tapestation 2200 (Agilent, USA) gel electrophoresis. The DNA concentration was measured with Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, USA) and on an Infinite® M1000 PRO (Tecan, Switzerland) microplate reader. Amplicon libraries were generated with V1-V3 primers with the following sequences: 27F 5′-AGAGTTTGATCCTGGCTCAG-3′ (Lane, 1991); 534R 5′-ATTACCGCGGCTGCTGG-3′ (Muyzer et al., 1993). Amplicon library PCR was run in duplicates for each sample with approximately 10 ng of extracted DNA. The PCR program was run at 95°C for 2 min, then 30 cycles of 95°C for 20 s, 56°C for 30 s, 72°C for 60 s, followed by at 72°C for 5 min. The amplicon libraries were cleaned up and the samples were pooled. The pooled library was sequenced on MiSeq (Illumina, USA), and the generated raw amplicon sequences were processed using AmpProc5.1 workflow (https://github.com/eyashiro/AmpProc).

Taxonomy was assigned to each amplicon sequencing variant (ASV) using the MiDAS 3 database and the AutoTax automated method (Dueholm et al., 2020) to obtain MiDAS 3 taxonomy (Nierychlo et al., 2020b). The database relies on high-quality full-length 16S rRNA gene sequences derived from activated sludge and anaerobic digester systems, and it proposes unique placeholder names for almost all micro-organisms to species level, allowing proper identification and characterization of the microbial composition in WWTPs. In case of presence of unclassified species, the species name was assigned combining the ASV level together with the first available taxonomic level (Dottorini et al., 2021).

2.4. Bioinformatics

Visualization, ordination, and data analysis were performed in R-Studio. The *ampvis2* R-package (Andersen et al., 2018) was used to load the ASVs read counts table into R, with its corresponding taxonomy and samples metadata. Samples below a minimum of 10,000 reads as well as positive and negative PCR control samples were removed, yielding a dataset of 135 samples with 24,967 - 93,612 read counts (mean = 54,087 ± 14,765 read counts per sample) including 12,344 classified and unclassified species. The main analysis was carried out as follow. Non-metric multidimensional scaling (NMDS) visualization analysis was performed with the *ampvis2* package using Bray–Curtis dissimilarity distance measure without data transformation; visualization of time-series data was performed with the *ggplot2* package (Wickham, 2016); heatmaps were generated with *ampvis2* package (Andersen et al., 2018); Venn-diagram were generated with *ggVennDiagram* package (Gao et al., 2021); the scatterplots were generated with *ggpubr* package (Kassambara, 2019); principal component analysis were performed using the *mdatools* R-package (Kucheryavskiy, 2020). Detailed codes used to analyse the data and to generate the plots are available at https://github.com/GiuDott/Transplantation_study.

2.5. Data pre-processing

Prior to formal analyses, the read count table was transformed to relative read abundance calculated within-sample. Afterwards, the dataset was grouped into ten sample groups (Table S2 - SI), based on specific time intervals of interest within the donor and recipient plant. Species with a mean normalized abundance smaller than 0.01% in all ten sample groups were filtered out. The species *Nocardioides* midas_s_1344 and midas_g_67_ASV4043 were excluded because they had increasing abundance only in the final 8 samples taken from the recipient plant (denoted as R-AS (after-late) in Table S2 - SI) and therefore were considered not relevant for the evaluation of transplantation. The filtered dataset used for the analysis resulted in 135 samples composed of 2118 classified and unclassified species.

2.6. Statistical methods

Principal component analysis (PCA) modeling was used (*i*) to monitor the development of the community structure in the recipient community in response to transplantation, and (*ii*) to evaluate the impact of immigration. In all PCA models used in this manuscript the abundance values were standardized and the number of principal components was selected to explain 70% of the total variance of the standardized values. Each PCA model, in addition to conventional results, such as scores and loadings, also contained vector with mean values and vector with standard deviations computed for the trained data.

To study the development of recipient community to transplantation *(i)*, samples from the donor and recipient activated sludge tanks *before* transplantation, namely D-AS(before) and R-AS(before), where each considered to represent the steady states of each microbial community before the onset of disturbance. We assumed that the state of the recipient microbial community following transplantation could be captured by studying the variance-covariance structure of its abundance profile (i.e., relative abundance data) using principal component analysis. Specifically, two independent PCA models were developed, one from the D-AS(before) and the other from the R-AS(before). Then, the measurements of species abundances from each of the three sample groups corresponding to activated sludge of recipient *after* transplantation, namely R-AS (after-early), R-AS (after-intermediate) and R-AS (after-late), were projected onto each PCA model. The measurements were standardized using mean and standard deviation of the models. The inter-relationship between the individual species measurements and each model were assessed using three statistics - orthogonal, score and total distances (i.e. Cooman's plot) - and visualized through distance plots (Pomerantsev and Rodionova, 2014; Rodionova et al., 2021). All three distance statistics were used to assess from a qualitative point of view how similar the abundance profile of a given sample was to the set of abundance profiles used for creating a corresponding PCA model. In this way it was possible to assess, in relation to the entire dataset, how close the abundance profile of a sample group of interest, e.g. a sample from R-AS(after-late), was to the profile of each of the two reference systems, R-AS(before) and D-AS(before). The preliminary projection of species abundance from R-AS(after) to D-AS(before) model highlighted the presence of an outlier sample (activated sludge of recipient on day -22), characterized by abnormally high relative read abundance of some species, which was removed from the dataset and the analysis was

repeated.

To evaluate the impact of immigration *(ii)*, independent PCA models were created for each of the 10 sample groups (Table S2 - SI) using the same procedure as for the previous analysis. A matrix with model distances was then computed for each pair-wise combination of models by calculating a ratio of residual variances. Let MA be a PCA model created for a dataset A and MB is a model created for a dataset B. In this case one can compute following residual variances:

- VAA — residual variance computed for dataset A being projected to model MA
- VBA — residual variance computed for dataset B being projected to model MA
- VAB — residual variance computed for dataset A being projected to model MB
- VBB — residual variance computed for dataset B being projected to model MB

The distance between the models A and B can be then computed as:

$$d_{AB} = \sqrt{\frac{VAB + VBA}{VAA + VBB}}$$

In this analysis, a small distance between two models indicates that PCA model created for one training set (e.g., MA) will largely explain the variation of the second training set (e.g., B), and vice versa. This is possible when the two datasets have similar variance-covariance structure (*i.e.*, two identical models have distance close to a value of 1).

In order to gain insight into how individual species contributed to the model distances, a discrimination power analysis was employed (Wold and Sjöström, 1977). We identified species that contributed to similarity in variance-covariance structure of the two pairs of models, R-IWW/-R-AS(before) and R-IWW/R-AS(after-late), by performing a discrimination power analysis and selected species with discrimination power below a threshold of 10 in both pairs. In order to avoid the selection of inert species (*i.e.*, those which do not contribute to the individual models and thus will also have small discrimination power), we also calculated for each species a variable of importance on projection score (Chong and Jun, 2005). Only species with non-zero VIP-scores were taken into account in the discrimination power analysis. Finally, we evaluated the Pearson linear correlation of the scaled mean-centered relative read abundance of these selected species in 17 IWW and AS paired samples of recipient; these samples were paired by date with maximum 3 days apart, considering that the variation in IWW would be reflected in AS within 3 days.

2.7. Species residence time calculation

We grouped the species added to recipient activated sludge based on their residence time after the transplantation, by modeling the change in the relative read abundance of added species according to a first-order kinetic model. We considered activated sludge process tanks as ideal continuously stirred tank reactors and we assumed that the total biomass concentration was constant, so the change of the concentration of a species over time was proportional to the species's relative read abundance. Therefore, the relative read abundance of the added species-j (X_j) could be described as follow:

$$\frac{dX_j}{dt} = -k\ X_j \tag{1}$$

where:

- X_j is the model-fitted relative read abundance of the species-j;
- t is the time in days of the sampling point which the relative read abundance corresponds to;
- k is first-order rate constant;

Integrating Eq. (1) for the rate constant over time, the species relative read abundance fitted in the model is calculated as follow:

$$X_j = X_{j,min} + X_{j,max} * exp[-k(t-t0)] \tag{2}$$

where:

- $X_{j,min}$ is the lowest relative read abundance of the species measured after transplantation;
- $X_{j,max}$ is the highest relative read abundance of the species measured after transplantation;
- t0 is equal to 0 days, referring to the day of transplantation.

The model was implemented in R-Studio using the non-linear regression function *nlm* from the *stats* package. The first-order kinetic constant (*k*) was estimated by minimizing the sum of squared errors. The species residence time was then calculated as inverse of the first-order kinetic constant from Eq. (2), as follow:

$$species residence\ time = 1/k \tag{3}$$

To evaluate the correspondence between data and model, the Pearson linear correlation coefficient (function *cor.test()* in R package *stats* (R Core Team, 2019) was computed between the species relative read abundance and the model-fitted relative read abundance obtained from Eq. (2), along with the corresponding *P*-value. The species with significant correlation coefficients were used to visualize the density distribution of residence times to find a meaningful threshold for grouping the species. Detailed codes used for also this analysis are available at https://github.com/GiuDott/Transplantation_study.

2.8. Total sludge retention time calculation

In order to compare the species residence time with the process operation of the recipient plant, we calculated the daily total sludge retention time (SRT) of the recipient plant as follow:

$$SRT = \frac{X_i\ *\ V_R}{WAS_i} \tag{4}$$

where:

- X = sludge biomass in the process tank expressed in total solids (TS) [kgTS/m^3] and measured daily for each i-sampling day from -475 to 203 days of transplantation.
- V_R = total volume of the two process tanks (here, 1750 m^3 for each of the two process tanks)
- WAS_i = wasted activated sludge biomass expressed in TS [kgTS/d] and measured for each i-sampling day from -475 to 203 days of transplantation.

3. Results

3.1. Process performance of recipient plant remained stable with transplantation

The transplantation of 75% of the activated sludge biomass in one single day was followed closely in the recipient plant to monitor possible changes in plant performance or effluent quality. The effluent quality, in terms of total COD, total N, NH_4^+, NO_3^-, total P, PO_4^{3-} and TSS, was not affected and was stable after the transplantation (Fig. 1). Some variations in the effluent quality were observed, but they resembled the variations observed during the pre-transplantation stage. Settling properties of the AS in the process tank of the recipient plant (represented by the DSVI) started to deteriorate just before the transplantation (rise in DSVI) and continued to do so afterwards, followed by an improvement. This indicated poorer settleability of the AS in the recipient plant, both right before and after the transplantation. The DSVI

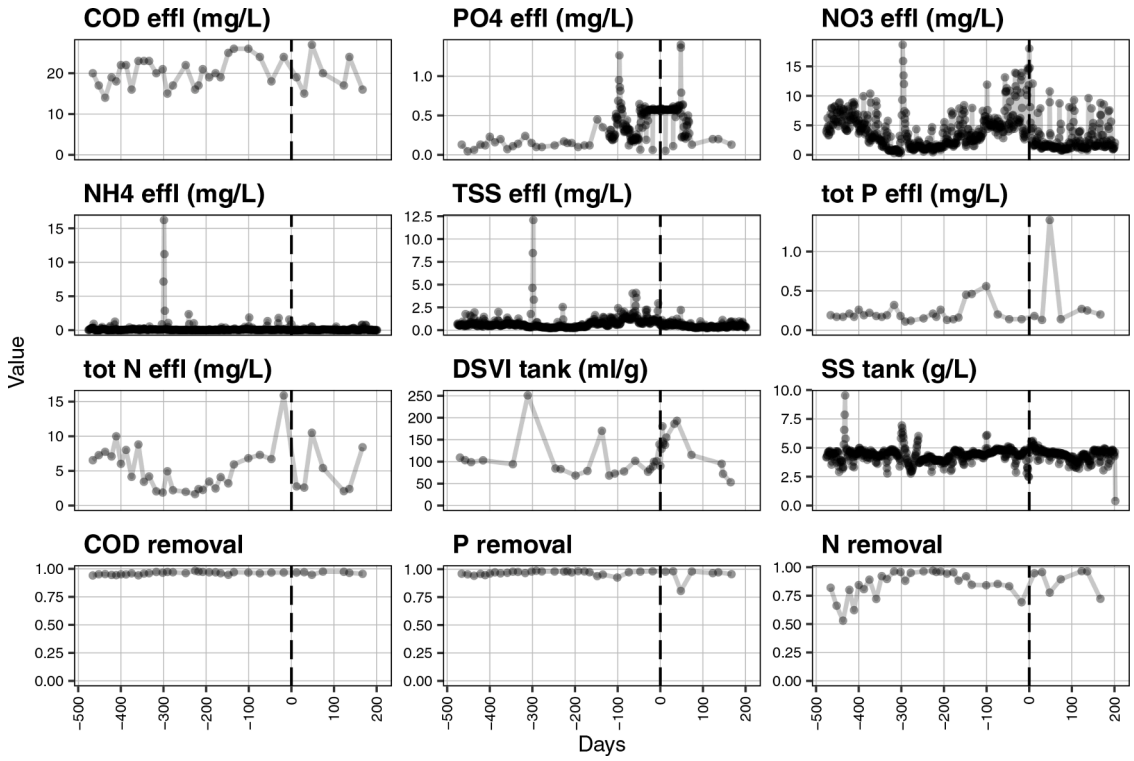

Fig. 1. Process performance parameters of recipient WWTP. Process performance is expressed through effluent quality, process tank biomass characteristics, and nutrient removal. Effluent quality is expressed by effluent COD (mg/L), effluent total N (mgN/L), effluent total P (mgP/L), effluent NH_4^+ (mgN/L), effluent NO_3 (mgN/L), effluent PO_4^{3-} (mgP/L), and effluent TSS (mg/L). Biomass characteristics in process tanks are expressed by DSVI (mL/g) and SS (g/L). Nutrient removal is evaluated by calculating COD removal, N removal, and P removal. The vertical dashed line shows the time of the transplantation. The x-axis shows the days before and after the transplantation (negative: before transplantation, positive: after transplantation).

in the donor plant was higher, between 130-200 ml/g, than the recipient plant prior to the transplantation (Fig. S3 - SI). The transplantation of this AS biomass likely contributed to the rise in DSVI and worsening of the settling conditions in the recipient plant right after the transplantation. Removal of COD, N, and P remained stable after the transplantation. Overall, these results indicated that the transplanted AS biomass could carry out all key processes also in the recipient plant.

3.2. *The overall microbial community of recipient was resilient to transplantation*

3.2.1. *Community structure of donor and recipient AS*

The AS microbial community structure in recipient (R-AS) and donor (D-AS) was different before and after transplantation, except for an increased similarity in the early stage after transplantation (days from 0 to 40), as shown by the complementary results from the NMDS

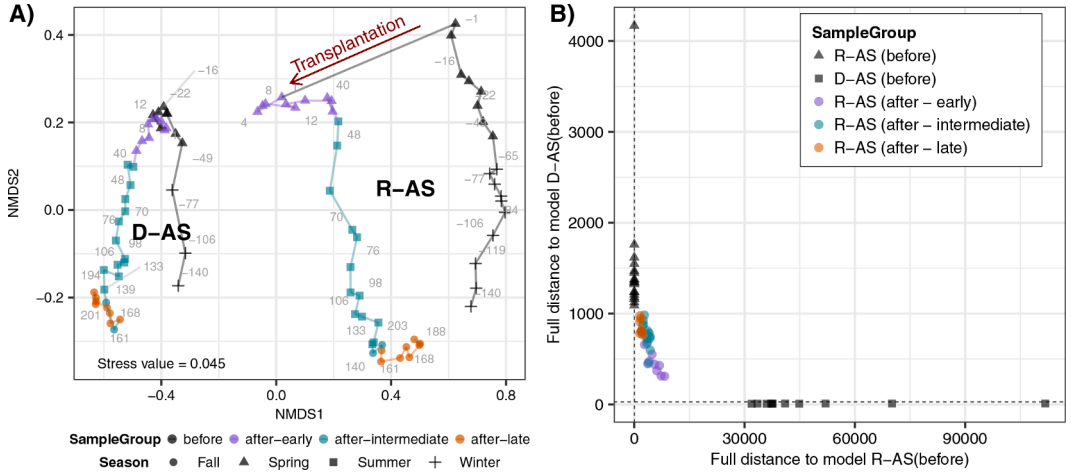

Fig. 2. Effect of transplantation on the overall microbial communities in recipient and donor. Each mark represents a sample coloured according to the days of sampling of AS: black refers to days from -140 to -1 ("before"); purple refers to days from 0 to 40 ("after-early"); green refers to days from 41 to 150 ("after-intermediate"); orange refers to days from 151 and onwards ("after-late"). (A) Non-metric multidimensional scaling (NMDS) of the microbial composition at species level of AS in the recipient (R-AS) and donor (D-AS) plant. Each sample is shaped by season (circle, Fall; triangle, Spring; square, Summer; cross, Winter). Some samples are labelled with the number of days before (negative numbers) and after (positive numbers) the transplantation. (B) Cooman's plot of full distances of recipient AS community after transplantation PCA model built for to the recipient (black triangles) and donor (black squares) AS samples before transplantation.

visualization (Fig. 2A) and PCA models (Fig. 2B). The microbial community structure of R-AS and D-AS before transplantation (between days -140 and -1) was substantially different, as highlighted by the separate clustering of all R-AS and all D-AS samples in NMDS plot (Fig. 2A) and by the largest orthogonal distances within and across the PCA models (Fig. 2B, Figs. S4A - SI, S5A - SI).

These differences were reflected by the presence of species only ever seen in one plant with an average relative read abundance > 0.01% (referred here as unique species) (Fig. 3A), and by the different abundance of shared taxa (Fig. 3B). However, the plants were characterized by approximately the same number of species (with average abundance > 0.01%) and most of them were present in both plants (Fig. 3A). These shared species included the top-5 most abundant genera typically found in Danish AS WWTPs (*Trichococcus, Ca.* Microthrix, *Tetrasphaera, Rhodoferax*, and *Rhodobacter*) (Fig. 3B). These genera, as examples of the whole communities, showed before transplantation either similar changes of abundance over time in both plants (e.g., *Tetrasphaera*), or showed abundance variations over time in one plant only (e.g., *Rhodoferax*) (Fig. S6 - SI). The community structure in IWW of both plants was also overall similar with distinct differences (Fig. S7 - SI) despite the different percentage of industrial load.

The transplantation of AS from donor to recipient (between days -1 and 1) induced a temporal change in the microbial community of the R-AS, which was greater than the seasonal change observed over time in both AS communities (Fig. 2A). In fact, in both plants the first and last collected samples cluster close to each other after a time span of approximately 365 days, indicating the recurrency of AS microbial communities over the years (Nierychlo et al., 2020a; Peces et al., 2022); however, the transplantation disrupted in R-AS the temporal development observed in D-AS. Moreover, we observed an increase of the number of shared species between R-AS and D-AS (approx. 100 species more after transplantation) and a decrease of the number of unique species in D-AS (approx. 50 species). The number of unique species in R-AS remained, instead, approximately unchanged (Fig. 3A). Both NMDS and PCA models suggested three stages of the overall microbial community in R-AS (Fig. 2, Figs. S4 - SI, S5 - SI): in the early stage (from day 1 to 40) R-AS had the highest similarity to D-AS community, but R-AS started to diverge quickly from D-AS; in the intermediate stage (days from 40 to 150), R-AS diverged slowly from D-AS, progressively increasing the similarity to R-AS before transplantation, thus slowly restoring the pre-transplantation community structure and establishing a new community "steady state" in the late stage after transplantation (from days 150 onwards). In the intermediate-late stage after transplantation (from day 40) the top-5 most abundant genera of AS (including process critical, e.g., *Tetrasphaera*) were still present in high abundance in both plants, suggesting that the main process functions were maintained (Fig. 3B). Throughout all the sampling points after transplantation, the differences between R-AS samples and R-AS model progressively decreased while, conversely, the differences between R-AS samples and D-AS model progressively increased (Figs. S4 - SI, S5 - SI). These results suggested that, although the AS community composition of R-AS became more similar to D-AS community as a consequence of the

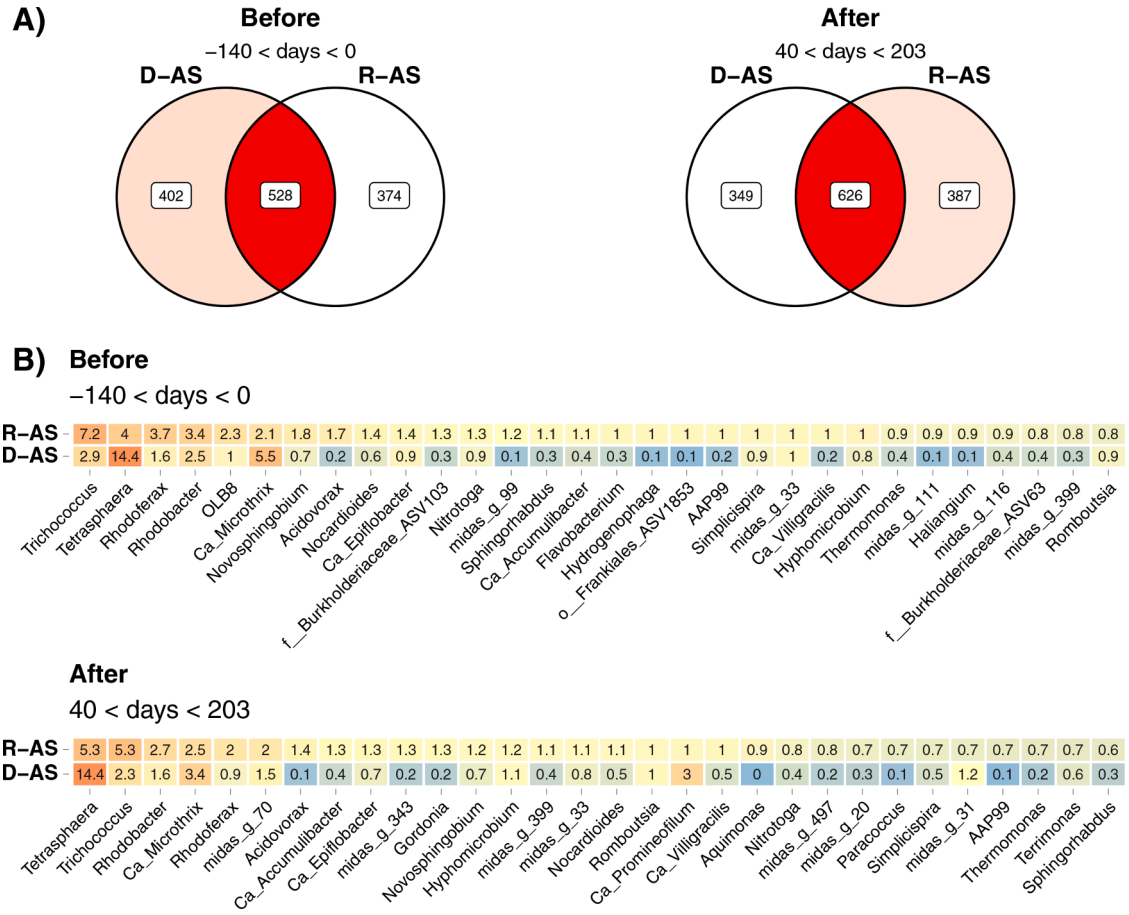

Fig. 3. Community richness and structure in activated sludge of donor and recipient before and after transplantation. (A) Venn Diagram of number of species in activated sludge samples of donor and recipient (D-AS and R-AS, respectively), before and after transplantation. The diagram refers to classified and unclassified species present with a relative read abundance > 0.01% on average in each group (D-AS before, R-AS before, D-AS after, R-AS after). The samples included after transplantation are from day 40 and onwards. (B) Heatmap of the top 30 genera of activated sludge in the donor (D-AS) and recipient (R-AS) sorted by relative read abundance in the recipient. The abundance values are an average across samples before and after transplantation, respectively. The samples included after transplantation are from day 40 and onwards.

transplantation, the microbial composition of R-AS showed resilience, quickly transitioning towards the pre-transplantation R-AS community structure all through the sampling points.

3.3. Transplanted species in recipient plant showed different patterns of resilience

3.3.1. Transplantation of unique and abundant species was retained only temporarily

To evaluate the impact of transplantation at species level, we investigated the possibility to add and maintain in R-AS abundant species which were not present before transplantation (unique species in D-AS). Among the 402 unique species in D-AS (Fig. 3A), we identified species in D-AS with a relative read abundance higher than 0.1% (49 species) on average before transplantation.

The unique species could be grouped based on their residence time within the recipient plant into species with low, medium and high residence time, including 27, 14, and 8 species, respectively. After transplantation, most of these species (41 out of 49, 83.7 %) had an overall residence time of 42.7 ± 25.5 days (Table S3 - SI), corresponding to approximately 3 times the SRT of ~15 days of the recipient plant (Fig. S7 - SI). Therefore, these species with low and medium residence time (Fig. 4) were removed from the R-AS almost like inert particles in AS. Among the added species with high residence time (Fig. 4), only a few unique species (4-5) were maintained in R-AS at the introduced abundance, which was anyway low in R-AS (~ 0.1%). Other species with high residence time showed a progressively decreasing abundance in R-AS, indicating inability to be retained in the recipient plant (Fig. 4, Fig. S8 - SI). Whether or not these species were ultimately removed from R-AS is not known due to the lack of further sampling points.

To evaluate the possibility to mitigate bulking problems of full-scale WWTPs, we investigated as well the possibility to remove abundant but unwanted bacteria, such as certain filamentous bacteria. We selected 14 unique and abundant (> 0.1%) species present in R-AS (including filamentous species), which were removed by the transplantation. Also in this case, most species restored the pre-transplantation abundance after 1-2 months, while few other species stayed in R-AS, but in low abundance (Fig. S9 - SI).

3.3.2. The functional redundancy of species within guilds could not be boosted

To evaluate the possibility to increase and boost the functional redundancy of microbial communities in WWTPs, we investigated the abundance variation over time of species belonging to guilds (PAOs, GAOs, nitrifiers, filaments) which were added to R-AS with transplantation. These species were either not present in R-AS before transplantation (unique species of D-AS) or were present in both R-AS and D-AS (shared species). In the first case, transplantation would increase the functional redundancy within their guild; in the second case, transplantation would boost the abundance and the redundancy within the guild. The 53 shared species belonging to guilds were present in similar and/or low abundances in both plants, except for some of the most abundant species (Fig. 5).

We focused on species within the PAO guild because they had well pronounced differences in abundances in R-AS and D-AS (Fig. 5), and because of the key importance of PAOs for the two EBPR AS systems. The added unique and shared species belonging to PAOs did not remain in R-AS at the transplanted abundance, but gradually restored their pre-transplantation abundance within ~200 days (Fig. 6). This indicated that unique and shared PAO species (e.g., *Tetrasphaera* midas_s_220 and *Tetrasphaera* midas_s_5) could not thrive in the R-AS at the transplanted abundance. The results for PAOs confirmed previous observations at species-level, and we found similar results for species belonging to the other guilds (Figs. S10 - SI, S11 - SI). In particular, we consistently observed the lack of establishment of: transplanted abundances (i.e., resilience of microbial community in R-AS) for different species within the same guild (Fig. 6, Fig. S10 - SI - e.g. *Tetrasphaera* midas_s_5 and *Tessaracoccus* midas_s_307 within PAOs); different species within the same genus (e.g., *T.* midas_s_45 and *T. elongata* within *Tetrasphaera* genus Fig. S10 - SI); different genera within the same guild (e.g., *Tetrasphaera, Dechloromonas, Ca.* Accumulibacter, *Tessaracoccus* among PAOs - Fig. S10 - SI).

3.4. Immigration from the wastewater may affect the community resilience of recipient

To evaluate if the resilience of R-AS community to transplantation was influenced by microbial mass-immigration, we investigated the overall microbial community structure of influent wastewater of donor (D-IWW) and recipient (R-IWW) in relation to D-AS and R-AS sample groups (Table S2 - SI).

D-IWW and R-IWW were more similar to each other throughout the whole sampling period than compared to their corresponding D-AS and R-AS samples (model distance ~ 20). Comparing AS models to each of the two IWW models, the overall microbial community structure of R-AS and D-AS throughout time (early, intermediate, and late stage) was more similar to its own influent (R-IWW and D-IWW, respectively), as indicated, for example, by a lower distance of all R-AS sample groups to R-

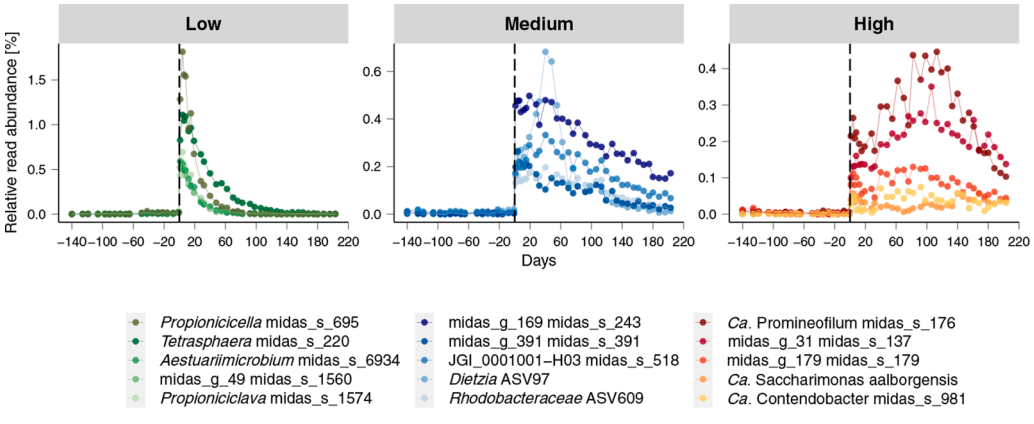

Fig. 4. Removal in R-AS of added unique and abundant species of D-AS. Temporal variation in R-AS of abundant species of donor (relative read abundance ≥ 0.1% on average before transplantation) not present in recipient (abundance before transplantation < 0.01% on average). The displayed species are the top 5 most abundant on day 1 in R-AS and are grouped according to their residence time in R-AS using a threshold of 50 days: (1) low residence time (species residence time lower than 50 days with significant Pearson linear correlation coefficient between abundance data and model-fitted abundance); (2) medium residence time (species residence time higher than 50 days with significant Pearson linear correlation coefficient between abundance data and model-fitted abundance); (3) high residence time (non-significant Pearson linear correlation coefficient between abundance data and model-fitted abundance). On x-axis: days before and after transplantation; the vertical dashed line shows the time of the transplantation. On y-axis: relative read abundance at species level. Colours indicate the top 5 species within each group of residence time.

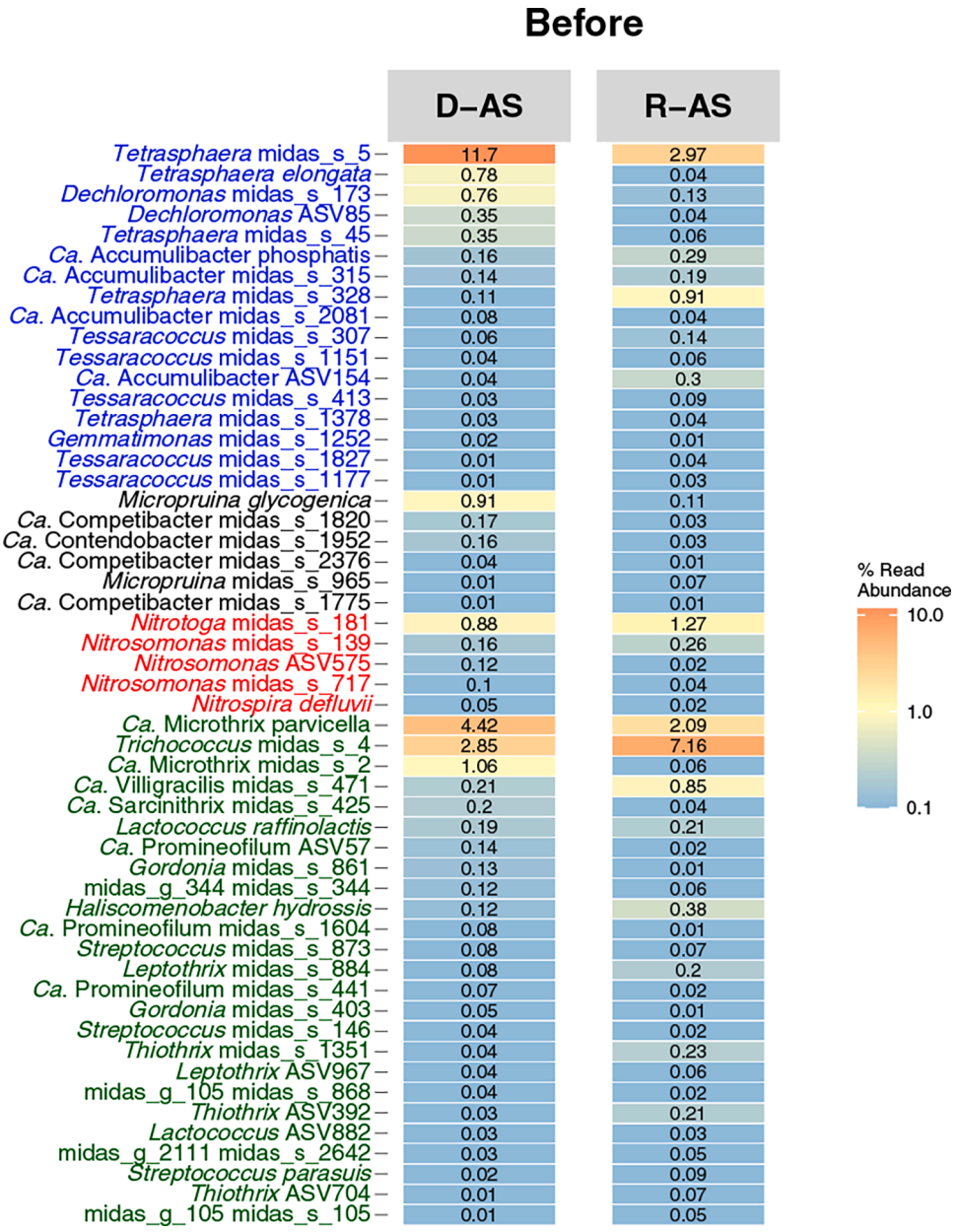

Fig. 5. Heatmap of shared species within guilds. The 53 species were present in both recipient activated sludge (R-AS) and donor activated sludge (D-AS) before transplantation with an average abundance higher than 0.01% and belonging to known functional guilds (PAOs, GAOs, nitrifiers, filaments). Species abundance is sorted within each guild by abundance in D-AS. Colours indicate: PAOs species (blue); GAOs species (black); nitrifier species (red); filamentous species (green).

IWW model compared to the distance of D-AS samples to R-IWW model (Fig. 7). Even if this is less obvious for the donor plant, the results suggested that the microbial structure of recipient AS was reflected by the structure of IWW from the same plant. After transplantation, the microbial community of R-AS in the early stage became more similar to both R-IWW and D-IWW compared to before transplantation [the distance of R-AS(before) and R-AS(after-early) to R-IWW and to D-IWW model decreases from 95.6±2.9 to 65.2±0.9 and from 222.5±11.3 to 139.5±2.3, respectively] probably as a consequence of transplantation. However, from the intermediate until the late stage after transplantation, R-AS community already restored the similarity to R-IWW almost to the same level as before the transplantation [R-AS(before)]. This confirmed the results seen previously for R-AS sample groups (Fig. 2, Figs. S4 - SI, S5 - SI) and suggested that continuous immigration of bacteria from R-IWW might play a role in maintaining the overall community structure of R-AS also after transplantation.

To evaluate at species-level the impact of immigration for transplantation, we investigated the abundance-trend of species in both R-IWW and R-AS. Interestingly, we found that for some species the abundance trend in AS resembled the abundance trend in IWW over time, as indicated by the Pearson linear correlation coefficient of their scaled abundance, which was significantly high for some species (e.g., *Ruminococcaceae* ASV3228, *Ca.* Microthrix parvicella, *Tetrasphaera* midas_s_5) (Fig. 8). The relative read abundance of species in IWW was generally much lower than in the AS, but even when lower than 0.01%, it showed a strong linear correlation with abundance in R-AS. Among the top 5 species contributing the most to the similarity between R-AS (before) and R-AS(after-late), some had a significant strong correlation with IWW abundance (e.g., *Ruminococcaceae* ASV3228) (Fig. 8A). For the unique species of donor added to R-AS (e.g., *Ca.* Amarolinea midas_s_1), the linear correlation of abundances was not strong, probably because these species were generally absent in the R-IWW and could not

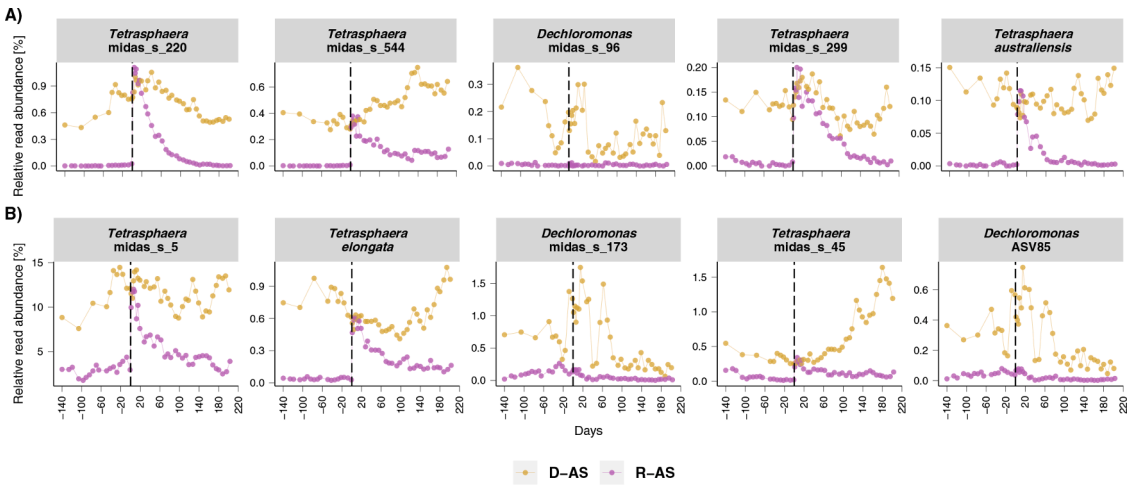

Fig. 6. Abundance variation over time of PAO species added to R-AS. On x-axis: days before and after transplantation; the vertical dashed line shows the time of the transplantation. On y-axis: relative read abundance at species level. Ochre and pink colors refer to samples of D-AS and R-AS, respectively. (A) Unique PAO species of donor added to R-AS. The selected species were the top 5 most abundant in D-AS among the ones present with an average abundance $\geq$ 0.01% before transplantation only in D-AS. (B) PAO species shared between R-AS and D-AS. The selected species were the top 5 most abundant on average in D-AS among the ones present in both R-AS and D-AS with average abundance $\geq$ 0.01% before transplantation.

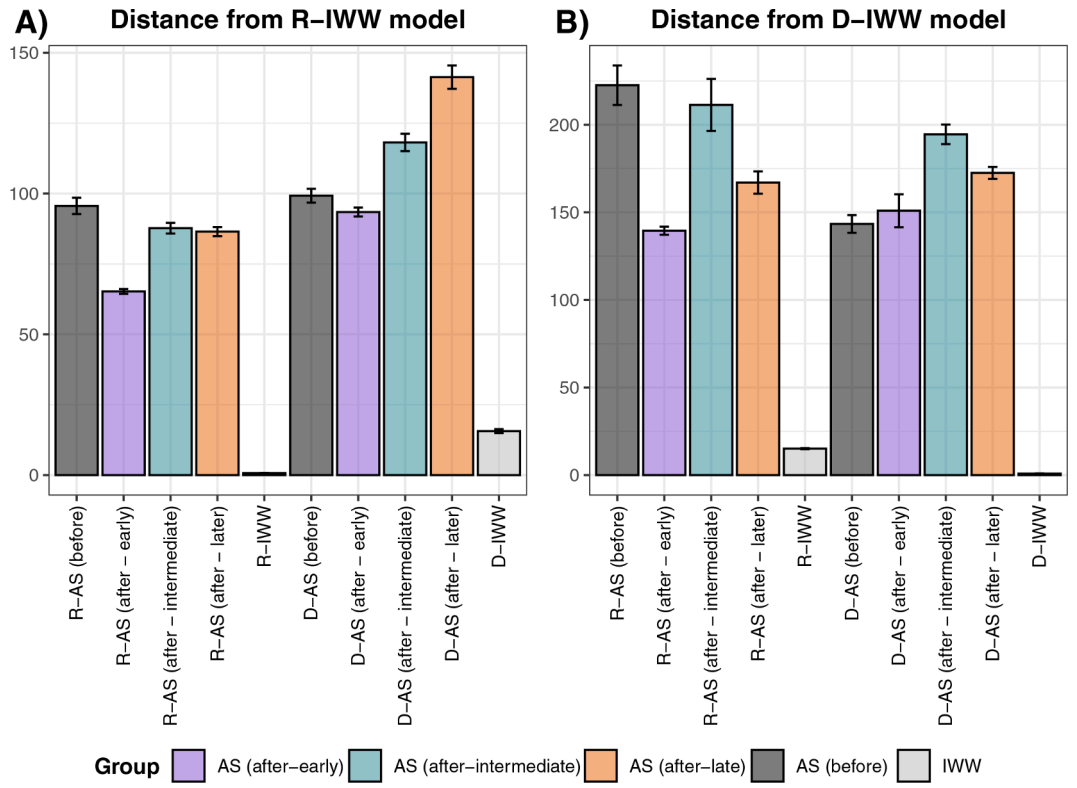

Fig. 7. Evaluation of immigration by independent PCA modeling. Barplot of the distances between the PCA models built independently for each sample group. The colour of the bars indicates the type of sample (AS or IWW) from either donor (D) or recipient (R) and the days of sampling: black, AS samples before transplantation (days from -140 to 0); purple, AS samples after transplantation in the early stage (days 0 to days 40); green, AS samples after transplantation in the intermediate stage (days 41 to days 150); orange, AS samples after transplantation in the intermediate stage (days 151 and onwards); gray, all IWW samples. On x-axis, sample groups. On y-axis: distance to the models from recipient influent wastewater R-IWW model (A) and from donor influent wastewater D-IWW model (B). The standard deviation refers to cross-validation with 20 iterations with Jack-Knifing approach with corresponding 95% interval for each distance value.

contribute to maintaining the transplanted abundance in R-AS (Fig. 8B, Fig. S12 - SI). The well-known filamentous and foaming genera *Ca.* Microthrix and *Gordonia* included species with a strong abundance correlation between R-IWW and R-AS (R $\backsimeq$ 0.7) (Fig. 8C) indicating a strong impact of the IWW.

4. Discussion

To explore the impact of disturbance on the microbial community structure of a full-scale WWTP, we carried out a massive bio-augmentation in the form of AS biomass transplantation between two full-scale WWTPs with nutrient removal. The two WWTPs had overall

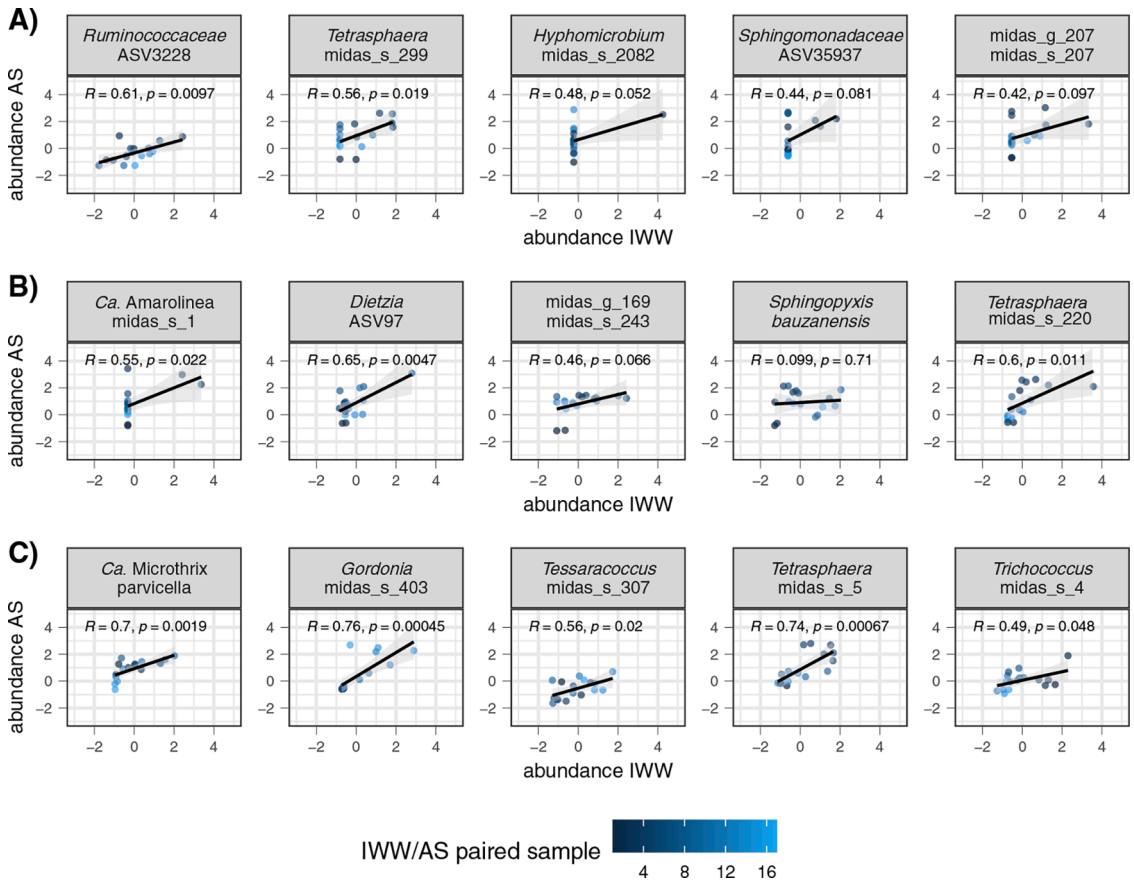

Fig. 8. Scatterplot of mean-centered relative read abundance in IWW and AS of selected species in R-AS. On x-axis, scaled abundance in IWW; on y-axis, scaled abundance in AS. The colour of the points refers to 17 IWW/AS sample pairs from -16 to 98 days of transplantation. The coefficient of Pearson linear correlation (R) and its associated *p*-value (p) are shown for each species; the grey area surrounding the black regression line corresponds to the 95% confidence interval for the coefficient "R". (A) Selected species that contributed the most to the similarities between independent PCA models of R-IWW/R-AS(before) and R-IWW/R-AS(after-late); (B) Selected unique and abundant species of D-AS added to R-AS; C) Selected species belonging to functional guilds: *Ca.* M. parvicella, *Gordonia* midas_s_403, *Trichococcus* midas_s_4 (filamentous microorganisms); *Tessaracoccus* midas_s_307, *Tetrasphaera* midas_s_4 (PAOs).

the same design, operation, and functional guilds, representing a perfect test case for biomass transplantation with the aim to evaluate how a massive disturbance affects resilience and functional redundancy of the recipient microbial community. This provided practical insights about the possibility to exchange taxa to increase or boost the functional redundancy within functional guilds (introducing new species) and to change the abundance levels of wanted or unwanted species to improve process performance. The microbial community of AS recipient demonstrated overall an unexpectedly strong resilience to the transplantation disturbance, and restored almost completely its pre-transplantation structure (composition and abundance) within a short time (1-2 months), contrary to what was found in other disturbance responses of AS systems (Vuono et al., 2015). We hypothesize that a longer period of observation than the one of 6 months shown in this study would have shown a full restoration of the pre-transplantation community structure, even if minor differences could be expected. Moreover, despite the temporary changes of community structure after transplantation, the recipient community was able to ensure stability of process performances, likely through functional redundancy of the local and transplanted community, as shown in other studies (Wang et al., 2021). The transient worsening of the settling properties in the recipient plant right after the transplantation, were probably due to an increasing content of the filamentous *Ca.* Microthrix both in the transplanted AS from donor (which also had a higher DSVI than the recipient) and in the recipient itself (where the abundance of *Ca.* Microthrix started to increase before transplantation) (Nierychlo et al., 2020b; Wágner et al., 2022). Our overall results were in contrast to studies of successful establishment of new immigrating colonizer bacteria in WWTP after disturbance (Vuono et al., 2016), but are in accordance with studies that acknowledged the difficulty to successfully implement bioaugmentation in full-scale WWTPs (Herrero and Stuckey, 2015; Park et al., 2008).

Our results indicated that the resilience of the AS recipient community was characterized by the lack of establishment of the vast majority of the added new species. By removing 75% of the original AS biomass, the added new species transplanted from a plant with similar conditions ("EBPR"-niche) were expected to be able to grow and to outcompete the local communities in recipient (25% of biomass) as a consequence of higher population size (both relative read abundance and biomass concentration) (Herrero and Stuckey, 2015; Kinnunen et al., 2016). However, the results showed that most of the unique species of donor plant added to the recipient plant (new species for the recipient) were progressively removed from the recipient with a removal rate often resembling the one of inert particles, that is by following the SRT of the recipient plant. This suggested that the newly added species added were not able to occupy a niche and/or to compete with the local existing community. In fact, even if the same function can be carried out by alternative taxa as an effect of functional redundancy, it is known that environmental factors can shape the possibility to grow of a certain taxonomic group within a functional group (Louca et al., 2018). In our case, environmental conditions, such as the organic load, the inorganic composition of influent wastewater and the operational parameters, were very similar in both donor and recipient plant. The industrial load of the plants was different and we do not have detailed information about the organic composition, but we do not expect this to play a major

role in shaping the communities in AS and IWW, as the overall community structure was very similar in donor and recipient plant before transplantation. Moreover, the "engineering resilience" of the AS system against a disturbance was already observed and it was proposed to occur through "ecological resilience", which is the selection of bacteria able to restore the condition before disturbance (Pérez et al., 2019). Similarly, our results indicated that the local community was resilient, and we did not observe the boost of functional redundancy within guilds by permanently changing the abundance of transplanted species shared between the two plants, such as unwanted filamentous bacteria (*Ca.* Microthrix and *Gordonia*), and species from functional guilds, such as the PAOs.

An alternative explanation for the outcome of the transplantation is the role of microbial immigration. Recently, we described that immigration strongly affects both composition and abundance of community members in AS (Dottorini et al., 2021), which could explain the very strong resilience of AS systems and, consequently, the unsuccessful bioaugmentation studies reported also elsewhere. In particular, the abundance of species in AS correlated well either with the abundance of the same species in IWW or with the abundance of a group of species in IWW, the latter being the case of the majority of species in AS (Dottorini et al., 2021). Similarly, our results are partially in accordance with the first case, where the abundance of some species in AS of recipient supported by immigration correlated well with the species abundance in IWW (such as for species belonging to guilds); while the second case was not investigated here. The unique species found in AS of each plant were presumably only present in the AS plants because they were continuously added by their respective IWW, as indicated by the high similarity in PCA model between communities of AS and IWW within the same plant. We hypothesize that when the unique species of activated sludge from the donor were transplanted to the recipient plant, they were either washed out or progressively removed because their presence in AS was not supported by continuous immigration from the recipient IWW. Similarly, the abundance of some species in AS of recipient supported by immigration correlated well with the species abundance in IWW (such as for species belonging to guilds).

Disentangling the factors affecting the outcome of transplantation was, however, difficult for several reasons. First, it was practically not possible to remove the whole activated sludge biomass of the recipient plant but 25% of the biomass remained in the recipient plant, thus potentially masking the real impact of transplantation at species level. Secondly, the microbial community of many AS plants exhibit strong temporal seasonal variations (Griffin and Wells, 2017; Peces et al., 2022) that may mask other processes, such as the transplantation. In our study, both AS plants showed some seasonal variations both at the overall community level and at species-level, as for *Ca.* Microthrix which is indeed known to increase in abundance in spring (Nierychlo et al., 2021). Thus, it was difficult to assess whether abundance variations after transplantation for some species were caused by transplantation or normal seasonal variations. Finally, but not less importantly, analyses of the microbial community structure in IWW is difficult, as the species that are growing in the AS are present only in very low abundance in the IWW (often close to detection level) and therefore difficult to quantify (Dottorini et al., 2021). However, as discussed above, our observations provided support to the hypothesis that immigration could potentially be responsible for the outcome of transplantation, where the lack of persistence of a newly added species to the AS recipient could be explained by the lack of continuous immigration of that species from IWW.

The strong resilience of the recipient AS community implies that the application of a one-time transplantation in a suspended growth system such as AS-WWTPs does not represent a proper long-term solution to possible operational problems. As our results suggest, this is likely due to the effect of mass-immigration from the recipient plant, but the influence of organic compounds in influent, unknown in this study, could not be excluded. However, importantly, the fact that the overall operational performance of the recipient plant was not impaired by the transplantation, indicated that the activity of the transplanted community worked very well over time despite the disturbance, probably because of intrinsic functional redundancy, partially determined by the immigration. This suggested that, for example, severe operational problems at WWTPs related to the microbial activity (e.g., community deterioration caused by the presence of toxic chemicals), could be temporarily solved altering a compromised or problematic community structure by adding significant amounts of "compatible" biomass and so possibly mitigating full-scale problems. In addition, transplantation could be applied to speed up the performance of newly constructed AS plants, similarly to bioaugmentation approaches (Guo et al., 2010). In both examples, the transplanted community should ideally be as similar as possible ("compatible") to the receiving AS community before the break-down or start-up; however, when this is not possible, AS from a plant with similar process design and/or similar functional guilds could also help to restore or initiate its function. For these applications, a database covering community composition of WWTPs in the same region or country would be useful to evaluate the best "match" between plants with the criteria just mentioned. Such database exists for Danish WWTPs as part of the MiDAS project, where we have recorded the normal community structure and temporal variations for several years (Nierychlo et al., 2020a). The database represents the starting point to explore theoretical and practical aspects of microbial ecology in AS systems, such as AS transplantation.

5. Conclusions

- We conducted a massive transplantation of activated sludge between two full-scale WWTPs with similar process type (biological removal of nitrogen and phosphorus) and performance, but with some differences in microbial community structure.
- The microbial community in the recipient plant showed a strong resilience, and most of the community structure was restored within 1-2 months. The treatment performance was not compromised, and the effluent quality remained stable. We did not observe a stable addition of new species to potentially introduce new functions or to boost functional redundancy; moreover, we did not observe a permanent increase of the abundance of shared species (e.g., the PAOs), nor a reduction of the abundance of unwanted bacteria (e.g., filamentous bacteria) in the recipient plant.
- The microbial community structure of AS suggested a close connection to their respective IWW communities, which may explain the high resilience of the plant. Microbial immigration is therefore an important aspect to consider for the application of transplantation applications and bioaugmentation in general in AS systems.
- Even if the community structure cannot be permanently altered, massive bioaugmentation of AS across different WWTPs may solve acute operational problems by selecting a compatible AS donor plant, which should have the same functional guilds, similar process design and operation, and, if possible, similar immigrating community.

Authors' contributions

PHalN, MSB and MN designed the full-scale experiment; PHenN and MSB arranged the transplantation; MSB performed the analysis of the process parameters and the initial community analyses; DSW and PHalN carried out the initial data analysis, data interpretation, and wrote the first paper draft; SK conceived and performed the statistical analysis; GD performed the molecular work, carried out new data analysis, and supported the statistical analysis; TYM and RW assisted with the statistical analyses; KSA performed initial bioinformatic analyses, quality checks and uploaded the sequences; MP suggested and supported the species residence time modeling; GD, MN, and MP supported data interpretation; GD and PHalN wrote the final version of the manuscript.

All authors read and commented on various draft stages of the manuscript text.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The bacterial amplicon sequences are available at NCBI Sequence Read Archive with accession number PRJNA815941. The raw amplicon data and the samples metadata are available as Supplementary Material (Raw amplicon data and metadata). The R scripts to analyze the data and to generate the Fig.s are available at: https://github.com/GiuDott/Transplantation_study.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.119454.

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