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Genome-level insights into the operation of an on-site biological wastewater treatment unit reveal the importance of storage time

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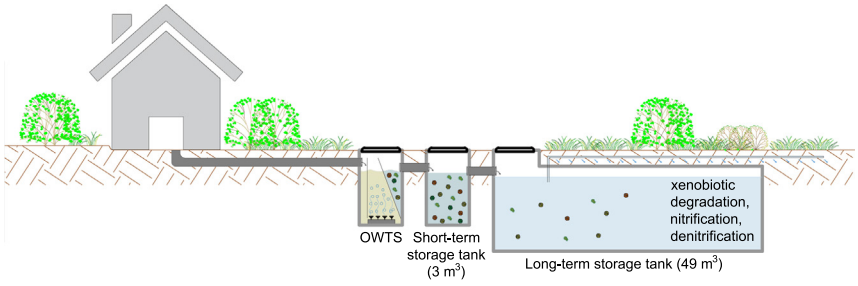
HIGHLIGHTS

- On-site wastewater treatment systems benefit from long term storage of effluent.
- Long-term storage reduces the abundance of pathogenic organisms.
- Long-term storage aids in the degradation of organic micropollutants.
- Denitrification continues during storage.

GRAPHICAL ABSTRACT



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ABSTRACT

On-site wastewater treatment systems are gaining popularity in areas where centralized wastewater treatment is not available. In the current case study a domestic activated sludge system was investigated, where treated effluent was stored in a short-term (1 week turn-over time) and a long-term (over 2–3 months) storage tank and was then used for irrigation. This design provided a unique opportunity to assess the chemical and microbial changes of the effluent upon storage. Long-term storage greatly improved both the chemical quality and the degradation efficiency of most organic micropollutants examined, including petroleum hydrocarbons and the pesticide diethyltoluamide. Taxonomic profile of the core microbiome of the effluent was also influenced upon storage. Relative abundance values of the members of *Azoarcus* and *Thauera* genera, which are important in degrading polycyclic aromatic hydrocarbons compounds, clearly indicated the biodegrading activity of these microbes across samples. The abundance of xenobiotics degradation functions correlated with the observed organic micropollutant degradation values indicating efficient microbial decomposition of these contaminants. Functions related to infectious diseases also had the highest abundance in the short-term storage tank corresponding well with the relative abundance of indicator organisms and implying to the significance of storage time in the elimination of pathogens. Based on these results, small, on-site wastewater treatment systems could benefit from long-term storage of wastewater effluent.

1. Introduction

Scarcity of freshwater is a growing problem worldwide. As a consequence, it is expected that treated wastewater will be increasingly used for food crop irrigation (Poustie et al., 2020). Water quality of treated wastewater gains high priority as wastewater reuse increases in arid regions (Bunke et al., 2019). Thus, sustainable and cost-effective wastewater treatment technologies are of increasing importance. The basic principle of municipal wastewater treatment in cities and large communities is to collect and transport sewage into centralized wastewater treatment plants (WWTP) that are capable of treating large volumes of wastewater. A number of Central and Eastern European countries have initiated intensive development of wastewater treatment

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processes since the mid 2000's to meet EU standards and requirements. Approximately 75% of the population in these countries is connected to centralized public sewage systems (European Environmental Agency, 2020). Small settlements are often remote and affected by outward migration and an aging population. Above all, they are heavily burdened by high investment costs in sewerage construction for common wastewater disposal. Therefore, many of these settlements are without proper wastewater treatment.

In rural areas decentralized approaches provide alternative treatment options. Decentralized clustered or onsite wastewater treatment systems (OWTS) are considered cost-effective by collecting, treating and disposing sewage at the site of generation. Properly functioning onsite systems represent a viable alternative in locations where pipeline construction is not possible or financially not feasible (Whitehead and Geary, 1999).

In Central and Eastern Europe about 30% of the population, in Western Europe less than 20% of the population live in settlements with less than 2000 inhabitants, while globally about half of the world population live in rural areas (Capodaglio et al., 2017). Many of these areas have been utilizing onsite solutions. In the US, more than one in five households depend on individual onsite or small community cluster systems (septic systems) to treat their wastewater (EPA, 2020). In Canada 10%, in Australia 12%, in Germany about 15% of the population use OWTS (Abbassi et al., 2018).

There is a wide spectrum of onsite solutions from pit privies through septic tanks, aerobic treatment units (ATU) and composting toilets to sophisticated installations producing water suitable for human consumption (Massoud et al., 2009; US Environmental Protection Agency, 2002). Conventional OWTSs comprise an anaerobic septic tank and a soil absorption system (Bradley et al., 2002). They are considered as poor pretreatment systems and sources of groundwater pollution with nutrients (Cogger, 1988) (Humphrey Jr. et al., 2013) and organic micropollutants (Elliott et al., 2018), though innovative modifications of septic tanks may overcome some of these challenges (Abbassi et al., 2018). Numerous alternative technologies to the septic tank system exist where microbes are incorporated in the aerobic system either in the form of activated sludge (AS) or attached biomass (Capodaglio et al., 2017). Microbes play a crucial role in system performance (Stevik et al., 2004). They have an important role in treating wastewater even in conventional OWTS, mostly in the soil treatment unit; microbes form biofilms at the soil infiltrative surface and remove or destroy pathogens and nutrients. Tomaras et al. (2009) analyzed this biofilm and found that Proteobacteria, Bacteroidetes and Acidobacteria were present in significant abundance and the biofilm effectively removed *E. coli* (85–90%). Still, there is little information about biofilm structure and composition on the removal of undesirable bacteria from infiltration systems.

On the other hand, the microbial diversity of municipal activated sludge wastewater treatment plants has recently been well characterized. Wu et al. (2019) collected ~1200 AS samples from 23 countries from 6 continents and revealed a small, global core bacterial community with 28 operational taxonomic units linked to AS performance with Proteobacteria and Bacteroides being the two most abundant phyla. The microbial composition of treated wastewater effluent is greatly influenced by the activated sludge microbiome (Cai et al., 2014). Studies revealed that the majority of microbial community of conventional and natural wastewater treatment system effluents is composed of Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes (Adrados et al., 2014; Cai et al., 2014; Do et al., 2019; Mansfeldt et al., 2020; Numberger et al., 2019). Microbes in the effluent may colonize receiving waters, as has been shown for nitrifiers (Mußmann et al., 2013) or alter the microbial community of receiving waters (Mansfeldt et al., 2020). Although most wastewater treatment reduces pathogen number during treatment, the efficiency greatly depends on the treatment type and residence time. Indicator organisms removal, such as *E. coli, Enterococci* and *P. aeruginosa* was more efficient in membrane bioreactor (MBR) systems compared to conventional systems, though total removal was not achieved in either case (Ng et al., 2019). It has been shown that OWTSs with activated sludge systems are more efficient in terms of treatment efficiency compared to septic tanks (Garcia et al., 2013), but less efficient than biofilm-based systems (Moelants et al., 2008). When the effluent is used for irrigation, soil or plants contamination with pathogens may pose a health hazard (Ibekwe et al., 2018), hence, it is important to regularly monitor and follow the fate of microorganisms in wastewater effluents.

The microbial communities of ecological WWTPs and the microbes' potential to metabolize pharmaceuticals were considered by Balcom et al. (2016). However, there has been no in-depth analysis of any domestic OWTS that would analyze the effluents' microbial community using high-throughput shotgun metagenome sequencing and link the presence of specific microbes to selected functions. In the current case study, the quality and microbial composition of the effluent from a typical onsite activated sludge system was analyzed that has been in use at more than 1000 homes and several small settlements in Hungary. The small wastewater treatment system is unique because owners supplement the system with a short and a long term storage tanks, allowing for the analysis of treated effluent after storage.

There have been indications that storing wastewater effluent might improve the quality of water (Ávila et al., 2013), nevertheless, this is the first in-depth study to analyze the effect of storage in a real domestic set-up.

2. Materials and methods

2.1. Small equipment and treatment process

The wastewater treatment unit (Ökotech-Home Ltd.) analyzed in the study is an activated sludge system. After entering into the unit, the sewage flows through a sieve located on the top of the anaerobic chamber, removing debris and larger materials, e.g. toilet paper, fecal matter which due to bubbling break up and enter the anaerobic chambers where fermentation and denitrification take place. The activated sludge continuously circulates between the two anaerobic chambers. At the lower region of the anaerobic chamber, the sewage together with the sludge moves into the anoxic chamber and then, by the help of two tilt-openings and a transfer tube, flows into the bottom of the aerobic chamber. The aerated wastewater enters the bottom of the post-settler from the top of the aerobic chamber. Treated wastewater is drained from the post-settler through a flow-controlled effluent pipe into the recipient, e.g. infiltration unit, surface water (requires permit), or a storage tank.

The activated sludge produced is continuously circulated by a pump, while excess sludge is pumped into a sludge thickening compartment. A photo and the schematic of the unit are shown in Fig. 1.

The main technical parameters of the unit are shown in Supplementary Table 1. The effluent quality was certified by an independent organization (values are shown in Supplementary Table 1). The Hydraulic Residence Time (HRT) is 1.41 d, whereas the sludge age is 14 d thanks to sludge recirculation, which keeps the major part of the biomass within the unit. Food to microorganism (F/M) ratio reflects that the system investigated has a low substrate load to one unit of biomass, operating as an extended aeration system. F refers to BOD in kg/day, while M refers to the biomass in kg (Mishoe, 1999). Despite the complex inner geometry and resistances caused by inner walls and baffles, 0.33 m head is enough to transfer the fluid through the system even at peak hydraulic load.

2.2. Characteristics of study site

The small wastewater treatment unit analyzed is located in a region of a municipality where sewer network is not available. The house belongs to a family of four who decided to use small equipment with an

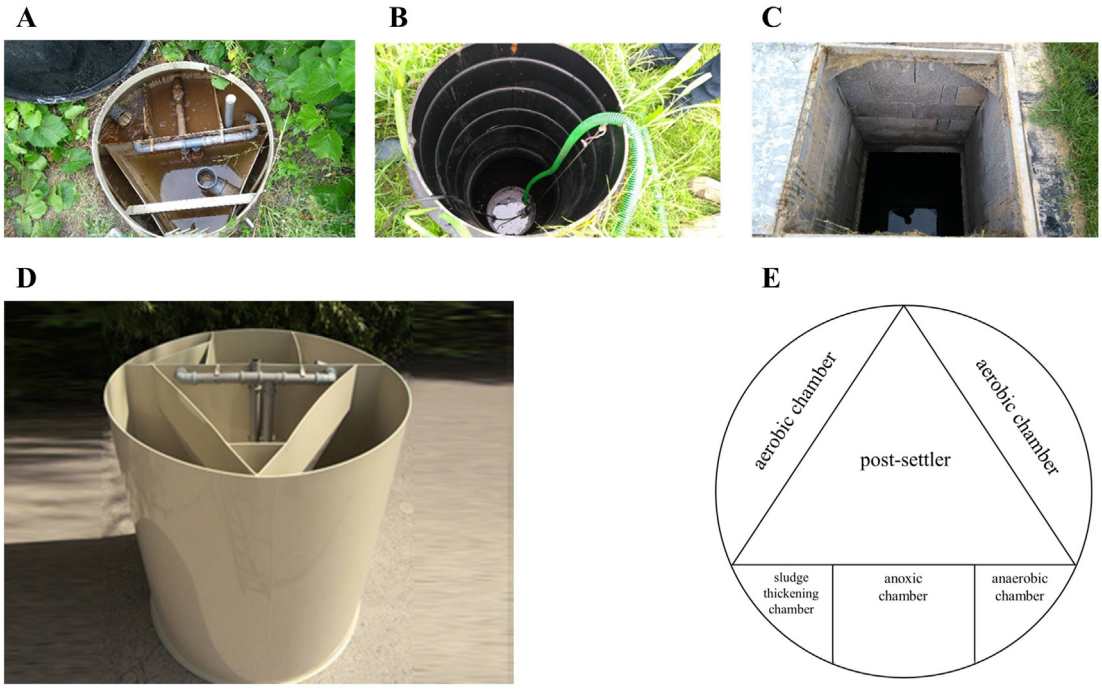


Fig. 1. Study site and the wastewater treatment unit. A. Treatment unit. B. 3 m^3 short-term storage container. C. 49 m^3 long-term storage container. D. Wastewater treatment unit. E. Schematic of the unit. Treated wastewater flows from the post-settler of the treatment unit (A) to a short-term storage tank (B) then into a long-term storage tank (C).

activated sludge system partly because of environmental awareness as well as of economic considerations; they use the treated water for irrigation. The site was used for the case study because it provides an opportunity to test several setups. The treatment unit (Fig. 1A) is designed for 6 population equivalents (PE). In many cases only this unit is installed and the effluent is either infiltrated into the soil root zone or could be discharged into a recipient, though this requires a permit. Those users who plan to use their water for toilet flushing, car washing, or irrigation collect the effluent in a storage tank (a few m^3, Fig. 1B). In the current site, the storage tank capacity is 3 m^3 with a turnover time of approximately 1 week. The study site is unusual in that it has an additional storage tank of 49 m^3 (Fig. 1C) allowing a long-term storage of treated water. Therefore, effluent from the post-settler flows into the short-term storage container from which the effluent flows over to the long-term storage tank. The rationale behind this large tank was that during winter the family cannot use the water for irrigation; instead they store and use it when needed during spring and drought season.

The family's daily water use is 0.54 m^3/day, the daily peak flow ($Q_{average}$) is 0.0675 L/s, and the maximum single load is 0.2 m^3. The access sludge is collected twice a year; after composting it is used as a fertilizer in the garden for bushes and trees.

This site provides a good opportunity to assess the effect of short and long term storage on chemical parameters as well as the microbial composition of treated water.

2.3. Sampling of treated wastewater

According to national regulations, qualified grab samples are required for sampling effluents from small equipment (Ministry of Environmental Protection and Water Management, 2008); thus, qualified grab samples were taken in a weekday in June 2019. A stainless steel sampler attached to a telescopic rod was used. The sampler was rinsed with ddH_2O, dried and disinfected by ethanol between sample points. Using the sampler, the water in the tanks was mixed by stirring before taking five grab samples with three to five minutes intervals. The grab samples were pooled and the pools were used for further analyses. The order of sample collection was long-term storage container (sample PC), short-term storage container (sample point PB), and the treatment unit (sample PA). Sampling from the long-term storage tank could only be performed at the only opening of the tank, i.e. ~ 1.5 m from the inlet of the short-term storage tank. The pooled samples were used for microbial community analysis, field tests and analytical tests. Water samples were transported into plastic or glass bottles and stored at 4 °C or frozen (for metagenome sequencing based community analysis) until use.

2.4. Analytical methods

On-site measurements of pH, dissolved oxygen (DO), conductance, oxidation reduction potential (ORP), and temperature were done by using the portable HQ40d digital two channel multi meter (Hach Lange) by applying the appropriate electrodes according to the manufacturers' instruction. Sludge volume index (SVI) was determined by the following. 1 L of mixed liquor from the aeration basin was allowed to settle for 30 min in an Imhoff tank. The suspended solids concentration of the same mixed liquor (MLSS) was determined. SVI (mL/g) was calculated by dividing the measured wet volume (mL/L) of the settled sludge by the dry weight concentration of MLSS in g/L.

Measurements of COD, BOD_5, dissolved organic carbon (DOC), organic nitrogen, total nitrogen (TN), ammonium, nitrate, nitrite, Kjeldahl nitrogen, total phosphorus (TP), total suspended solids (TSS), organic solvent extracts (oils, fats), total salt (105 °C and 600 °C), anion active detergents, cation active detergents, non-ionic detergents, organic micropollutants, and metals were performed by the Bálint Analitika Ltd. accredited laboratory (Budapest, Hungary).

2.5. Ecotoxicology tests

Measurements were performed by the accredited laboratory of National Public Health Center (Budapest, Hungary). Undiluted and 2×, 5× and 10× diluted samples were used for each test. Germination test

assessing the toxicology of water for irrigation using white mustard (*Sinapsis alba*) was performed according to standard MSZ 22902–4:1991. The test duration was 72 h. After germination, root length was measured, the average of parallel samples was calculated and expressed as a percent compared to control root length and evaluated according to the following: 0–9,9% - very potent inhibitor (highly toxic); 10–29,9% - potent inhibitor (very toxic); 30–59,9% - inhibitor (moderately toxic); 60–84,9% - mild inhibitor (mildly toxic); 85–114,9% - no effect (non-toxic); > 115% - stimulating effect.

Acute toxicity test was performed on *Daphnia magna* Straus neonates (<24 h) according to standard MSZ EN ISO 6341:2013. The test duration was 48 h. The number of Daphnia immobilized ($EC50_i$) is recorded after 24 and 48 h. Twenty Daphnia were used for each dilution. 10% immobility is accepted in the control, too. *Daphnia magna* at the age of 4–6 days were also used according to standard MSZ 21978–13:1985.

Algal growth inhibition test was performed by using *Pseudokirchneriella subcapitata* according to standard MSZ EN ISO 8692:2012. Test duration was 72 h. Results are expressed in percent growth inhibition compared to control.

2.6. ATP bioluminescence assay

Second generation adenosine triphosphate (ATP) measurement assay (Luminultra Technologies Ltd., Canada, Quench-Gone Aqueous Test Kit, Product #: QGA-100) was used to detect intracellular ATP indicating living biomass. Measurements were performed according to the manufacturer's instructions (Luminultra, 2010) using a PhotonMaster Luminometer (Luminultra). Measurements of the same samples were repeated twice to get the standard error of method performance.

2.7. Bacteria enumeration

Measurements were performed by the accredited laboratory of a regional municipal waterworks (E.R.Ö.V Ltd., Szekszárd, Hungary). Samples were collected in sterile bottles and immediately cooled to and stored at 4 °C. Samples were measured in 24 h following sample collection. Heterotrophic plate counts were performed according to the MSZ ISO 6222:2000 standard. Detection of *Salmonella* sp. was done following the protocol of standard MSZ 318–27:1986. Enterococci and thermotolerant coliforms were analyzed according to standards MSZ 318–27:1986 and MSZ ISO 9308-2:1993, respectively.

2.8. DNA extraction

2-mL liquid fermentation samples were collected for total community DNA isolation by applying a cetyltrimethylammonium bromide-based DNA extraction buffer (Miller et al., 1999; Wirth et al., 2012). Cell lysis was carried out at 55 °C overnight. Phenol:chloroform (1:1) was used to extract contamination; the genomic DNA was precipitated with ethanol (90%). The DNA pellet was resuspended in 100 μL of Tris–EDTA buffer. The DNA content and quality was determined in a Tapestation 2200 System (Agilent Technologies). The described isolation method yielded pure (A260/A280⩾ 1.8) and sufficient amount of total DNA (200–800 ng/μL).

2.9. DNA sequencing and data handling

Isolated total metagenome DNA was used for library preparation. In vitro fragment libraries were prepared using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq® 500/550 High Output Kit v2 (300 cycles). The read cluster numbers were the following: 413.160 for the post-settler (PA), 402.384 for short-term storage tank (PB) and 465.400 for long-term storage tank (PC) (Accession number: PRJNA666055). Primary data analysis (base-calling) was carried out with Bbcl2fastq^ software (v2.17.1.14, Illumina). Reads were quality and length trimmed in CLC Genomics Workbench Tool 9.5.1 using an error probability of 0.05 (Q13) and a minimum length of 50 nucleotides as threshold. Trimmed sequences were further analyzed for taxonomic identification and functional assessment.

2.10. Read trimming and taxonomic classification

Reads were trimmed using BBDuk tool within the Bbmap utilities (version 38.34). Trimmed reads were classified first with Kraken2 (version 2.0.8) followed with species level estimation using Bracken (version 2.5.0). To remove false positives, bracken was run with a threshold of 5 reads. Any taxonomic classification that had fewer than 5 reads were removed and named as unclassified.

A custom database was created with genomes from Archaea, Bacteria, Fungi, Plasmid, Protozoa, Viral, Nt and Chlorophyta databases for both kraken2 and bracken classification.

All analysis and graphs were created in Rstudio (version 3.6.1). Species richness was calculated with the Divnet package. Graphs were built using ggplot2.

2.11. Functional analysis of metagenomics samples

All reads were first assembled using Megahit. Generated contigs were functionally and structurally annotated using Prokka (version 1.11). Trimmed reads were then mapped against assembled contigs and used to quantify abundance annotated contigs with EC numbers using Bowtie2 (version 2.3.4.3). Metabolic pathways were predicted using Minpath (version 1.4) and EC number of annotated contigs. Abundance was estimated using Htseq (version 0.11.3).

All counts were normalized and converted into TPM (Transcripts per million reads). Normalization using TPM allows us to take into account both the total number of reads mapped along with the length of the gene to which the reads are mapped to. Functional maps were generated using Krona and an in-house Rscript.

2.12. Metagenome co-assembly, gene calling, binning

After simplifying the header of contig FASTA file and filtering the contigs by length (min. Contig length: 1500) using the Anvi'o script "reformat-fasta", Bowtie2 (v.2.3.4) was equipped to map back the original sequences to the contigs (Langmead and Salzberg, 2012). Following the "metagenomics" workflow (Eren et al., 2015), Anvi'o (v.6.1) was used. The main steps include the following. Contig database was generated, open reading frames were identified by Prodigal (v.2.6.3) and each contig k-mer frequencies were computed (Hyatt et al., 2010). The Hidden Markov Model (HMM) of single-copy genes (SCGs) were aligned by HMMER (v.3.3.1) using GTDB database. InterProScan was used (v.5.31–70) on Pfam for the functional annotation of contigs (Finn et al., 2017; Finn et al., 2014; Jones et al., 2014; Menzel et al., 2016). The outputs were imported into the contig database by using the "anvi-import" command. BAM files by Bowtie2 were used to profile contig database to obtain sample-specific information about contigs (i.e. mean coverage) (Langmead and Salzberg, 2012). The sample-specific information was merged using the "anvi-merge" command. Three automated binning programs, namely CONCOCT (v.1.1.0), METABAT2 (v.2.3.0) and MAXBIN2 (v.2.2.4) were employed to reconstruct microbial genomes from contigs (Alneberg et al., 2013; Kang et al., 2015; Wu et al., 2015). The combination of Metawrap (v.1.2.4) and Anvi'o human-guided refine option were used to improve the quality of metagenome assembled genomes (MAGs). For taxonomic assignment of bins, GTDB and Miga genomic databases were utilized. Consensus results were applied to name specific MAGs.

2.13. Estimation of functional pathway completions

Prokka was employed to translate and map protein sequences (create protein FASTA file of the translated protein coding sequences) (Seemann, 2014). For the calculation of module completion ratio (MCR) MAPLE (v.2.3.2) (Metabolic And Physiological potentiaL Evaluator) was used (Arai et al., 2018). This automatic system is mapping genes on an individual genome and calculating the MCR in each functional module defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Guto, 2000). Only MAGs having medium or above quality were included in the calculation.

3. Results and discussion

The main goal of this study was to analyze the microbial composition of an OWTS effluent and its microbial and chemical change over storage. We aimed to identify and correlate specific microbes and pathways contributing to organic matter degradation. The hypotheses driving the present study were the followings: (1) organic matter, including organic micropollutants removal could be improved by storing the effluent, (2) storage decreases the abundance of pathogenic organisms, (3) the effluent produced by the domestic OWTS is suitable for irrigation when appropriate storage conditions are applied.

3.1. Effluent quality

The post-settler as well as the short-term and long-term storage tanks were sampled and analyzed for various chemical parameters. Sludge settling was also measured in the aerobic chamber (600 mL/L) and the sludge volume index (SVI) was calculated (153.97 mL/g; excellent: <80, moderate: 80–150, poor: >150), indicating poor sludge settling quality. All parameters regulated by Decree No. 28 of 2004 (XII.25.) (Ministry of Environmental Protection and Water Management, 2004) in wastewater treatment systems below 600 PE, i.e. COD, BOD_5 and TSS were met in each sampling points. However, they were all above the typical effluent values the unit should achieve, according to the manufacturer. Storage improved most chemical parameters examined, except total salts and cation active detergents.

Effluent TN (156 mg/L), TP (14.7 mg/L), and ammonium (158 mg/L) content also exceeded typical effluent values, though they decreased over storage (Table 1). From an environmental point-of-view, TN and TP values are important effluent parameters, as they can cause eutrophication upon entering surface waters. Many regulations do not include limits for small units; rather, local limits are applied if the recipient is surface water. When effluent water is used for irrigation, higher TN and TP values may even be beneficial as they provide important nutrients for plants (Poustie et al., 2020).

Removal efficiency of various components can only be determined if both raw and treated sewage are analyzed. Grab sampling does not give an accurate result, as raw sewage composition greatly differs depending on family activities; thus, composite sampling should be performed. As there was no opportunity to perform composite sampling, design parameters described in Metcalf and Eddy (2003) were used to assess treatment efficiency for the removal of COD, BOD, TSS, TN and TP (Table 1). In the literature, there is a wide range of data on design parameters, e.g. for TN= 24–750 mg/L (Metcalf and Eddy, 2003), explaining why effluent parameters for TN and TP are higher than the design parameters (Mikola et al., 2009).

Although the effluent complies with legal limits, the momentary performance lags behind the aimed performance of the unit. The reasons behind this may be various. One possible explanation is that real time situations may differ from a testing situation. However, another

Table 1
Chemical parameters measured in water samples from the post-settler (PA), after short (PB) and long term (PC) storage.

Parameters	Unit	Raw influent design parameters	Typical effluent values of the unit[c]	Legal limits of effluents below 600 PE[a] in Hungary	Post-settler (PA)	Short-term storage tank (PB)	Long-term storage tank (PC)
pH			–		8.1	10.72	9.37
Dissolved oxygen	mg/L		–		1.89	1.43	2.68
	%		–		23.1	17.4	30.6
Conductance	μS/cm		–		1209	1111	1087
ORP	mV		–		45.6	91.2	126.5
Temperature	°C		–		24.9	24.3	25.5
COD	mg/L	1000	55	300	159	80	<30
Estimated removal efficiency[b]					84%	92%	100%
BOD_5	mg/L	500	15	80	48	22	5
Estimated removal efficiency[b]					90%	96%	99%
DOC	mg/L		–		25.3	21.5	14.6
Total inorganic nitrogen (ammonium, nitrite, nitrate)	mg/L		–		123	99	77
Organic nitrogen	mg/L		–		33	7	5
Total nitrogen	mg/L	116	20		156	106	82
Estimated removal efficiency[b]					0%	9%	29%
Ammonium	mg/L		10		158	127	96
Nitrate	mg/L		–		0.5	0.6	4
Nitrite	mg/L		–		0.39	0.05	4
Kjeldahl nitrogen	mg/L		–		156	106	80
Total phosphorus	mg/L	10	5		14.7	7.72	7.7
Estimated removal efficiency[b]					0%	23%	23%
Total suspended solids	mg/L	625	20	100	80	30	4
Estimated removal efficiency[b]					87%	95%	99%
Organic solvent extracts (oils, fats)	mg/L		–		8	4	<2
Total salt 105 °C	mg/L		–		1164	1144	1228
Total salt 600 °C	mg/L		–		744	676	728
Anion active detergents	mg/L		–		1.7	0.6	0.2
Cation active detergents	mg/L		–		<0.2	0.3	0.3
Non-ionic detergents	mg/L		–		0.4	0.6	<0.3

[a] According to Decree No. 28 of 2004 (XII. 25.) KvVM of the Ministry of Environmental Protection and Water Management concerning emission standards of water-pollutant substances and laying down rules of application.
[b] Estimated removal efficiency is calculated based on design parameters.
[c] According to the manufacturer.

study analyzing a unit of the same type at a different municipality indicated that strict owner behavior results in quality parameters meeting all typical effluent parameters except TP in the post-settler, but a short-term storage (2 m^3) tank resolved the TP value as well (Knisz et al., unpublished results). Indeed, a questionnaire addressing the maintenance behavior of the owner of the unit described in the current study indicated that the owner looks at the unit once per month instead of weekly check-ups as recommended by the manufacturer and almost never fills in the maintenance log sheet (data not shown). Moelants et al. (2008) have shown that treatment units with a maintenance contract perform significantly better than those without one. The exact cause behind the observed phenomenon, however, requires further analyses.

3.2. Presence of organic micropollutants and toxic chemicals

Next the presence of some organic micropollutants was analyzed in the treated wastewater. By using a questionnaire, the antibiotic and chemical use of the owners was assessed. As they had not used antibiotics or any other medication, pharmaceuticals were not measured in the water. Total petroleum hydrocarbons (TPH), including polycyclic aromatic hydrocarbons (PAH), as well as heavy metal content of the water were analyzed. As the house is located next to an agricultural field, pesticide content was also assessed (Table 2).

All the measured micropollutants with environmental limits were below the limit values except PAH, which is limited between 0.015 and 0.03 μg/L depending on the recipient (Ministry of Environmental Protection and Water Management, 2004). Among naphthalenes, naphthalene contributes the most to higher PAH values. Naphthalene has been identified as a public health concern. Naphthalene concentrations are usually higher indoors than outdoors and moth balls were associated with increased levels. However, building materials, furnishing, especially vinyl furniture and wall paints could be the dominant sources of household exposure (Kang et al., 2012), but how it entered the present wastewater system is unclear. Degradation of PAH compounds and naphthalenes is much less pronounced over storage (~20%) compared to other micropollutants measured. Indeed, naphthalene has been shown to meet the P (persistent) and vP (very persistent) criteria in PBT assessment, i.e. persistent, bioaccumulative, and toxic, but does not meet the B and T criteria (ECHA, 2007), which may explain its persistence over storage.

Out of the 100 pesticides measured, only two were detected in the samples at concentrations of 3.5, 2.8 and 0.12 μg/L in the post-settler, short-term and long-term storage tanks, respectively. Diethyltoluamide (DEET) is a widely used insect repellent and likely comes from the household and continues to degrade during storage. It is often detected in sewage plants as well as in groundwater (Montes-Grajales et al., 2017) and considered as an emerging pollutant (NORMAN Network). The other pesticide, MCPA is a systemic hormone-type selective herbicide, readily absorbed by leaves and roots (WHO, 2003). It was only measured in the short-term storage tank in very low (0.03 μg/L) concentration, suggesting that it might have entered the storage tank from the surrounding agricultural environment.

Toxic metal discharge into rivers, irrigation with water containing heavy metals can pose a threat to aquatic life, agricultural products as well as to groundwater. Domestic wastewater usually contains less metals as commercial or industrial wastewater (ICON, 2001), and their use for irrigation is considered a safe wastewater disposal practice (Kim et al., 2015). Still, certain chemicals, residential practices may result in the presence of toxic metals in the effluent in concentration exceeding limits. To assess the presence and the amount of toxic metals in the effluent, 59 metals were analyzed in all three sampling points. Out of the metals analyzed, 18 were detected and they were all below environmental limits (Supplementary Table 2).

Table 2
Organic micropollutants detected in the treated wastewater.

Components	Unit	Post-settler (PA)	Short-term storage tank (PB)	Long-term storage tank (PC)
PAHs				
naphthalene	μg/L	0.022	0.022	0.017
2-methyl-naphthalene	μg/L	0.009	0.011	0.01
1-methyl-naphthalene	μg/L	0.008	0.006	0.005
fluorene	μg/L	0.004	0.004	0.003
phenanthrene	μg/L	0.011	0.01	0.007
fluoranthene	μg/L	0.002	0.003	0.002
pyrene	μg/L	0.003	0.003	0.002
Total naphthalenes	μg/L	0.039	0.039	0.032
Total PAH without naphthalenes	μg/L	0.02	0.02	0.014
Total PAH	μg/L	0.059	0.059	0.046
TPH				
C 5–12	μg/L	34.3	10.1	1.9
C 13–40	μg/L	136	58.7	31.7
TPH-GC		170	68.8	33.6
Pesticides				
Diethyltoluamide (DEET)	μg/L	3.5	2.8	0.12
MCPA	μg/L	nd	0.03	nd
Total pesticides	μg/L	3.5	2.83	0.12

nd: not detected.

3.3. Ecotoxicological quality of treated wastewater

Ecotoxicological tests were performed to assess the toxicity of treated wastewater. Samples from the post-settler (PA) and from the long-term storage tank (PC) were analyzed. Data were not obtained for the short-term storage tank (PB). The 10× diluted sample from the post-settler had a stimulating effect on the germination of mustard seeds; all the other samples had neither stimulating nor inhibiting effect (Supplementary Fig. 1). According to the standard, wastewater effluent can be used for irrigation, even before storage.

Since the treated wastewater was used for irrigation, its toxicity to aquatic organisms was investigated. 72 h algal growth inhibition test revealed, that the undiluted and 2× diluted samples from both the post-settler and the long-term storage tank inhibited algal growth, however, after dilution no inhibition was observed, thus, the effluent can be discharged into surface recipient after dilution (Supplementary Fig. 2).

In the case of *Daphnia* immobility test, non-diluted samples showed 100% immobility in both age groups (*Daphnia* neonates and 4–6 days old), while the 2× diluted sample of the effluent from the post-settler (PA) showed 10% immobility in 4–6 day-old *Daphnia*. The rest of dilutions did not result in *Daphnia* immobility; 10% immobility is allowed even in the control (Supplementary Fig. 3). Based on the results, after dilution the examined treated water is not considered toxic to the aquatic environment.

3.4. Microbial components of the treated wastewater

Conventional culturing techniques, e.g. heterotrophic plate counts can only measure less than 1% of the total biomass (heterotrophic cultivable organisms). To get information about the total living biomass of the samples, intracellular ATP testing of treated wastewater was applied. 2nd generation ATP testing methods were originally designed for use in biological wastewater treatment plants and have been shown to provide accurate results in wastewater treatment processes optimization (Whalen et al., 2018). As a single *E. coli* cell contains 1 fg of cATP, ATP concentration values can be used for putting ATP results on the same scale of measurements as colony forming units (CFU), often expressed as microbial equivalent or ME. Thus, 1 ME/mL equals to 10^3 pg/mL of cATP. ATP measurements revealed a steady, 2.5 and 2.7 fold decreases in total biomass as the effluent was stored in the

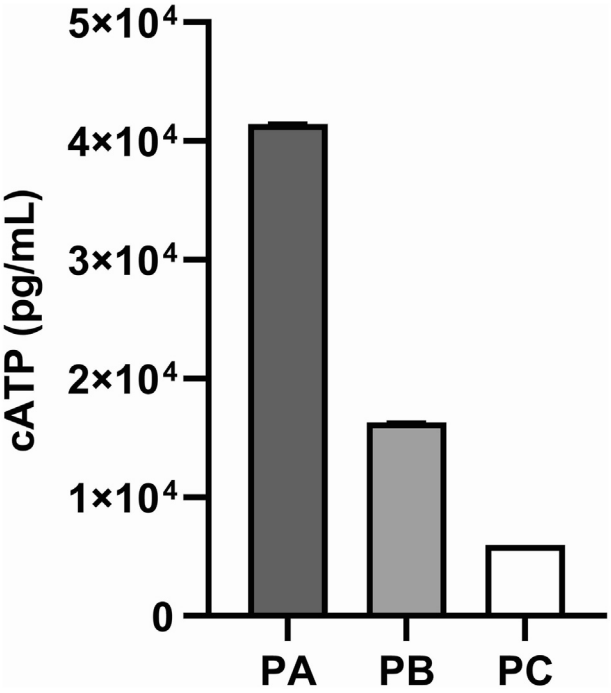


Fig. 2. Total biomass of treated wastewater measured by ATP bioluminescence assay. Error bars represent two parallel measurements of the same sample. PA: post-settler, PB: short-term storage tank, PC: long-term storage tank.

short-term and long-term storage tanks, respectively (Fig. 2). Heterotrophic plate counts revealed a similar 2.5 fold decrease between the post-settler and the short-term storage tank, but a more pronounced, 10 fold decrease of cultivable organisms was observed between the two storage tanks, suggesting that the decrease of cultivable microorganisms is more pronounced between the two storage tanks compared to the decrease of other, non-cultivable microbes (Supplementary Fig. 4).

3.5. Read classification and identification of microbial species

Next generation sequencing (Illumina NextSeq) was performed to analyze the entire microbiome present in the effluent that can come into contact with surface waters, plants, soil, or possibly with humans. The statistics for raw and trimmed reads are shown in Supplementary Table 3. About 40–50% of all reads were taxonomically identified (Supplementary Table 3).

The post-settler had the highest number of unclassified reads. The high ratio of unclassified reads suggests that the post-settler has a more diverse community compared to the other tanks. The number of reads taxonomically classified increases through storage, indicating that bulks of rare/transient species are lost as the effluent gets processed and nutrients are consumed. This result is also confirmed by the values of the Shannon's diversity index used to calculate species richness (Supplementary Fig. 5). This estimation also takes the abundance of different species into account.

The overall taxonomic distribution was obtained along the path of the effluent at phylum and family levels. A few important patterns emerge in the microbiome composition as the effluent moves from the initial post-settler to the long-term storage tank.

At phylum level (Fig. 3A) the long-term storage tank harbors the least diverse microbial community, with Proteobacteria being the most abundant phylum, just as in the other two tanks. Proteobacteria was also the most prominent phylum of the global core bacterial community (82%) (Wu et al., 2019).

Family level profile provides a more in-depth view of the microbial composition of the three tanks (Fig. 3B). Members belonging to the *Comamonadaceae* family reduced in abundance as the effluent was treated. The *Comamonadaceae* family is a diverse group with striking metabolic diversity including anaerobic denitrifiers, Fe^{3+} reducing bacteria, and aerobic organotrophs among many others (Willems, 2014). Recently, they have been identified as key polyphosphate accumulating organisms (PAOs) under low sludge retention time (SRT) conditions (Ge et al., 2015). Other PAOs *(Ge et al., 2015)* were also found in the samples analyzed, including *Tetrasphaera*, *Actinobacteria, Pseudomonas* and the well-characterized *Candidatus Accumulibacter phosphatis,* the last two with the highest abundance in the long-term storage tank. Along with the *Comamonadaceae* family, the family of *Rhodobacteraceae* also reduced in abundance.

As opposed to the two previous families, the abundance of the *Moraxellaceae, Pseudomonadaceae, Campylobacteraceae, Bradyrhizobiaceae,* and *Zoogloeaceae* family increased, among which the first two showed the most prominent increase. The family *Moraxellaceae* includes the *Acinetobacter* genus which can degrade petrochemicals (Teixeira and Merquior, 2014) and have been implicated in phosphorus removal (Kim et al., 1997). Some members of the genus *Acinetobacter* are human pathogens that can develop and transfer multidrug resistance. The human pathogen *A. johnsonii* and *A. baumannii* were found in all the three sampling sites with the highest abundance in the long-term settler. Indeed, it has been shown that *A. baumannii* is emitted from wastewater treatment systems through the effluent (Higgins et al., 2018), hence, their presence is a concern both environmentally and to the health of the OWTS users.

Members belonging to the family *Campylobacteraceae (Epsilonproteobacteria),* specifically the emergent pathogens of the *Arcobacter* genus, such as *A. cryaerophilus* and *A. butzleri* need to be monitored as these can cause infections in humans and animals. Their abundance increased in the samples during storage and they were highly abundant in the long-term storage tank. *Arcobacter* is abundant even in the effluents of large, well-working plants with advanced biological and chemical treatment (Kristensen et al., 2020).

The family *Rhodocyclaceae* and *Zoogloeaceae* belong to the order of Rhodocyclales together with the third family *Azonexaceae*. Members of these families increased in abundance as the effluent moved into the long-term storage tank. Members of the Rhodocyclales order are abundant in wastewater treatment systems and perform various functions. Many of its members participate in denitrification (Heylen et al., 2006) e.g. *Dechloromonas* which play an integral role in most treatment plants (Wu et al., 2019). *Candidatus Accumulibacter* participates in enhanced biological phosphorus removal (EBPR), while *Azoarcus* and *Thauera* are important in degrading PAH compounds (Wang et al., 2020; Zhang et al., 2018), all of which were present in the post-settler and storage tanks.

The abundance of *Pseudomonaceae* including *P. guangdongensis, P. alcaligenes, P. aeruginosa,* etc., many of which can be human pathogens, increased exponentially from the post-settler to the long-term storage tank. *Pseudomonas* species have also been implicated in the degradation of aliphatic hydrocarbons, and *P. putida* has been shown to be able to degrade DEET (Rivera-Cancel et al., 2007), which is an insect repellent commonly found in wastewater treatment plants and was also detected in each of the samples (Table 2). The increase of *Pseudomonas* spp. is closely followed by an increase in phages that infect *Pseudomonas* (Supplementary Fig. 6). Thus, phages could also be used to monitor the health of the microbiome community in future studies, as it has been suggested by McMinn et al. (2017).

3.6. Functional classification

The diversity of biological functions within the three tanks was studied using KEGG functional annotations. The KEGG annotations were hierarchically divided into Level 1 (Basic; e.g. metabolism, cellular

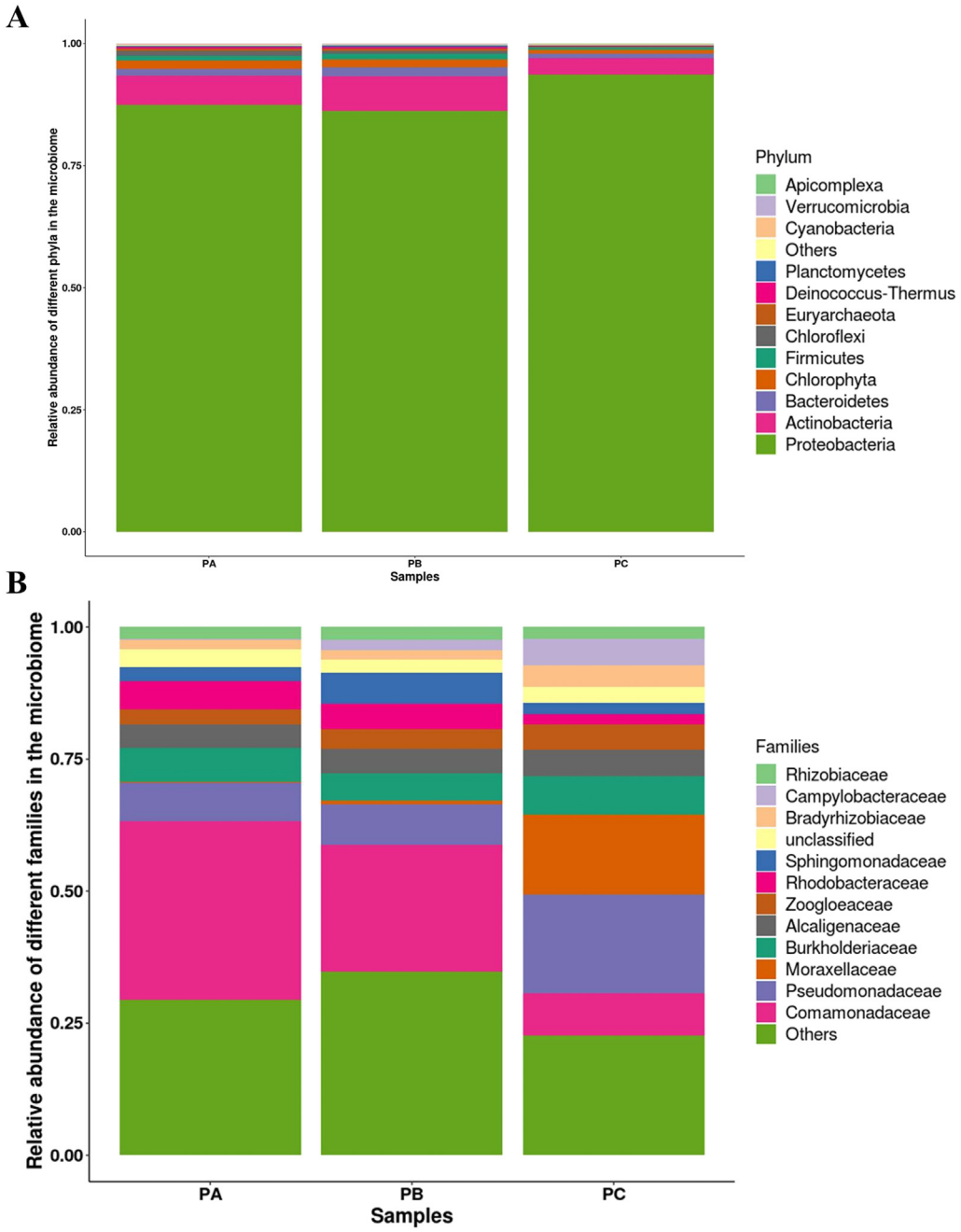


Fig. 3. Taxonomic profile of a household wastewater treatment unit and storage tanks at phylum (A) and family (B) levels. PA: post-settler, PB: short-term storage tank, PC: long-term storage tank.

processing), Level 2 (intermediate: e.g. lipid metabolism, Carbohydrate metabolism) and Level 3 (Specific: e.g. fatty acid degradation, lysine biosynthesis). By focusing on the second level of KEGG pathways, clear variations were observed between the 3 samples in terms of functional distributions (Fig. 4). The relatively low numbers of mapped reads are explained by the applied approach, that only contigs with EC numbers were utilized (Supplementary Table 3). The numbers of reads with EC numbers identified is shown in Supplementary Table 3.

Importantly, functions related to infectious diseases (parasites, bacteria and viruses) had higher abundance in the short-term storage tank compared to the post-settler and the long-term storage tank. This indicates that conditions in the short-term storage tank were more suitable for pathogens than in the other tanks. Furthermore, abundance of functions related to xenobiotic biodegradation and metabolism gradually reduced as the effluent moved from the post-settler to the long-term storage tank. This is an indication that pollutants in the effluent are slowly being degraded as they move into the long-term storage tank, which is consistent with the decreased concentration of most organic micropollutants in the long-term storage tank. Other functions that have a lower abundance in the long-term storage tank belong to lipid metabolism and the metabolism of terpenoids and polyketides.

3.7. Presence of indicators of sanitary quality

As the short-term storage tank seemed to be a suitable environment for pathogenic microorganisms, the presence of indicator organisms was analyzed that have been used to predict the risk associated with water usage over 100 years. The most commonly used indicator bacteria include thermotolerant (or fecal) coliforms (*Klebsiella, Enterobacter, Citrobacter, Esherichia),* enterococci, and *Clostridium perfringens*

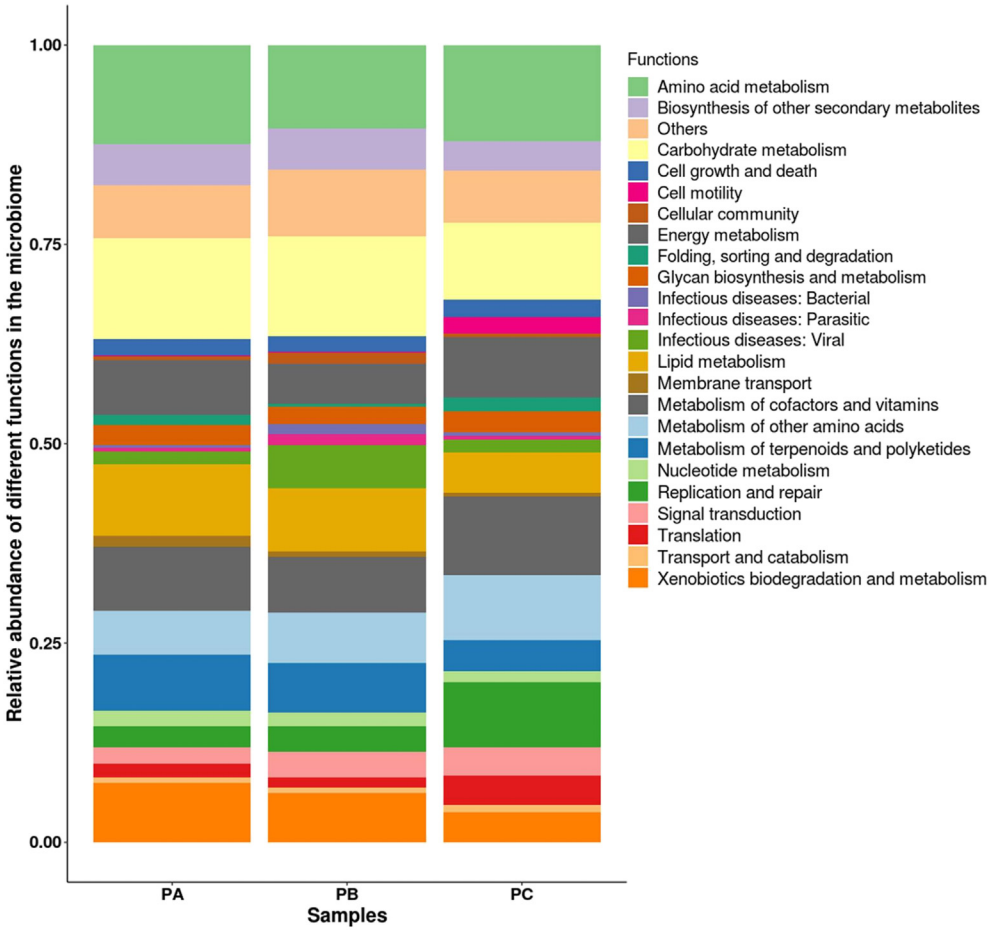


Fig. 4. Barplot of abundance across different functions at Level 2. PA: post-settler, PB: short-term storage tank, PC: long-term storage tank.

(Ashbolt et al., 2001). These indicators fail to detect non-bacterial pathogens; thus, viruses and protozoa indicators have been proposed (Ashbolt et al., 2001; De Luca et al., 2013), e.g. F-RNA coliphages, *Bacteroides fragilis* bacteriophages (Ashbolt et al., 2001), pathogenic viruses (norovirus GI and GII, adenovirus), and protozoan parasites (*Giardia* and *Cryptosporidium*).

Traditional culture tests were performed to enumerate enterococci and thermotolerant coliforms (Fig. 5B) as well as the abundance of

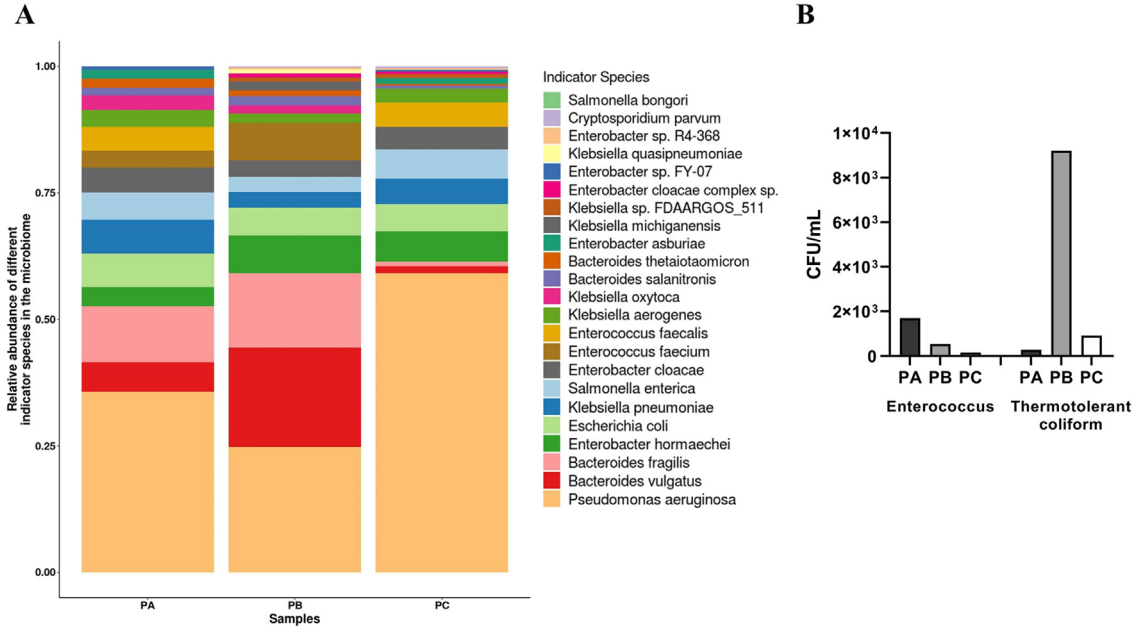
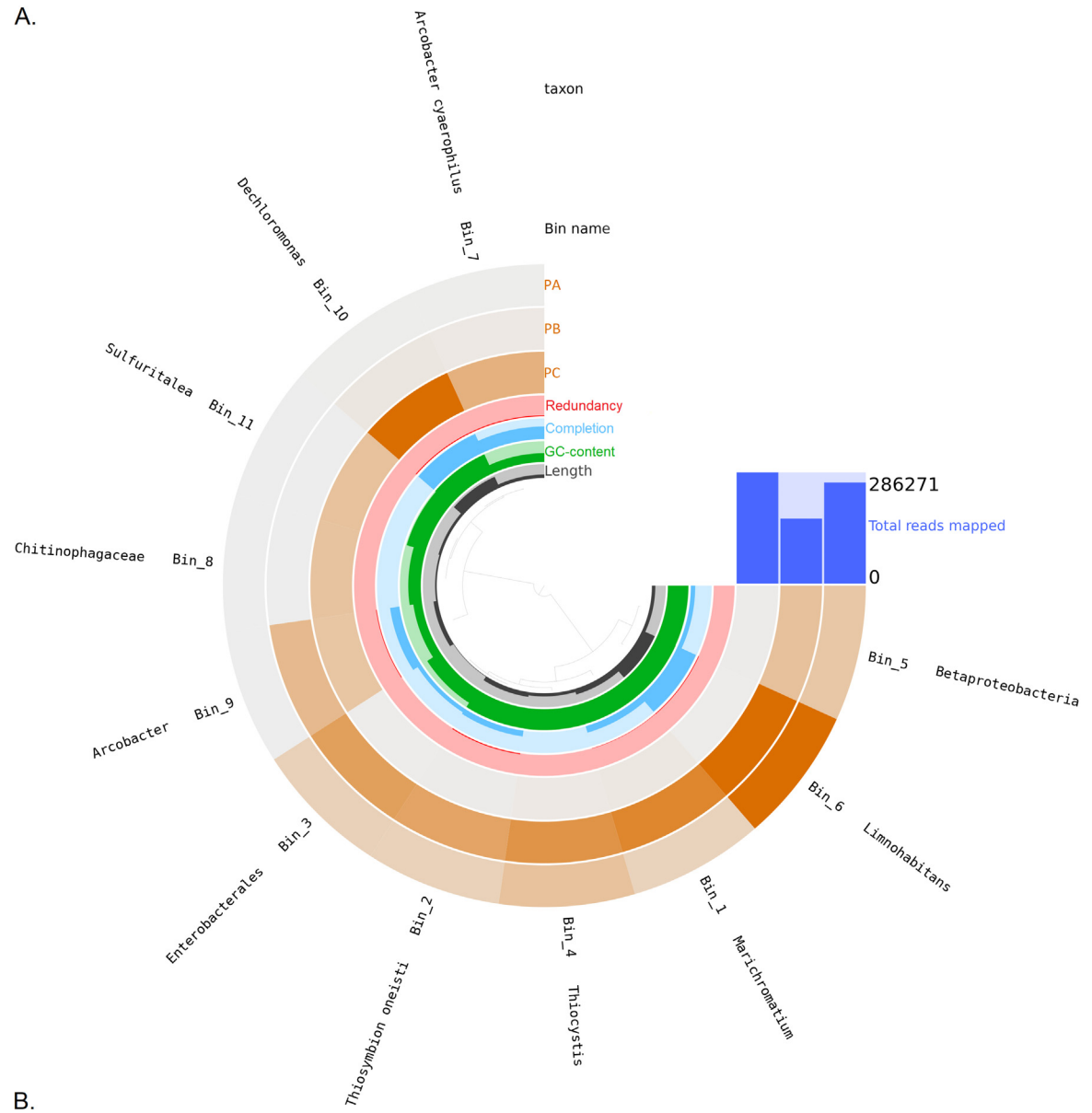
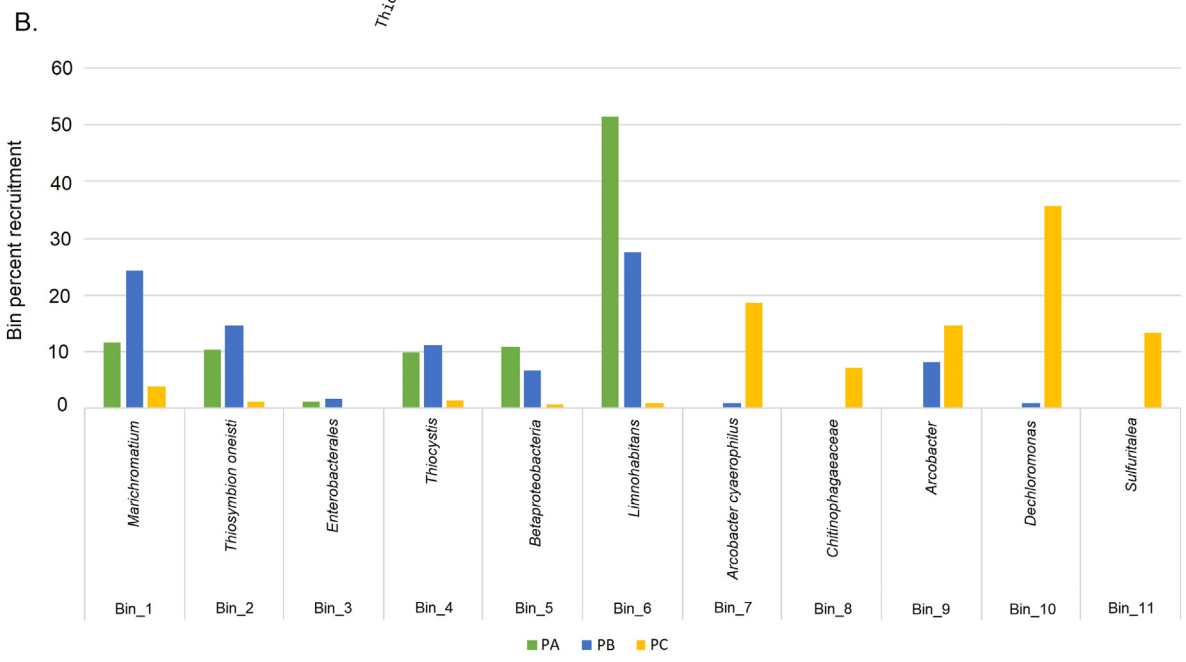


Fig. 5. Abundance of prokaryotic indicator species in the post-settler (PA), short-term (PB) and long-term storage tanks (PC). A: Abundance of prokaryotic indicator species. B: Enumeration of indicator organisms.


A.
taxon
Bin name
PA
PB
PC
Redundancy
Completion
GC-content
Length
286271
Total reads mapped
0
Arcobacter cyaerophilus
Bin_7
Dechloromonas
Bin_10
Sulfuritalea
Bin_11
Chitinophagaceae
Bin_8
Arcobacter
Bin_9
Enterobacterales
Bin_3
Thiosymbion oneisti
Bin_2
Thiocystis
Bin_4
Marichromatium
Bin_1
Limnohabitans
Bin_6
Betaproteobacteria
Bin_5


B.
Bin percent recruitment
60
50
40
30
20
10
0
Marichromatium
Thiosymbion oneisti
Enterobacterales
Thiocystis
Betaproteobacteria
Limnohabitans
Arcobacter cyaerophilus
Chitinophagaceae
Arcobacter
Dechloromonas
Sulfuritalea
Bin_1
Bin_2
Bin_3
Bin_4
Bin_5
Bin_6
Bin_7
Bin_8
Bin_9
Bin_10
Bin_11
PA
PB
PC

Pseudomonas aeruginosa, *Klebsiella, Enterobacter, E. coli, Enterococcus, Bacteroides*, *Salmonella, Giardia, Cryptosporidium*, norovirus, adenovirus, rotavirus, and enterovirus were analyzed. Fig. 5A depicts the abundance of those bacterial species that were present either in the post-settler or in the storage tanks.

The colony number of enterococci decreased over storage, which corresponds well with the abundance of the *Enterococcus* genus. Interestingly, the colony number of thermotolerant coliforms was the lowest in the post-settler. As thermotolerant coliforms also detect non-fecal coliform bacteria, enumeration of *E. coli* provides a more reliable indicator of fecal pollution (Odonkor and Ampofo, 2013; Paruch and Mæhlum, 2012). Indeed, the abundance of *E. coli* slightly decreased over storage, together with a decrease in total biomass. Although enumeration of *E. coli* was not performed, its presence in the storage tanks is a potential threat if water is used for surface irrigation.

Among indicator organisms, *Pseudomonas aeruginosa* was the highest in abundance in all tanks. It was followed by the *Bacteroides* genus which represents a major constituent of the human gut microbiome. The abundance of *Salmonella* sp. was the lowest in the short-term storage tank; interestingly, laboratory culture tests were unable to detect *Salmonella* in either the post-settler or in the storage tanks.

Among enterobacters, *Enterobacter faecium* was the most abundant. It was absent in the long-term storage tank, but was highly abundant in the short-term storage tank. The lowest number of reads against indicator organisms were observed in the long-term settler (1.74% of the total reads compared to 2.15% in the post-settler, see Supplementary Table 3), while the highest number of reads mapping to indicator strains were found in the short-term storage tank (2.70%) corresponding well with the identified functions related to infectious diseases (Fig. 4).

According to the results, as treated wastewater flows into the short-term storage tanks, the total biomass decreases similarly to the ratio of organisms specific to wastewater treatment. At the same time, the abundance of indicator pathogens as well as the functions related to infectious diseases show an increase in abundance in the short-term storage tank. By storing the effluent further, both the total biomass and the abundance of indicator pathogens decreases as other environmental bacteria gather ground.

According to the latest European reclaimed water quality requirements for agricultural irrigation, treated wastewater can be used for irrigation if *E. coli* number is below 10 to 10^4 CFU/100 mL, depending on the food crop. The strictest criterion (10 CFU/100 mL) is related to food crops consumed raw where the edible part is in direct contact with reclaimed water and root crops consumed raw, while 10^4 *E. coli*/100 mL is allowed if the water is used for the irrigation of industrial, energy or seeded crops (2020/741 EU regulation (European Parliament and the Council of the EU, 2020)). In a domestic setting, as the number of *E. coli* cannot be strictly controlled, unless chemicals are used, the safest if drip irrigation or other irrigation methods are used by avoiding direct contact with the edible part of the specific crop.

3.8. Taxonomic distribution and functional characteristics of metagenome assembled genomes

Trimmed reads were assembled with Megahit, which resulted 226,673 contigs (Supplementary Table 4). These contigs were used to recruit metagenome assembled genomes (MAGs) by Anvi'o program in metagenomics mode.

The collection describes 11 Bins accounting for 16,534,907 nucleotides, which represent 99.12% of all nucleotides stored in the contigs database (length filtered contigs). The recruited MAGs (A) and their percentage recruitments (B) are shown in Fig. 6.

Based on read-based data the *Comamonadaceae* family was the most widespread taxon, among which the *Limnohabitans* (Bin 6) MAG was the most abundant; it was frequent in the post-settler and the short-term storage tank and rare in the long-term storage tank. *Limnohabitans* are "not-easily cultivable" bacteria (Kasalický et al., 2013), they are part of the ammonia-oxidizing bacterial (AOB) consortia with versatile metabolic activity, including photosynthesis, ammonium and sulfur oxidation (Baskaran et al., 2020). From the *Campylobacteriaceae* family, two bins were recruited, i.e. *Arcobacter cyaerophilus* (Bin 7) and another *Arcobacter* sp. (Bin 9) MAGs. These are identified only in the storage tanks and more abundant in the long-term storage tank. The remaining families of the read-based data are represented in genome-centric data by the families of *Chromatiaceae* (*Marichromatium* sp., *Thiosymbion oneisti,* and *Thiocystis* sp.), *Chitinophagaceae, Rhodocyclaceae*, and *Sterolibacteriaceae*. The genus *Dechloromonas* was recruited from the storage tanks with an especially high abundance in the long-term storage tank. Based on these results, read-based and genome-centric data correlated well with each other.

The functional characteristics of MAGs were also assessed. Module completion ratio (MCR) was calculated from high and medium quality MAGs. High quality MAGs include *Limnohabitans* (Bin 6) and *Dechloromonas* (Bin 10); while *Arcobacter cyaerophilus* (Bin 7) represent a medium quality MAG. The main metabolic pathways with 100% MCR with the related genomes are shown in Supplementary Table 5. The majority of identified metabolic pathways represent basic cellular functions (e.g. glycolysis, fatty acid biosynthesis). Additionally, four specific pathways were identified at genome level which are important in wastewater treatment, i.e. nitrogen fixation, dissimilatory nitrate reduction, denitrification, and thiosulfate oxidation in the MAG of *Dechloromonas,* while dissimilatory nitrate reduction was identified in the *Limnohabitans* MAG. *Dechloromonas* was found to be the most abundant MAG in the long-term storage tank further indicating that denitrification continues over storage.

The hypotheses concerning the significance of wastewater storage time were partly supported by the data presented herein. Microbial diversity is consistently reducing as the high nutrient content in wastewater degrades. This is evidenced by the lower number of unclassified reads in the storage tanks compared to the post-settler. This observation is confirmed by both the bioluminescence assay data and the species richness assessment of the samples. However, the applied storage was still insufficient to fully get rid of some potentially pathogenic species (especially *E. coli*). The treated water contains some pathogens even after long-term storage, thus it is not recommended to be used directly on plants. Data, however, clearly demonstrated that wastewater storage improved effluent quality and that small, on-site biological wastewater treatment systems can efficiently be used to improve the quality of raw wastewater.

There are several alternatives for post-treatment of wastewater effluents to prevent the pollution of surface or ground waters, such as chemical post-treatment (e.g. chlorination), membrane filtration, or constructed wetlands as reviewed in Almuktar et al. (2018). Membrane filtration approaches efficiently remove organic matter and pathogenic bacteria, but their high investment cost and operability make them uneconomic in domestic settings. Most chemical treatments as well as UV radiation also require special operational skills and are labor and cost-intensive. Constructed wetlands represent great means of wastewater post-treatment. These systems can decrease pathogen concentration of raw wastewater by 1–6 log units, though they also cannot completely eliminate pathogenic microbes (Almuktar et al., 2018). Constructed wetlands are able to remove nutrients to meet irrigational standards; however, their large footprint and sensitivity to weather changes, as well as their maintenance demand to ensure the survival of plants make them suboptimal for domestic use.

Fig. 6. Recruited metagenome assembled genomes (A) and their percentage recruitments (B). A: Light to dark colors represent frequency. The inner circle represents MAGs contig lengths and GC contents. The completion and redundancy were estimated by CheckM indicating MAGs quality. The PA, PB and PC circles indicate the coverage of specific MAGs in the post-settler, short-term and long-term storage tanks, respectively. The numbers of mapped reads are also distributed. The name of MAGs and their Bin number are shown in the outer circles. PA: post-settler, PB: short-term storage tank, PC: long-term storage tank.

Subsurface storage tanks, either concrete or plastic may provide a viable alternative to constructed wetlands as they require less surface area which might be an important aspect in a smaller backyard. Also, they do not require maintenance or operation, only a pump to obtain the treated water for irrigation. However, their moderate to high investment cost may limit the use of storage tanks, as it could exceed the installation cost of the treatment unit. Also in areas where subsurface construction is not feasible, they might not be the best option. A feasibility study might be needed to decide whether it is worth to invest into an OWTS. In cases, however, where centralized wastewater treatment is not available and discharge of the effluent to a recipient or to a soil infiltration unit is not possible or allowed, some form of storage, e.g. a pond, a constructed wetland or a storage tank is indispensable.

4. Conclusions

This case study presented the chemical and microbial analysis of a unique OWTS setup. Degradation of organic matter and bioremediation of organic micropollutants continued during storage. This was supported by the applied genome-level analyses of the microbial communities and metabolic functions throughout the wastewater storage process. High abundance of microbes utilizing nitrogen compounds and the identified relevant decomposition-related functions in the post-settler and short-term storage tank clearly suggested an ongoing denitrification and biodegradation of organic pollutants. Similarly, the decreased relative abundance of xenobiotic degradation pathways in the long-term storage tanks correlated well with the lowered concentration of micro-pollutants, and indicated the active decomposition of these contaminants during storage. The analysis of indicator microbes revealed that the presence of pathogenic organisms poses a health risk for the owners if directly using the effluent for surface irrigation. Overall, the data suggest that long-term storage of household wastewater effluent might be highly beneficial before further use.

CRediT authorship contribution statement

J. Knisz: Conceptualization, Methodology, Investigation, Validation, Visualization, Writing – original draft. **P. Shetty:** Methodology, Software, Visualization, Writing – review & editing. **R. Wirth:** Methodology, Software, Visualization. **G. Maróti:** Funding acquisition, Resources, Data curation, Writing – review & editing. **T. Karches:** Writing – review & editing. **I. Dalkó:** Investigation. **M. Bálint:** Resources. **E. Vadkerti:** Writing – review & editing, Supervision. **T. Bíró:** Writing – review & editing, Supervision.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.144425.

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