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Insights into the microbial community of treated wastewater, its year-round variability and impact on the receiver, using cultivation, microscopy and amplicon-based methods

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Abstract: Apart from chemical constituents, wastewater treatment plant (WWTP) effluents also release microorganisms that can be important to the receiving water bodies either from a sanitary point of view, or taking to the account the biogeochemical potential of the recipients. However, little is known about the treated wastewater microbial community, its composition, seasonal changes, functions and fate in the waters of the receiver. Thus, this study presents a synergistic approach coupling new and traditional methods: analytical chemistry, classical microbiology (cultivation- and microscopy-based methods), as well as Next Generation Sequencing and a quantitative real-time polymerase chain reaction (qPCR). The results show

that in terms of bacterial community composition, treated wastewater differed from the environmental samples, irrespectively if they were related or unrelated to the WWTP effluent discharge. The canonical correspondence analysis (CCA) taking into account chemical parameters and taxonomical biodiversity indirectly confirmed the seasonal deterioration of the treated wastewater quality as a result of temperature-driven change of activated sludge community structure and biomass washout (observed also by DAPI staining). Despite seasonal fluctuations of total suspended solids and inter-related parameters (such as COD, BOD, TN, TP), the treated wastewater quality remained within current discharge limits. It was due to treatment processes intensively adjusted by WWTP operators, particularly those necessary to maintain an appropriate rate of autotrophic processes of nitrification and to support biological phosphorus removal. This can explain the observed microbiome composition similarity among WWTP effluents at high taxonomic levels. Obtained data also suggest that besides wastewater treatment efficiency, WWTP effluents are still sources of both human-related microorganisms as well as bacteria equipped in genes involved in N-cycling. Their potential of participation in nutrients cycling in the receivers is widely unknown and require critical attention and better understanding.

Keywords: sewage treatment; wastewater treatment plant effluent; environmental health; bacterial community composition; nitrogen cycling genes; nutrient discharge

List of abbreviations

WWTP-W	Wastewater Treatment Plant Gdansk-Wschod
WWTP-D	Wastewater Treatment Plant Gdynia-Debogorze

TW	Treated wastewater
TW-W	Treated wastewater from WWTP-W
TW-D	Treated wastewater from WWTP-D
MO	Marine outfall
MO-W	Marine outfall of WWTP-W
MO-D	Marine outfall of WWTP-D
VIS	Vistula River estuary
GD	Gdansk Deep
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
TSS	Total Suspended Solids
TN	Total Nitrogen
TP	Total Phosphorus
DEFT	Direct Epifluorescent Filter Technique
TCN	Total (Prokaryote) Cell Number
PB	Prokaryote Biomass
ACV	Average Cell Volume
OTU	Operational Taxonomic Unit

NGS	Next Generation Sequencing
CCA	Canonical Correspondence Analysis

1. Introduction

In urban areas, wastewater treatment plants (WWTPs) usually receive wastewater from households, offices, hospitals, and local industries. Regardless of the type of sewage network, it is clear that WWTPs are crucial in protecting the water resources and other ecosystems from chemical contaminants as well as human-related fecal material (Crini and Lichtfouse, 2019). Thus, adopted in 1991 the Urban Waste Water Treatment Directive (UWWTD, 91/271/EEC) has already aimed to protect the environment from untreated or inadequately treated wastewater, settling the standards for collection and discharge. In general, the Member States have been required to treat the wastewater in agglomerations of $\geq$ 2 000 population equivalents (PE) to reduce, suspended solids, organic matter (measured as biochemical and chemical oxygen demand; BOD and COD, respectively) and nutrients (nitrogen and phosphorus compounds).

To fulfill the current requirements the wastewater is usually treated by combining mechanical and biological processes. The latter ones are mainly based on activated sludge technology, which employs mixed microbial consortium, enables degradation of the organic pollutants, and is involved in nutrient cycling. Simultaneously wastewater treatment processes reduce the fecal bacteria load with effectiveness reaching usually > 90% (Garcia and Bécares, 1997; Reinoso et al., 2008). But even if the removal rate reaches 99.99%, the bacteria of human intestines' origin are not completely removed, since their initial load, expressed by fecal indicators, varied in the

general range of 10^6 - 10^9 per 100 mL (George et al., 2002; Lucena et al., 2004; Łuczkiewicz et al., 2010). For this reason, it is reported that conventional treatment systems are still the source of pathogens (Dias et al., 2019; Ju et al., 2016; Lucena et al., 2004; Ottoson et al., 2006) and other bacteria of concerns, also those carrying resistance genes (Łuczkiewicz et al., 2010; Rizzo et al., 2013; Sadowy and Luczkiewicz, 2014; Tennstedt et al., 2003; von Wintersdorff et al., 2016). Even so, the sanitary quality of treated wastewater is obligatorily analyzed only when reused in agriculture (Dias et al., 2019). Thus, disinfection of wastewater frequently is not required.

Until now, the attention of WWTP operators and scientists has been focused mainly on wastewater treatment processes efficiency, which allows keeping the chemical discharge requirements. Therefore the community composition and biochemical potential of the activated sludge in bioreactors has been investigated more frequently (Albertsen et al., 2012; Saunders et al., 2016; Wagner et al., 2002) than the WWTP outflow (Mansfeldt et al., 2020), which is still largely unexplored area. It is also important to note that in temperate climate zones, cold winter months are highly challenging for activated sludge processes. Especially seasonal decrease of nitrification rate, which is performed by autotrophic bacteria, is observed. The disruptions of wastewater processes may, among others, also hinder the activated sludge settling and its separation from the final effluent in the secondary clarifier, e.g.: due to the presence of filamentous bulking or small, easily sheared pin-flocks (Guo et al., 2016; Morgan-Sagastume et al., 2008). As a consequence, the flocks enter the WWTP effluents and deteriorate their chemical and microbial quality. The composition of dispersed biomass (Do et al., 2019) and the fate of functional genes, carried by those bacteria in the receiver, are almost unknown.

Additionally, it has been already proofed, that the composition of bacterial communities in WWTP effluent can potentially alter the receiving ecosystem (Atashgahi et al., 2015). Indeed

in reservoirs the WWTP effluents resulted in both an increase (García-Armisen et al., 2014; Kalinowska et al., 2020; Price et al., 2018; Wakelin et al., 2008) and a decrease (Drury et al., 2013; Lu and Lu, 2014) of bacterial communities diversity. Evaluation of the Urban Waste Water Treatment Directive, prepared by the European Commission, indicated that load of the targeted pollutants discharged via treated wastewater from urban point sources decreased significantly between 1990 and 2014 (BOD by 61% nitrogen by 32% and phosphorus by 44%) and improved the quality of bathing sites across the EU (SWD 700, 2019). Nonetheless, EU waters fail to achieve good status under the Water Framework Directive and the inappropriately treated urban wastewater has been still pointed as an area for improvement. Additionally, there is growing evidence that contaminants of emerging concern are not targeted and are continuously discharged to the environment, even via appropriately treated wastewater. Especially pharmaceuticals, microplastics, human-related bacteria and antimicrobial resistance are recognized as a global threat (Everaert et al., 2020; Marano et al., 2020; Roca et al., 2015), however activated sludge-related bacteria are also disseminated. Their potential of participation in C, P, and N cycling in the receiver requires attention. Nitrification and denitrification are one of the most frequently investigated wastewater treatment processes. The abundance of bacteria responsible for these processes can be quantified using specific genes as molecular markers: *amoA* and *nxrA* genes for nitrifiers, and *nirK*, *nirS* and *nosZ* for denitrifying bacteria (Huang et al., 2019; Tang et al., 2020; Wang et al., 2014; Zhang et al., 2019).

This study covered two largest municipal WWTPs (WWTP-W and WWTP-D) located upon the Baltic Sea in northern Poland. They receive wastewater generated by a relatively large metropolitan area of Tricity (over 1 mln inhabitants, area around 400 km^2), with various branches of industry. In this study, it has been hypothesized that treated wastewater disposal can shape the microbial community of the recipient, especially by discharge of human related bacteria, washout of activated sludge community and release of functional N-cycling genes,

increasing the nutrient cycling potential of the receiver. A wide range of complementary microbiological methods were applied to (1) investigate the year-round fluctuations in the microbial community of the WWTP effluent and (2) to elucidate the impact of its discharge on the marine waters. Microscopic observations and analysis provided information about prokaryotic cells abundance and morphology, cultivation on selective media enabled fecal bacteria enumeration, next generation sequencing revealed the taxonomic composition of the microbial community and quantitative PCR gave the information about the abundance of nitrogen-related genes and provided the insight into the nutrient cycling potential of both the WWTP effluent and its recipient.

2. Materials and Methods

2.1. Study area, sampling, and WWTP characteristics

The 24h composite samples of the influent and final effluent were collected from the two WWTPs (WWTP-W and WWTP-D, Fig. 1). Both WWTPs operate on conventional mechanical and biological treatment with advanced nutrient removal followed by secondary settling tanks with activated sludge recirculation. Detailed WWTPs characteristics are presented in schematic technological diagrams in Fig.1 and Supplementary Table S1. Influent and effluent samples (10 L) were collected twice a week for two years (from January 2012 to December 2013), transported to the laboratory in cooler boxes and immediately analyzed.

Figure 1. Schematic technological diagrams, aerial photos and location of two WWTPs: Gdansk-Wschod (WWTP-W) and Gdynia-Debogorze (WWTP-D). Samples of treated wastewater were taken from both WWTPs (TW-W and TW-D, respectively), together with their marine outfalls into the Gulf of Gdansk (MO-W and MO-D, respectively) and two reference points – Vistula River estuary (VIS) and Gdansk Deep (GD).

Both WWTPs discharge treated wastewater into the Gulf of Gdansk (Natura 2000 area), approx. 2.3 km from the coastline at a depth of about 8 m via submarine collectors equipped with diffusers (Fig. 1). Samples of the marine waters at the point of treated wastewater discharge (marine outfalls: MO-W and MO-D, respectively to the name of each WWTP) were sampled three times (August and September 2012, February 2013). Additionally, two reference points: Vistula River estuary (VIS) and Gdansk Deep (GD) were sampled twice (summer 2012 and winter 2012/2013). Vistula River was chosen due to the ecological importance of its flow on the Gulf of Gdansk quality. It is the longest river in Poland (1047 km) as well as in the area of the Baltic Sea, with its catchment equal to 194,424 km^2 (87% in Poland). The Vistula flows directly into the Gulf of Gdansk through a straight, man-made outlet, with an average annual flow of about 1×10^3 m^3/s at the mouth. Gdansk Deep (GD) is located in the open sea and is assumed to be isolated from the anthropogenic impact (Maksymowska et al., 2000). All environmental samples (points MO-W, MO-D, VIS, and GD) were collected with a Niskin bottle and transferred to sterile polyethylene bottles. The bottles were rinsed with the sampled water three times before sample collection. Samples were immediately transported in the cooler box at +4°C to the laboratory.

2.2. Chemical analysis

Chemical analyses were conducted in all influent and effluent samples (twice a week for two years). Parameters were determined according to the Standard Methods (APHA, 2005): total nitrogen (TN), ammonium nitrogen ($N\text{-}NH_4$), nitrate nitrogen ($N\text{-}NO_3$), total phosphorus (TP), phosphate phosphorus ($P\text{-}PO_4$) and chemical oxygen demand (COD) were analyzed by a XION 500 spectrophotometer (Dr. Lange, GmbH, Germany); 5-day biochemical oxygen demand (BOD_5) – by a manometric respirometric BOD OxiTop® method; total suspended solids (TSS) – by a gravimetric method.

158 **2.3. Microbiological analysis**

159 Microbiological analyses of treated wastewater samples were conducted once a month, from
160 June 2012 to May 2013 (12 samples per WWTP). Additionally, 10 environmental samples were
161 collected: from marine outfalls of WWTP effluents (MO-D and MO-W, three times each),
162 Vistula estuary (VIS, sampled twice), and Gdansk Deep (GD, sampled twice).

163 **2.3.1. Microscopic methods**

164 Direct Epifluorescent Filter Technique (DEFT) was used for the microscopic analyzes: DAPI
165 staining (Porter and Feig, 1980) and Live/Dead assay (Boulos et al., 1999). Samples for
166 microscopic enumeration with use of DAPI staining (50 ml) were fixed with particle-free
167 buffered formaldehyde (Merck, Germany) to a final concentration of 2% and stored at +4°C
168 until the analysis. Subsamples of treated wastewater (0.5 mL) and marine waters (2 mL) were,
169 stained with DAPI fluorescent dye (4',6-diamidino-2-phenylindole, Thermo Fisher Scientific,
170 US) to final concentration 1 $\mu g\, mL^{-1}$ and filtered on black Nucleopore polycarbonate filters
171 (Millipore Membrane Filter, 0.2 μm pore size, Merck, Germany). Filters were mounted on a
172 microscopic slide with non-fluorescent oil (Citifluor AF2: Agar Scientific, US) and stored
173 at -20°C until analysis. Microscopic slides were analyzed in triplicates under an epifluorescence
174 microscope Nikon Eclipse 80i under 1000-fold magnification (HBO-103W high-pressure
175 mercury burner, Osram GmbH, US, 330-380 nm excitation filter, 420 nm barrier filter, 400 nm
176 dichroic mirror). Triplicates of 10 microscope view fields, with a maximum of 60 thousand
177 objects, were digitized using Nikon DS-5Mc-U2 high-resolution color digital camera and NIS-
178 Elements BR 3.0 software. Image system analysis (MultiScan, v.14.02) with modification of
179 Świątecki (Świątecki, 1997) was applied to determine total prokaryotic cell number (TCN),

prokaryotic biomass (PB), and average cell volume (ACV). Bacterial biomass was calculated using conversion factor (170 fg C μm^3) by Norland (Norland, 1993).

Live/Dead assay was performed immediately after delivery to the laboratory on 0.5 mL treated wastewater and 2 mL marine water subsamples, stained in duplicates with fluorescent dyes SYTO9 and PI from the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, USA). Identical volumes of each dye were applied (0.1 mL), samples were incubated in darkness for 30 min and filtered through 0.2 μm polycarbonate filters (Whatman, UK). Filters were kept at -20 °C until further examination. The percentage of alive cells was determined using an epifluorescence microscope (400–440 nm and 450–490 nm excitation filters, 455 nm and 505 nm dichroic mirror, 470 nm and 520 nm absorption filter) under 1000-fold magnification. Green fluorescence (excitation/emission: ~495 nm /~515 nm) corresponds to live bacteria with undamaged cell membrane, while damaged (dead) cells produce a bright red fluorescence (excitation/emission: ~495 nm / ~635 nm). The bacteria in 2 repeats of 10 fields were counted and the result is given as a percentage of live cells in all observed cells.

2.3.2. Cultivation methods

In this study, Gram-negative enteric rods from the *Enterobacteriaceae* family were enumerated, including indicators of fecal contamination, such as fecal coliforms and *Escherichia coli*. Cultivation was performed immediately after sample delivery to the laboratory. Serial dilutions were applied: 10^{-4} to 10^{-1} mL for wastewater, and 10 to 500 mL for marine waters was filtered in triplicates on cellulose membrane filters (47 mm diameter, 0.45 μm pore diameter, Whatman, UK). Bacteria were grown on Chromocult® Coliform Agar and Membrane Fecal Coliform Agar (Merck, Germany) according to the manufacturer specifications, as is summarized in

detail in Supplementary Table S2. Based on bacterial colony growth, the amount of colony-forming units (CFU) per 100 mL was determined.

2.3.3. DNA extraction and bacterial 16S rRNA gene amplification

Subsamples of treated wastewater (100 mL) and marine waters (400 mL) were filtered on polycarbonate filters (0.2 µm pore diameter, Merck, Germany) and stored at -80°C for the DNA extraction. Duplicates of the filtered material for each sampling point were merged for DNA extraction and considered as one sample in further taxonomic analysis. The total community DNA was extracted and purified using Genomic Mini AX Bacteria+ (mod.5) isolation kit (A&A Biotechnology, Poland) and quantified by spectrophotometry at 260 nm using Nanodrop (Thermo Fisher Scientific, UK). The presence of bacterial DNA was confirmed by real-time PCR with SYBR Green fluorochrome, in Mx3000P thermocycler (Stratagene, USA). The following PCR conditions were used: initial denaturation at 95 °C for 3 min, followed by 40 cycles consisting of denaturation (95 °C for 15 s), annealing (58 °C for 30 s), fluorescence measurement, and extension (72 °C for 30 s). For amplification of 16S rDNA fragments, the universal primers were applied: 1055F and 1392R (Ferris et al., 1996). The final check on the DNA quality was done by determination of the PCR product melting curve and measuring fluorescence at temperatures from 65 °C to 95 °C. The PCR products were stored at -20 °C for sequencing.

2.3.4. Sequencing, taxonomic assignment, and data analysis

To establish microbial community composition of the analyzed samples, 16S rRNA gene V3-V4 region amplified with 341F and 785R primers pair (Klindworth et al., 2012). Paired-end sequencing was performed with an Illumina MiSeq 2×300 bp platform by the Macrogen

company (Macrogen Inc., South Korea) and following manufacturer's run protocols. FASTQ files were generated from MiSeq Reporter (Illumina) and the paired reads were initially joined with the FASTQ joiner and subjected to quality control with the FASTQC (at quality cut-off value=20). Tools are available at the UseGalaxy server (https://usegalaxy.org). Sequences shorter than 120 bp were excluded from further analysis. Classification of the reads on each taxonomic level was carried out with Silva NGS server (http://www.arb-silva.de) by the use of database release version 138 at the species similarity level of 90% and OTUs (operational taxonomic units) clustering at 97%. Alpha diversity was assessed based on richness and diversity indices: Chao1, Shannon (H') and Simpson (D), obtained in CLC Genomics Workbench software.

2.3.5. Quantitative PCR of nitrogen-cycling-related genes

Quantitative real-time PCR was used to validate the absolute abundance of 16S rRNA and some functional genes, including *amoA*, *nxrA*, *nirS*, *nirK*, and *nosZ*. The fragments coding these genes were amplified with specific primers listed in Table S3 (Supplementary Materials). Amplification of real-time PCR products was carried out with Stratagene Mx3000P thermocycler (Agilent Technologies, US) using SYBR Green as a detection system in a reaction mixture of 20 μL containing: 0.1 μL of each *nirS* and *nirK* primers, 0.4 μL for *nxrA* primers and 1 μL for *amoA* and *nosZ* primers; 10 μL of Real-Time 2xRT-PCR Mix SYBR A mixture (A&A Biotechnology, Poland), 1 μL of DNA diluted template corresponding to 10 ng of total DNA, and Rnase-free water to complete the 20 μL volume. All primer pairs amplifying gene fragments of *nirS* and *nirK* were run with an initial denaturation of the DNA at 95 °C for 3 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C. For *amoA*, *nxrA*, and *nosZ* a similar procedure was applied, with the only difference in primer annealing temperature (55 °C, 63 °C, and 65 °C, respectively).

2.4. Statistical analysis

PCA was performed in R software (version 3.6.2), using the FactoMineR package, on the scaled dataset. Canonical correspondence analysis (CCA), combining the basic properties of a typical correspondence analysis with those of a constrained ordination, was performed with the vegan package (Dixon, 2003). To observe the clusters of microbial taxa, ordered along the canonical axes, following their ecological optima, and to obtain a clearer model with a limited number of significant axes, a forward selection of explanatory variables was performed for each WWTP. To identify the explanatory variables that significantly explained variation in microbial communities, forward selection was performed using the ordistep function within the vegan package (999 Monte Carlo permutations, alpha < 0.05). For transparency, only taxa of the relative abundance over 3% at the family level in at least one sample were used for CCA analysis. For the heatmap with dendrogram, a hierarchical clustering was performed using the average (UPGMA) method on the Bray-Curtis dissimilarity matrix, evaluated on the reduced dataset.

3. Results and discussion

WWTPs tested in this study (WWTP-W and WWTP-D) are located near each other, serve similar urban catchments (equipped with a sanitary network) and perform enhanced simultaneous C/N/P removal (for details see Materials and Methods). Both also discharge the effluent into the coastal marine waters via marine outfalls (MO-W and MO-D, respectively). To better elucidate the characteristics of coastal areas impacted by treated wastewater (MO-W and MO-D), and non-impacted points were tested: Vistula River estuary (VIS) and Gdansk Deep (GD). Microbiological results were supported by physical and chemical parameters.

3.1. Chemical parameters

The wastewater profiles depend on many factors, e.g.: catchment size and type of sewer network, number of people served, and industrial discharges (Deblonde et al., 2011). This study focused on two WWTPs (WWTP-W and WWTP-D), which treat wastewater generated by a metropolitan area of Tricity (Fig.1). Chemical parameters (COD, BOD_5, TSS, TN, $N\text{-}NH_4$ and TP) in raw wastewater (Fig. 2a) were typical for the studied urban catchment (Krzeminski et al., 2012; Pasztor et al., 2009) and indicated high similarity between WWTPs (Supplementary Fig. S1A). Factors explaining the variability of raw wastewater were most importantly: TP, TN, and N_{org} (organic nitrogen) for WWTP-D, and COD, TSS, and TN for WWTP-W (Supplementary Fig. S1B and C).

Figure 2. Time series of basic chemical parameters in a) influent and b) effluent for both WWTPs (WWTP-D and WWTP-W) investigated in this study

Higher concentrations of the chemical parameters at the influent did not however always result in the higher concentration in the effluent (Fig. 2a, 2b). The principal component analysis (PCA) revealed the seasonality pattern of decreasing wastewater treatment efficiency in winter months (Fig. 3a, 3c), as PC1 correlates positively to the ambient temperature variability and negatively with BOD and TSS in treated wastewater (Fig. 3a). This trend was also shown in other studies (Xue et al., 2019). PC2 reflects mostly treated wastewater parameters and it strongly separates the treatment plants (WWTP-W and WWTP-D) from each other (Fig. 3b). The most important factors separating the WWTP effluents are nitrogen and phosphorus compounds concentrations and their corresponding removal efficiencies (Fig. 3a,b, Supplementary Fig. S2), which may result from the differences between applied treatment systems, WWTPs size and operator's management.

Figure 3. Principal component analysis (PCA): (a) loading plot, and score plots: (b) sampling colored by the WWTP supplementary categorical variable, (c) sampling colored by the average ambient temperature variable. Analysis was conducted on the entire dataset constituted by the chemical parameters of both raw and treated wastewater (indicated by *R* or *T* after the variable name), and relevant removal efficiencies (indicated by *eff*).

Note that deterioration of treated wastewater quality is usually connected with an increase in WWTP effluent turbidity, and inter-related parameters such as mainly COD and BOD (Fig. 2b, 3a) were mainly linked to the turbulence in the activated sludge process. The reason may be a seasonal change of activated sludge community structure (see Section 3.3) due to the drop of both wastewater and ambient temperature. As a consequence, sedimentation disturbance and activated sludge biomass washout may occur and lead to deterioration of the treated wastewater quality, causing the need to change the wastewater treatment strategy. A common practice is to increase the density and age of activated sludge by prolonging biomass retention time (necessary to maintain an appropriate rate of autotrophic processes such as nitrification), while dosing of coagulants (e.g.: PIX/PAX) prevents biomass washout with treated wastewater (Boguniewicz-Zablocka et al., 2020). Nevertheless, despite preventive measures undertaken at both studied WWTPs, microscopic analysis indirectly confirmed dispersed biomass being washed out during the winter season (Section 3.2.1 and Fig. S3), observed especially between October 2012 and April 2013. This phenomenon was also confirmed by NGS analysis of TW-D and TW-W (for details see Section 3.3.1). Despite the seasonal deterioration of the TW-D and TW-W quality, all indicators of treated wastewater quality remained lower than current discharge limits (COD < 125 mg O_2 L^{-1}, BOD < 15 mg O_2 L^{-1}, TN < 10 mg L^{-1}, TP < 1 mg L^{-1}, TSS < 35 mg O_2 L^{-1}) (Dz.U. 2019 poz. 1311).

316 Marine outfalls of studied WWTPs (MO-D and MO-W) are located 2.3 km from the coastline
317 (Fig. 1) to ensure proper dispersion of treated wastewater (TW-W and TW-D) in the receiver
318 (Gulf of Gdansk and its internal part - Puck Bay) and to avoid the deterioration of coastal
319 bathing areas. According to the obtained results, COD and BOD concentrations in TW-D and
320 TW-W were similar to these noted in environmental samples (VIS and GD) during the summer
321 (Supplementary Table S4), but the other parameters (TN, $N\text{-}NH_4$ and TP) were consistently
322 higher. These results confirm continuous supply of nutrients from WWTPs to the marine waters
323 via marine outfalls (MO-D and MO-W). Interestingly, at MO-D and MO-W all the tested
324 chemical parameters were on a similar or lower level than noted at Vistula estuary (VIS)
325 (Supplementary Table S4). This can be explained by the Vistula estuary geomorphological and
326 hydrological features, which receive on average 1081 m^3/s discharge from the Vistula River
327 (Wielgat-Rychert et al., 2013). Because Vistula River serves as wastewater receiver and its
328 catchment area is intensively cultivated cropland, its estuary is known to receive high nitrogen
329 and organic matter loads: 97000 t TN yr^{-1} and 600000 t C yr^{-1}, respectively (Bartl et al., 2019;
330 Maksymowska et al., 2000). This leads to high concentrations of nutrients and organic matter
331 (Pastuszak et al., 2012), high primary production rates (Witek et al., 1999; Wielgat-Rychert et
332 al., 2013), and thus to the eutrophied state (Maksymowska et al., 2000). On the other hand, high
333 riverine nitrogen loads are known to increase rates of microbial processes (Seitzinger et al.,
334 2006), which was confirmed by the level of N-cycling genes in VIS, similar as for MO-D and
335 MO-W (for details see Section 3.4).

336 **3.2. Enumeration of bacteria**

337 **3.2.1. Microscopic analysis**

338 Availability of nutrients and increased temperature of the wastewater are the conditions
339 supporting bacterial growth. Therefore, as suspected, all the parameters tested under the

microscope (TCN, PB, ACV, and Live/Dead) were on average higher in the treated wastewater (TW) than in the environmental samples: MO, VIS and GD (Fig. 4a, 4b). TCN and PB values in the treated wastewater were similar for both WWTPs (average 2.22 mln cells/mL and 69.22 µg C/mL for TW-D, 2.21 mln cells/mL and 68.27 µg C/mL for TW-W). They ranged between 1.65 – 2.91 mln cells/mL and 30.6 – 80.5 µgC/L, what is the same magnitude as observed in other WWTP effluents (Bray et al 2021, Kalinowska et al 2021).

Direct microscopic observations revealed some seasonal variability in the bacterial morphology in the treated wastewater. During the summer, the bacterial cells observed in TW-D and TW-W were rather larger and free-swimming, while in winter more numerous, smaller, and structured into small flocks (Fig. S3). It was already suggested that dispersed biomass in activated sludge expresses different aggregative properties than the biomass of settleable sludge. Additionally, factors that may negatively impact the production of extracellular polymeric substances (EPS) or may cause EPS destruction also influence the presence of floc-forming species. EPS are the structural backbone of the activated sludge flocs, and play a crucial role in activated sludge flocculation, settling, and dewatering. Note that EPS acts also as a survival mechanism for bacteria, protecting them from stress conditions, such as dehydration, presence of toxic substances, or nutrient deficiency (Laspidou and Rittmann, 2002). EPS destruction may be caused by turbulence in the activated sludge biomass, but technological processes and other factors important in this phenomenon are still not fully understood.

Environmental samples were characterized by larger fluctuations of TCN and PB than in the WWTPs' effluents, as wastewater is more stable in terms of temperature and availability of nutrients. The amount of prokaryotic cells varied between 0.18-2.30 mln cells/mL at the in marine outfalls, 0.19-2.30 mln cells/mL at Vistula estuary and was the lowest (0.15-2.07 mln cells/mL) at Gdansk Deep (Fig. 4a, 4b) what is in the range noted in the other studies from this

Baltic Sea area (Piwosz et al., 2013, Kudryavtseva et al., 2012, Ameryk et al., 2014). Prokaryotic biomass followed the same pattern and ranged between 3.7-81,5 µgC/L for MO, 2.5-82.8 µgC/L for VIS and 2.9-36.5 µgC/L for GD. Large fluctuations of microbial microscopic parameters in the marine samples result from sampling in warm (August, September) or cold period (November, February) and they correspond to the general seasonal trend confirmed by other authors (Ameryk et al., 2021; Freese et al., 2006; Piwosz et al., 2013). Gulf of Gdańsk is an eutrophicated water reservoir, rich in phyto- and bacterioplankton during the vegetation period from April to October (Witek et al. 1997) and several studies (Danovaro and Fabiano, 1997; La Ferla et al., 2014, 2010) indicate that the availability of nutrients affects the bacterial parameters. Both terrigenous organic matter and autochthonous matter of phytoplankton origin can also support the growth of heterotrophic bacteria (Ameryk et al. 2005). The lower ambient temperature and limited nutrient availability in the marine waters during winter were followed by one magnitude lower bacterial cell abundance and biomass (February) than in the summer season (August, September). Also the Vistula River introduces nutrients to the Gulf of Gdansk, therefore it stimulates the bacterial production in its internal part (Ameryk et al., 2005; Wielgat-Rychert et al., 2013) what is reflected by both high values and large fluctuations in the microscopic parameters at VIS. Gdansk Deep (GD) was characterized by the lowest values of all microbial parameters (Fig. 4a, 4b, 4c), which supports its choice as the reference point, being under the limited impact of anthropogenic and riverine origin.

Results of the Live/Dead assay showed that treated wastewater contained a higher ratio of the live cells than the environmental samples (Fig. 4b). This was according to expectations, as activated sludge can contain up to 80% of live and active cells (Kocwa-Haluch and Woźniakiewicz, 2011) which typically occur in sludge flocs. Only about 10% of activated sludge biomass tends to remain in suspension and/or to detach easily from average sludge flocs.

Thus, observed increased Live/Dead ratio in treated wastewater may indicate a poor sedimentation and biomass washout. In environmental samples, the share of active bacterial cells was not exceeding 15%, and the highest values were observed at marine outfalls (MO-D and MO-W). The quantification of alive bacteria is important in the context of microbial production, organic matter decomposition, and for assigning microbial activities to individual organisms (Kogure et al., 1979; Rodriguez et al., 1992; Schumann et al., 2003). Microscopic techniques, including Live/Dead assay, were in recent years superseded by metagenomic methods and currently are rarely used in environmental studies. However, these methods still provide significant information on cell viability, which is missed or biased when using methods based on DNA approach (Cangelosi and Meschke, 2014; Guo et al., 2014; Li et al., 2017; Nielsen et al., 2007; Nocker et al., 2010).

Figure 4. Results of the microscopic and cultivation analysis for treated wastewater (TW), its marine outfalls (MO), Vistula estuary (VIS), and Gdansk Deep (GD): a) total prokaryotic cell number (TCN) and prokaryotic biomass (PB) obtained in DAPI staining; b) average cell volume (ACV) obtained in DAPI staining and percentage of alive bacteria obtained in Live/Dead assay (LD; not carried out for GD samples); c) sanitary indicators in various sample types, d) comparison between tested WWTPs effluents (TW-D and TW-W). *Enterobacteriaceae* and fecal coliforms were not tested for GD. The abundance of various bacterial groups is expressed as a number of colony forming units (CFU) in 100 mL.

3.2.2. Bacteria cultivation

The bacterial community can be assessed by a variety of approaches. The culture-dependent methods have been in recent years dislodged by the high-throughput sequencing of the 16S rRNA gene due to less time-consuming procedure, ability to generate larger datasets and

improved access to the rare biosphere (Tytgat et al., 2014). However, the cultured organisms from a given sample might be important for determining some impacts, such as e.g.: anthropogenic stress on indigenous microbial communities. Moreover, short 16S rRNA gene reads lead to technical limitations to obtain species-level identification (Bibby et al., 2010; Ju et al., 2016; Luo and Angelidaki, 2014; Ye and Zhang, 2011). Thus in this study, the indicators of fecal contamination were additionally assessed by cultivation of gram-negative enteric rods from *Enterobacteriaceae* family together with the fecal coliforms and *E. coli* (Fig. 4c and 4d).

An average number of *Enterobacteriaceae* was similar in both WWTPs effluents (3.6×10^5 CFU/100 mL for TW-D and 3.3×10^5 CFU/100 mL for TW-W, Fig. 4d), but values in TW-W were more uniform throughout the year (from 2.6 to 3.8×10^5 CFU/100 mL versus from 1.7 to 6.1×10^5 CFU/100 mL in TW-D, Fig. 4d). No clear seasonal pattern was observed in terms of their variability. In environmental samples both *Enterobacteriaceae* and *E. coli* were on average three orders of magnitude lower than in WWTP effluents. Interestingly, *Enterobacteriaceae* were ten times less abundant at MO than at VIS (8.4×10^2 CFU/100 mL versus 8.7×10^3 CFU/100 mL) and not detected at GD (Fig. 4c).

Fecal coliforms, as well as their representative - *E. coli*, were detected also in environmental samples and presented a similar trend as the *Enterobacteriaceae* family: their average values were higher for the Vistula River estuary than the marine outfalls of the treated wastewater, but Gdansk Deep presented the lowest abundance of these bacteria. The number of *E. coli* varied from 1.5×10^4 to 6.4×10^4 CFU/100 mL in TW-D and from 7.0×10^3 to 6.9×10^4 CFU/100 mL in TW-W, respectively (Fig. 4d), which is rather typical for treated wastewater (Łuczkiewicz et al., 2010; Marano et al., 2020). It was also reported by others that fecal bacteria, even if removed with high efficiency of over 90%, are still released to the recipient with WWTP effluent as a result of their high initial number (Lucena et al., 2004; Marano et al., 2020). However, in none

of the environmental samples the number of *E. coli* exceeded the allowable standard for bathing sites (<500 CFU/100 mL), according to the New Bathing Water Directive (2006/7/EC). Previous studies of Polish rivers receiving treated wastewater also reported presence of fecal coliforms and *E. coli* in Vistula (Donderski and Wilk, 2002; Walczak, 2008) or other rivers (Bączkowska et al., 2022, 2021; Niewolak, 1998). Their abundance was in the similar range as presented in this study and very likely was supported by high availability of easily absorbed organic matter (Donderski and Wilk, 2002).

3.3. Metagenomic analysis of microbial community

Beyond laboratory-grown cultures, metagenomic tools have significantly enhanced our understanding of microorganisms associated with numerous habitats. In this study microbial composition of WWTP effluents (TW-D and TW-W) and environmental samples impacted (MO-D and MO-W) and not directly impacted by treated wastewater (VIS and GD) were analyzed (Fig. 5a,b,c). A total of 6 600 OTUs were identified from 2 726 599 sequences (average length of 301 bp) obtained from 34 samples (24 samples of treated wastewater collected from June 2012 to May 2013 - 12 for each WWTP; and 10 environmental samples). Alpha diversity was quantified using three richness and diversity indices: Shannon, Simpson and Chao1 (Fig. 5d). On average, they were the highest for treated wastewater and lower for the environmental samples, and for Shannon and Simpson the differences between these sample types were significant. WWTPs did not differ significantly from each other, but throughout the year the microbial community composition of the WWTP effluent fluctuated (Fig. 6a, 6b) and its biodiversity decreased in winter, what reflects the trend found in the activated sludge reactor (Wang et al., 2016).

For an open-sea sampling station GD, the alpha diversity was the lowest, with the exception of Chao1, which is highly influenced by the presence of rare taxa. Samples being under treated wastewater impact (MO) presented higher microbial diversity, what has been also noted in other studies (García-Armisen et al., 2014; Kalinowska et al., 2020; Price et al., 2018; Wakelin et al., 2008). This supports the hypothesis of increasing biochemical potential of the natural waters due to WWTP effluent discharge. Nevertheless, these changes may be temporary and may depend highly on the water mixing (Price et al., 2018). The share of treated wastewater in recipients may also play a significant role. Increased biodiversity indices for VIS may reflect the massive amounts of river waters introduced by the Vistula River to the Gulf of Gdansk (approx. 30 km^3 annually) and therefore the intensive mixing of marine and freshwater communities in the river estuary.

Figure 5. Taxonomic relative abundances at phylum level noted in WWTP effluents TW: a) TW-D and b) TW-W and in c) environmental samples of marine outfalls MO (MO-D, MO-W), Vistula estuary (VIS) and Gdansk Deep (GD). Names and colors are listed for phyla with relative abundance >3% in at least one sample. Figure 5d presents Chao1, Simpson, and Shannon indices for the sample types: TW, MO, VIS and GD. Figure 5e shows heatmap of main phyla with the dendrogram of the samples.

Taxonomy-based analysis indicated that *Bacteria* constituted a majority of the total microbial community, and *Archaea* less than 0.2% in a single sample (higher share in treated wastewater, while in environmental samples maximum 0.02%). *Archaea* were represented mainly by *Parvarchaeota*, class *Parvarchaea*, but smaller shares of *Crenarcheota* and *Euryarchaeota* (*Methanobacteria* and *Methanomicrobia* classes) were also found, however only in TW-D and TW-W, which is in agreement with other wastewater studies (Gonzalez-Martinez et al., 2018; Greay et al., 2019; Tiirik et al., 2021). Among 56 identified bacterial phyla, 38 were present in

minor shares (less than 1% in each sample). Fig. 5 shows the relative abundances of the most abundant phyla and 11 of them were common for all the samples analyzed, however their abundance varied significantly. They belonged to *Actinobacteria* (4.5-41.0%), Proteobacteria (5.5-56,9%), Bacteroidetes (0.5-12.4%), Firmicutes (0.03-16.6%), Verrucomicrobia (0.1-9.7%), and Planctomycetes (0.04-8.5%), with smaller shares of Acidobacteria (<0.01–1.3%), Chlamydiae (<0.01-2.3%), (0.01-3.9%), Cyanobacteria (0.05-49.4%) and Saccharibacteria/TM7 (<0.01-60.9%). Heatmap with dendrogram (Fig. 5e) confirms the higher similarity among the environmental samples, clearly separated from TW samples.

3.3.1. Microbial community of WWTP effluents (TW-D and TW-W)

In the case of TW-D and TW-W samples, 22 phyla formed the core microbial community. Among them, the most abundant in TW-D and TW-W were *Proteobacteria* (up to 57% and up to 38%, respectively), *Saccharibacteria/TM7* (up to 48% and up to 61%, respectively), *Acidobacteria* (up to 33% and up to 41%, respectively), *Firmicutes* (up to 17% and up to 15%, respectively), *Parcubacteria/OD1* (up to 16% and up to 12%, respectively) and *Bacteroidetes* (up to 12% and up to 10%, respectively), Fig. 5a and 5b. Most of those taxa, were also identified as core ones in the activated sludge (Wu et al., 2019). Similarity of microbial communities detected in TW-D and TW-W samples can be explained by similar urban catchments and sanitary networks but most of all by treatment processes served by the tested WWTPs (enhanced simultaneous C/N/P removal; for details see Materials and methods). Although, it should be noted that similar microbial communities in treated wastewater were also shown by others (Cai et al., 2014; Do et al., 2019; García-Armisen et al., 2014; Tiirik et al., 2021; Xue et al., 2019). For instance, Do et al. (2019), who tested WWTPs effluents in Ireland, reported among predominant phyla: *Proteobacteria* (67%), *Actinobacteria* (up to 50%), followed by *Bacteroidetes* (up to 18%) and *Firmicutes* (up to 16%). Similar phyla, but in different share

were noted in the outflows of WWTP located in Belgium (García-Armisen et al., 2014): *Proteobacteri*a (up to 74%), followed by *Bacteroidetes* (up to 37%) and *Actinobacteria* (up to 18%). In WWTP from Hong Kong (Cai et al., 2014) the following share of phyla were found: *Proteobacteria* (up to 60%), *Saccharibacteria/TM7* (up to 25%), *Bacteroidetes* and A*cidobacteria* (up to 20%), followed by *Firmicutes* (up to 14%). The microbial community of treated wastewater has been rarely studied, especially in terms of seasonal variations in composition and diversity (Wang et al., 2016), therefore this issue is not fully recognized and understood. However, worldwide similarities observed until now at high taxonomic levels may suggest that the microbiome composition of WWTP effluents is to some extent consistent among WWTPs (Adrados et al., 2014; Cai et al., 2014; Silva-Bedoya et al., 2016). Wastewater treatment is intensively adjusted by operators in winter as a response to the current effectiveness of microbiological processes, particularly those linked to nitrogen and phosphorus removal. Thus, the metagenomic approach indirectly confirmed the seasonal disruptions of wastewater processes, already confirmed by elevated chemical parameters (see Section 3.1) and presence of numerous, small-structured flocks in TW-D and TW-W during the cold season (see Section 3.2.1, and Fig. S3).

Figure 6. Canonical correspondence analysis (CCA) in respect to chemical parameters and NGS analysis of samples from a) WWTP-D and b) WWTP-W. The arrows represent the explanatory variables and the lines of corresponding color show their values. Representative bacterial taxa are given in black and the sampling date is given in grey. From the microbial community, the representative taxa of the relative abundance over 3% at the family level in at least one sample were chosen for the analysis.

Canonical correspondence analysis (CCA) analysis was done with regard to WWTPs' inflow and effluent chemical parameters and microbial communities in treated wastewater (TW)

samples. For WWTP-D (Fig. 6a), four parameters were chosen as explanatory variables: concentration of organic nitrogen in the WWTP influent (N_{org}.R), P-PO_4 removal efficiency (PO_4.eff), TN/COD.T ratio in the effluent and ambient temperature, explaining overall 73.0% of the total variance. The winter samples (January-February) were characterized by the worst PO_4 removal efficiency, combined with the lowest organic nitrogen concentration in the influent and lowest temperature. Together with early spring samples (March-April) they contained increased amounts of bacteria potentially related to foaming and bulking (family *Microthrixaceae*, mostly *Candidatus Microthrix*), originating from human gut (*Carnobacteriaceae* and *Lachnospiraceae*), or potentially pathogenic ones (*Campylobacteraceae* and *Legionellaceae*). *Campylobacteraceae* would be of special concern, as they were found to be positively correlated with occurrence of some antibiotic resistance vectors in the WWTPs effluents (mainly β-lactamases and integrons) (Fernandes et al., 2019).

The total variance for WWTP-W samples explained by CCA was lower than for WWTP-D and involved less variables: total phosphorus removal efficiency (TP.eff), nitrate nitrogen concentration in the effluent (N-NO_3.T) and ambient temperature (Fig. 6b). It showed the dominance of similar taxa in the WWTP-W effluent all over the year. Winter/spring samples were associated with presence of bacteria that imply treatment efficiency deterioration. In April and May, particularly high shares of *Gordonia* (an opportunistic human pathogen widely distributed in aquatic and terrestrial environments) were found. Summer/autumn samples, characterized by higher N-NO_3 concentrations contained microorganisms commonly found in activated sludge reactors (representatives of *TM7/Saccharibacteria* phylum, eg. *EW055*, Gómez-Acata et al., 2017) or in environmental samples: anoxic (*ZB2* and other representatives of *Parcubacteria/OD1*, Harris et al., 2004), or potentially associated with mammal presence (BD1.5 - representatives of *Gracilibacteria/GN02*, Dudek et al., 2017). It is worth noting that in the case of both WWTPs, the summer-autumn samples were usually grouped together, while

555 winter-spring were separated from them (Fig. 6a,b). For WWTP-W the samples from June-July
556 were clearly separated from the other samples regarding both dominating microbial taxa, as
557 well as chemical parameters (primarily N-NO_3 concentration).

558 During wastewater processes, nitrogen is usually removed via the nitrification-denitrification
559 pathway. Most nitrifiers are strongly associated with activated sludge, therefore the structural
560 integrity of flocs is an important factor for forming close spatial associations among ammonia
561 and nitrite oxidizers (Johnston et al., 2019). However, the disintegration of activated sludge
562 flocs in this study was observed during the cold season but no clear trend of the nitrifiers'
563 washout with the treated discharge (TW-D and TW-W) was found. According to the obtained
564 results, in TW-D and TW-W samples, the ammonia- and nitrite-oxidizing microorganisms
565 reach up to 1.1% of the total community, while in the bioreactor they reach up to 15% (Saunders
566 et al., 2013). Ammonia oxidizing bacteria (AOB) in TW-D and TW-W were represented mainly
567 by the *Nitrosomonadaceae* family, genus *Nitrosomonas* (up to 0.3%), while the nitrite oxidizers
568 (NOB) were mainly represented by *Nitrospiraceae* family, genus *Nitrospira* (up to 0.9%). Both
569 genera were found be the dominant in many bioreactors (Limpiyakorn et al., 2006; Park and
570 Noguera, 2004; Wang et al., 2012; Zhang et al., 2011). Note that *Nitrospira* is potentially able
571 to completely oxidize ammonia to nitrate in the comammox process (Daims et al., 2015). It is
572 worth noting that no ammonia-oxidizing archaea nor anammox bacteria were detected in
573 samples collected from WWTP effluents.

574 In the case of denitrification, a wide variety of heterotrophic facultative anaerobes are capable
575 of oxidizing organic compounds via nitrate respiration (Geets et al., 2007). In our study,
576 potential denitrifying bacteria were represented by a wide range of *Proteobacteria* members, as
577 well as some representatives from *Actinobacteria*, *Bacteroidetes*, and *Firmicutes*. They ranged
578 between 3.9.% - 24.6% in TW-D, 4.3% - 19.5% in TW-W and 3.0%-15.6% in environmental

samples. The denitrification bacteria community in treated wastewater was much more diversified and represented by more taxa than in environmental samples. WWTP effluent was dominated by *Acinetobacter*, with some *Dokdonella*, *Dechloromonas*, and other taxa, present in the recipient (*Flavobacterium*, *Rhodobacter*, *Pseudomonas*, *Hyphomicrobium* and *Stenotrophomonas*). The presence of *Hyphomicrobium* among abundant denitrifiers in WWTPs was confirmed by Wang et al. (Wang et al., 2014). On the contrary, *Thauera* and *Azoarcus*, reported by Wang as one of the main denitrifiers in tannery WWTP sludge, in this study were detected only in treated wastewater and only in minor shares (≤0.01%), which suggest their strong sludge association. Recently attention is given also to the bacteria such as *Agrobacterium* sp., *Raoultella* sp., *Alcaligenes faecalis*, *Paracoccus versutus*, as well as *Pseudomonas stutzeri*, *Pseudomonas tolaasii*, and *Acinetobacter* sp., that are capable of using ammonium, nitrate or nitrite as a inorganic source of nitrogen and carry heterotrophic nitrification and aerobic denitrification. Among these genera, only *Pseudomonas* and *Acinetobacter* were detected in this study in higher shares (up to 0.5% and 2%, respectively) in treated wastewater samples.

In addition to nitrogen removal, both WWTPs perform enhanced biological phosphorus removal, which is carried out by polyphosphate accumulating organisms (PAOs) that can accumulate P in amounts exceeding their growth requirement. *Candidatus Accumulibacter* and *Tetrasphaera* are most frequently identified PAOs in full-scale wastewater plants even geographically distinct (Nielsen et al., 2019; Onnis-Hayden et al., 2020). Among other bacterial PAOs connected with activated sludge *Actinobacteria* (*Friedmaniella*, *Candidatus Microthrix*, *Microlunatus*, *Tessaracoccus*), *Proteobacteria* (*Dechloromonas*, *Pseudomonas*, *Ca. Accumulimonas*, *Quatrionicoccus*, *Malikia*, *Lampropedia*), and *Gemmatimonadetes* (*Gemmatimonas*) are also mentioned (Akbari et al., 2021). In this study, only *Candidatus Microthrix* (up to 23.31%), *Dechloromonas* (up to 0.6%), *Pseudomonas* (up to 0.5%) and *Gemmatimonas* (below 0.1%) were detected in TW-D and TW-W. Enhanced presence in

604 treated wastewater of flocs forming bacteria, involved in N/P removal may indicate the
605 weakening of the sedimentation capacity of the activated sludge, as well as dissemination of
606 such biomass via treated wastewater to receivers.

607 Another important aspect of WWTP effluent discharge into the environment is dissemination
608 of pathogens or other emerging bacteria, because of correlation between recreational use of
609 surface waters, and the occurrence of various infections (Pruss, 1998; Witzig et al., 2002). In
610 this study, some potentially pathogenic genera were detected in the treated wastewater:
611 *Mycobacterium* (up to 3.15%), *Bacteroides* (up to 1.51%), *Acinetobacter* (up to 1.94%),
612 *Streptococcus* (up to 0.77%), *Arcobacter* (up to 0.5%) and *Pseudomonas* (up to 0.48%). Fecal
613 indicators such as *Escherichia* and *Enterococcus spp.* were detected in most TW-D and TW-W
614 samples and ranged between 0.01 - 0.11%, and up to 0.03%, respectively. No clear seasonal
615 dependence was found, what was in line with cultivation-dependant analysis (see Section 3.2.2).
616 In environmental samples these genera were observed sporadically, more frequently in winter
617 than in summer samples, and in values not exceeding 0.01%. Also other bacteria from human
618 gut microbial taxa (i.e. *Ruminococcus*) were found in all TW-D and TW-W samples (up to
619 0.8%), while in the recipient they were noted sporadically and in minor shares (<0.02%).

620 **3.3.2. Microbial community of environmental samples**

621 As already mentioned, the biodiversity of WWTPs effluents is rarely studied and not fully
622 understood, however even less is known about the fate of wastewater-related bacteria in water
623 reservoirs serving as WWTP effluent receivers. In this study, the microbial communities of
624 environmental samples (MO-D, MO-W, VIS and GD) were highly similar on phylum level,
625 irrespective of the presence or absence of wastewater discharge (Fig. 5c, e). These samples were
626 dominated by eleven taxa, and similarly to treated wastewater, they showed a high percentage

of *Actinobacteria* (classes *Acidimicrobiia* and *Actinobacteria*, together up to 41%), *Proteobacteria* (*Alpha–*, *Beta-* and *Gamma-* clades, together up to 57%) and *Bacteroidetes* (classes *Sphingobacteriia* and *Flavobacteriia*, together up to 12.5%). However, the most characteristic for the marine and estuarine samples were the high relative abundance of *Cyanobacteria* (21-49%), *Verrucomicrobia* (1-10%), and *Planctomycetes* (1-8%), which comprised 29-54% of the total microbial community, while in TW-D and TW-W these three phyla reached a maximum of 3.5%.

Most of the aforementioned bacteria (*Actinobacteria*, *Flavobacteriia*, *Sphingobacteria*, *Alpha-*, *Beta-* and *Gammaproteobacteria*) were also found by Berg et al. (2009) as accompanying cyanobacterial blooms. Interestingly, the several taxa dominating in environmental samples on genus level (constituting 48-75% of their total community) were also related to the bloom phenomenon. These genera included wide-spread marine clade *Pelagibacteracea*e, as well as *Synechococcus* and *Prochlorococcus* (small marine cyanobacteria) with *Flavobacterium* that contain strains capable of degrading cyanobacterial toxins (Berg et al., 2009), even if toxic *Nodularia* and *Anabaena* were not found. On the other hand, typically freshwater cyanobacterial genera were detected, eg. *Dolichospermum* (up to 5%) or *Microcystis* (0.04%), however this is not surprising given the low salinity of the Baltic Sea waters and the inflow of riverine waters. Cyanobacterial blooms in the coastal zones are triggered by warmer temperatures and high nutrient availability, especially in areas impacted by treated wastewater (here: MO-D and MO-W) or by river discharge (here: VIS). This was confirmed by *Prochlorococcus* cyanobacteria showing higher abundance (2.6-8.6%) in summer samples (August/September), while did not exceed 1% in winter months (Nov and Feb). Nevertheless, HELCOM reports (Hansson and Öberg, 2012) reported that the surface blooms in 2012 were lower than average, compared with previous years. The NGS analysis also showed a relatively high percentage of sequences aligned to chloroplast or *Stramenopiles*, common marine single-

cell eukaryotes (protists) that play a great role in the nutrient cycling in the oceans (Seeleuthner et al., 2018). Sequences identified as *Chloroplasts* covered even 19% of the microbial community in the environmental samples, while they did not exceed 3% in treated wastewater (with higher abundance in summer months, not exceeding 0.06% in winter). Despite their probable eukaryotic origin they were not excluded from the analysis, as they may correlate with the summer algal bloom or correspond to photosynthetic activity of some autotrophic bacteria.

Environmental samples on average presented lower abundance of nitrifying bacteria than treated wastewater. They were also represented by different taxa: Nitrospina (NOB) and Nitrosopumilus (AOA), which are ubiquitous in marine waters (Brown et al., 2013). They were more abundant in marine waters impacted by treated wastewater (MO-D and MO-W), where they reached up to 0.44%. In Vistula river estuary (VIS) they did not exceed 0.04%, while in Gdansk Deep 0.02%. Bacteria conducting anammox process were not detected, while ammonia-oxidizing archaea consisted up to 0.03%, and were represented by popular marine archaea *Nitrosopumilus*. In case of nitrite oxidizers, this group was represented mainly by the family *Nitrospiraceae*, genus *Nitrospina*, NOB, while *Nitrospira* was noted only in the Vistula river estuary (up to 0.01%). The denitrification community of environmental samples highly differed from treated wastewater. It was less numerous and dominated by two genera: *Flavobacterium* (phylum *Bacteroidetes*) and *Rhodobacter* (*Alphaproteobacteria*), only occasionally with the minor addition of other taxa, such as *Stenotrophomonas*, *Hyphomicrobium, Achromobacter, Pseudomonas* (mainly *Proteobacteria* representatives). It is worth noting that decaying blooms may also serve as an additional source of organic matter supporting the denitrification processes. Both nitrification and denitrification microorganisms were on average more abundant at the stations related to treated wastewater discharge (MO-D and MO-W) than on those not exposed to WWTP effluent.

3.4. Functional gene detection and quantification

As mentioned above, the N-cycle is transformed by a diverse microbial community, whose members are equipped in key genes. Thus, to fully recognize the nitrogen removal potential, the genes used as molecular markers of nitrification (amoA and nxrA) and denitrification (nirS, nirK, nosZ) were tested in this study together with 16S rRNA genes. It is worth to note that in environmental studies most of the available literature regarding gene abundance refers to soil or sediments rather than sea water.

Obtained data indicated that WWTPs effluent contained 16S rRNA target molecules ranging from 2.4×10^5 to 2.1×10^6 copies per µL of DNA (6.6×10^5 to 3.5×10^6 copies of 16S rDNA per 1 mL of the sample), while in environmental samples they varied in wider range from 4.2×10^4 to 1.4×10^6 copies per µL of DNA (1.6×10^4 to 2.5×10^6 copies of 16S rDNA per 1 mL of the sample), Fig. 7. These values, together with the abundance of N-functional genes, were compared with the data available in the literature (Fig. 8). In all samples of treated wastewater (TW-D, TW-W) the studied genes were present, with the highest abundance of nirS gene (encoding a haem-containing nitrite reductase) and nirK gene (encoding Cu-containing nitrite reductase), which are responsible for the reduction of nitrite to nitrogen oxide. Thus, both unrelated nir-genes, which never occur together in the same organism, are used as markers of denitrifiers. As expected, the nirS and nirK washed out from WWTPs with treated wastewater were noted on a similar level and ranged from 10^5 to 10^6 gene copies mL^{-1}, which was much lower than in activated sludge samples (Fig. 8c,d). In marine outfalls (MO-D and MO-W) and Vistula river estuary (VIS) both genes did not exceed 10^5 gc mL^{-1}, while in the Gulf of Gdansk (GD) they reached only 10^4 gc mL^{-1}.

698 The final step of denitrification, catalyzed by nitrous oxide reductase, was also analyzed by the
699 presence of the nosZ gene, frequently used as a process biomarker (Fernández-Baca et al., 2018)
700 and factor regulating the production of N_2O in different niches (Henry et al., 2006). In this
701 study, the nosZ-based community was present in each analyzed sample, at the same order of
702 magnitude from 10^4 to 10^5 gc mL^{-1}, except GD point, where they were less prevalent: 10^2 to
703 10^3 gc mL^{-1} (Fig.7f).

704 **Figure 7.** Relative abundance of various DNA fragments and functional genes in different
705 sample types (treated wastewater - TW, its marine outfall - MO, Vistula estuary - VIS, and
706 Gdansk Deep - GD); a) 16S rDNA fragment, and functional genes involved in nitrification: b)
707 amoA and c) nxrA, and denitrification: d) nirS, e) nirK and f) nosZ.

708 Since NGS analysis has indicated that no AOA nor anammox bacteria were detected in samples
709 collected from WWTP effluents (TW-W and TW-D), and AOA only occasionally appeared in
710 marine outfalls (MO-D and MO-W) and in GD, it indirectly confirmed that organic matter
711 stimulates the denitrification and suppress anammox community, which is overcompetited for
712 NO_2^-, and that amoA-base community mainly consists of AOB. The amoA gene was detected
713 in the same range as nosZ and linked mainly to *Nitrosomonadaceae* family. Gene nxrA, which
714 is present in NOB and encodes the NO_2^- oxidation (Rani et al., 2017), occurred in lower quantity
715 and could be linked mainly to family *Nitrospiraceae* (for details see Section 3.3.1)

716 **Figure 8.** Relative abundance of nitrification genes: a) amoA, b) nxrA, and denitrification
717 genes: c) nirS, d) nirK and e) nosZ in treated wastewater and marine samples, compared with
718 the literature data, expressed in various units.

719 **4. Conclusions**

Up to date, microbial community of the treated wastewater has still been rarely studied and is still largely unexplored area, especially in terms of its seasonal variability and the microbial influence on the receiving waters. In this study, the synergistic approach has been applied, combining chemical and microbiological analyses. Characteristics of the treated effluent from two major WWTPs in northern Poland were tested with a set of various cultivation-dependent and independent techniques.

The WWTPs' effluents showed some variations regarding the basic chemical parameters, however the mechanisms behind these changes and the link with microbial community composition fluctuations are not fully understood yet. Concentrations of the chemical parameters in the effluent seem to be more influenced by the season than the influent parameters. The results showed that not only the chemical quality of the effluents, but also their microbial community undergoes transformations throughout the year. Decreased wastewater treatment efficiency during winter was reflected in more numerous and smaller bacteria structured into small flocs in the treated wastewater. The most pronounced and unambiguous seasonal changes in the microbial community of the WWTP effluent can be seen in respect to temperature: in abundance of filamentous and bulking bacteria, and as a result of worse dispersed flocks sedimentation. Biomass washout appeared, however WWTP exploiters undertake measures to (e.g.: PIX/PAX dosing) to prevent this situation.

From the sanitary point of view, the abundance of fecal indicators in WWTP effluent did not present a clear seasonal pattern, neither it exceeded the current standard for bathing sites in the coastal waters impacted by treated wastewater. However, despite high treatment efficiency (<95%) in terms of chemical and microbiological parameters, the treated wastewater can still be a source of both nutrients and bacteria (also human-related ones) to the receiving waters. Treated wastewater discharge can also increase the biochemical potential of the receiving

waters. The samples subjected to higher anthropogenic impact (MO-D, MO-W, VIS) consequently showed higher abundance of all the tested, potentially wastewater-related nitrification and denitrification bacteria, as well as N-cycling genes. To the best of the authors knowledge, it is the first study that shows the presence of N-cycling genes in the treated wastewater and one of the few concerning their abundance in the marine water column.

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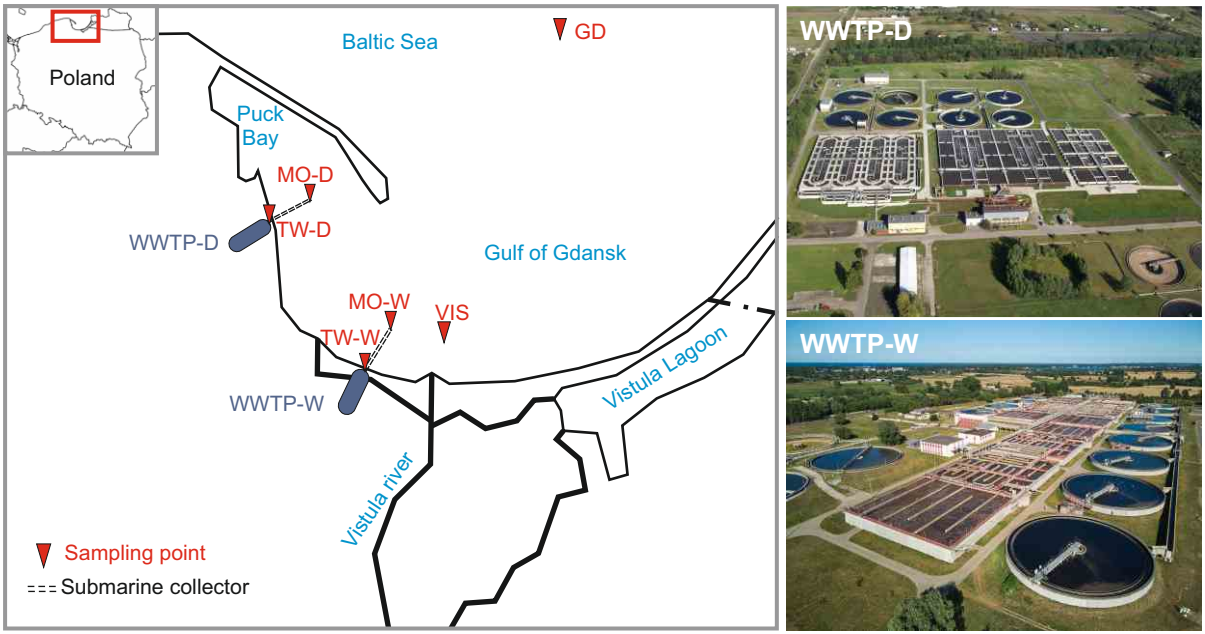

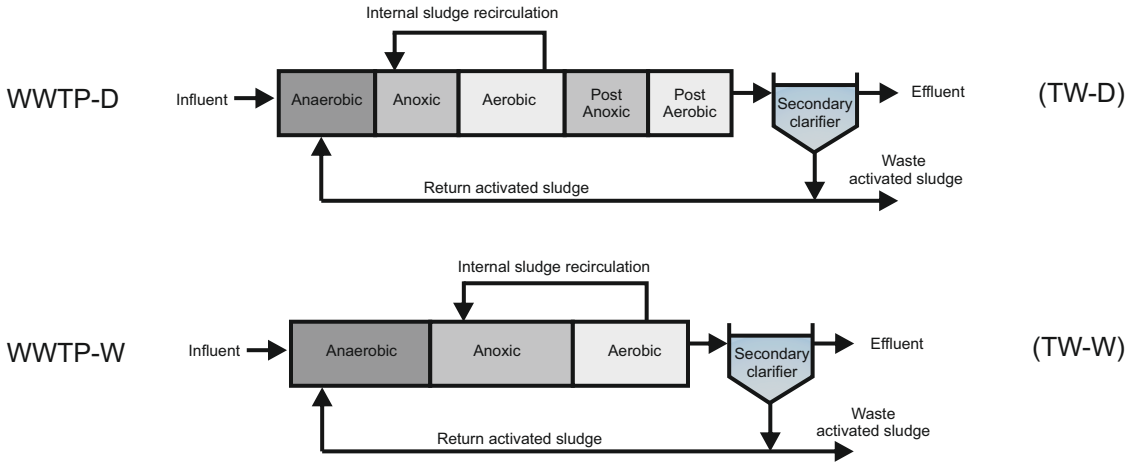

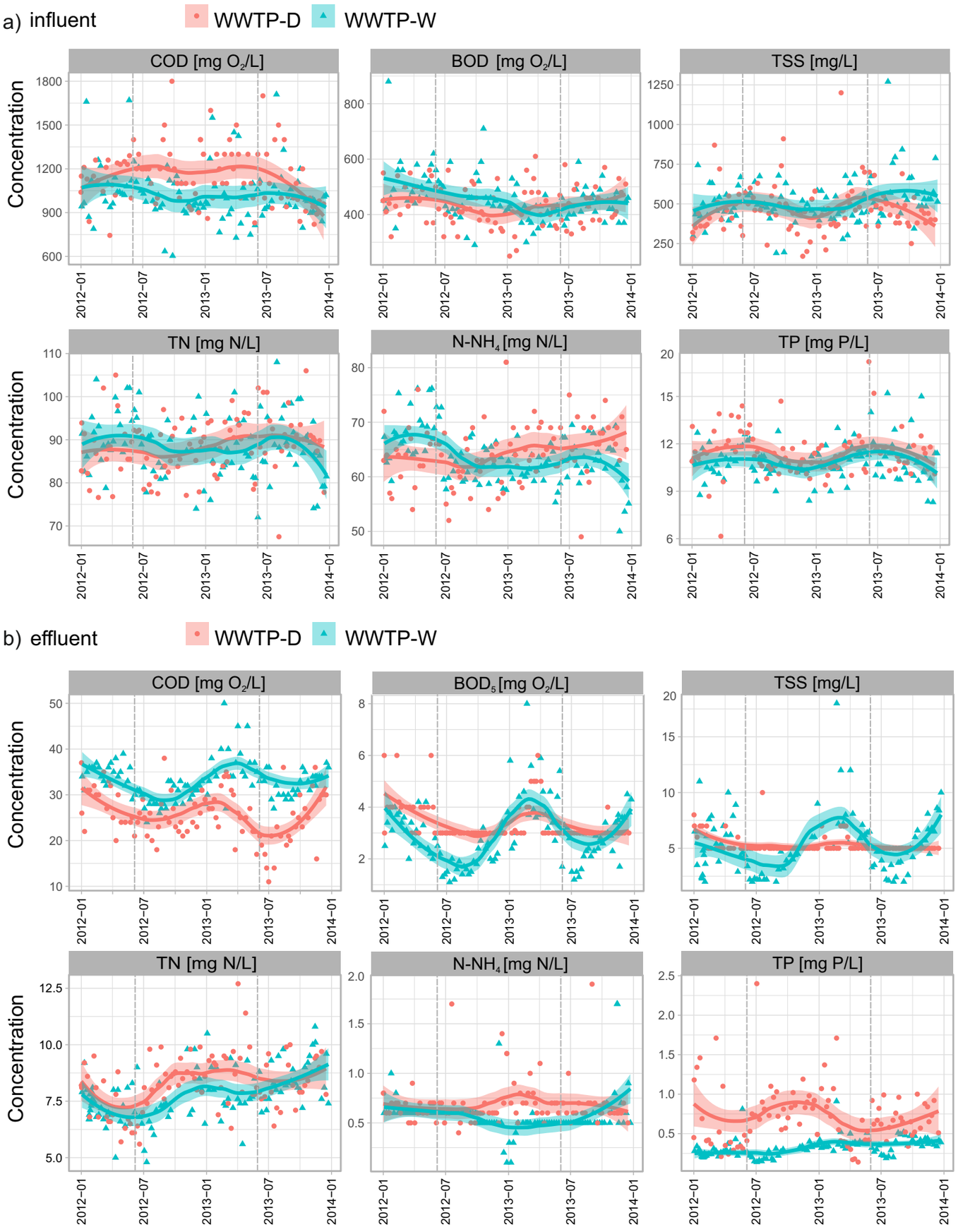

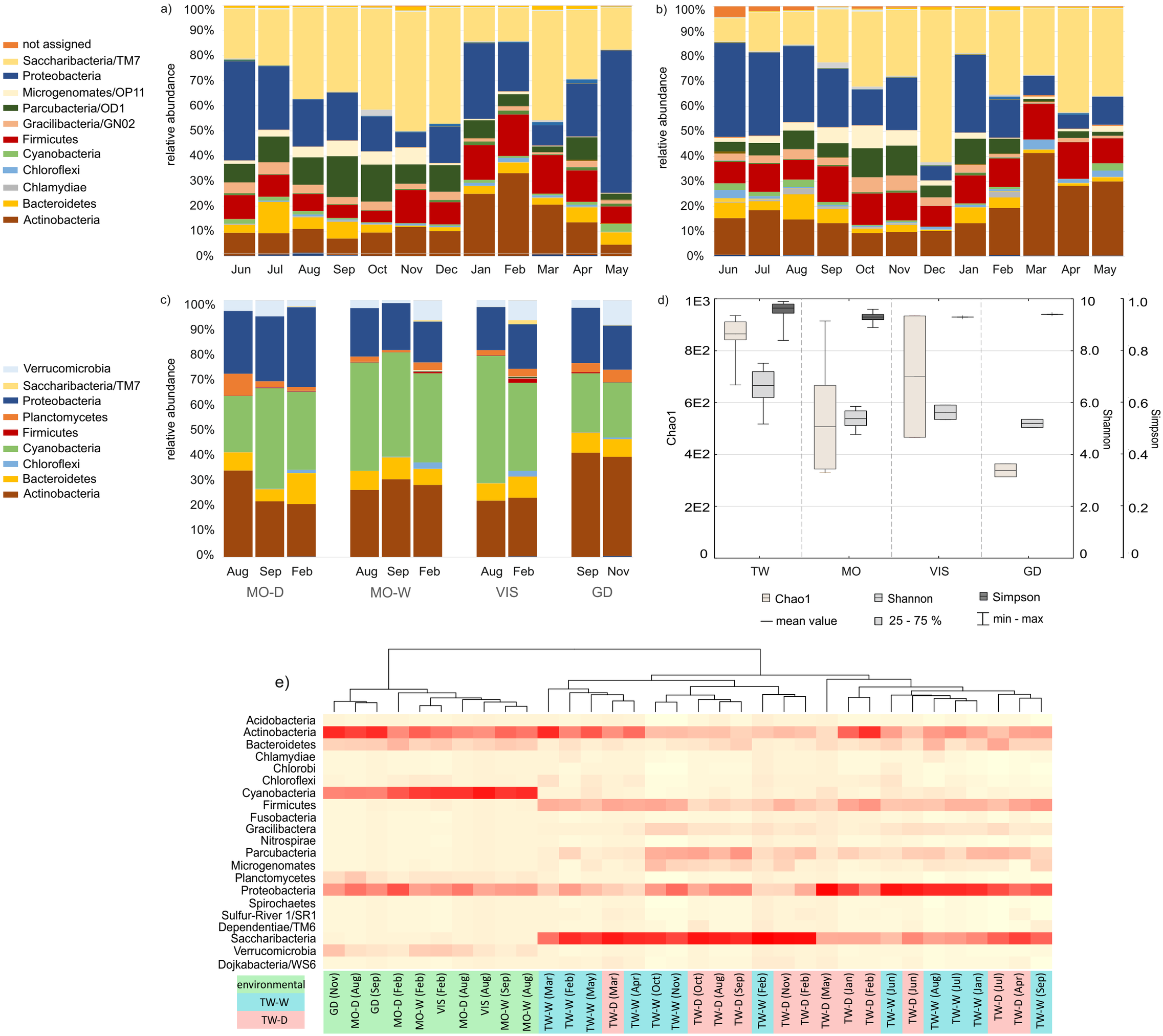

Figure6

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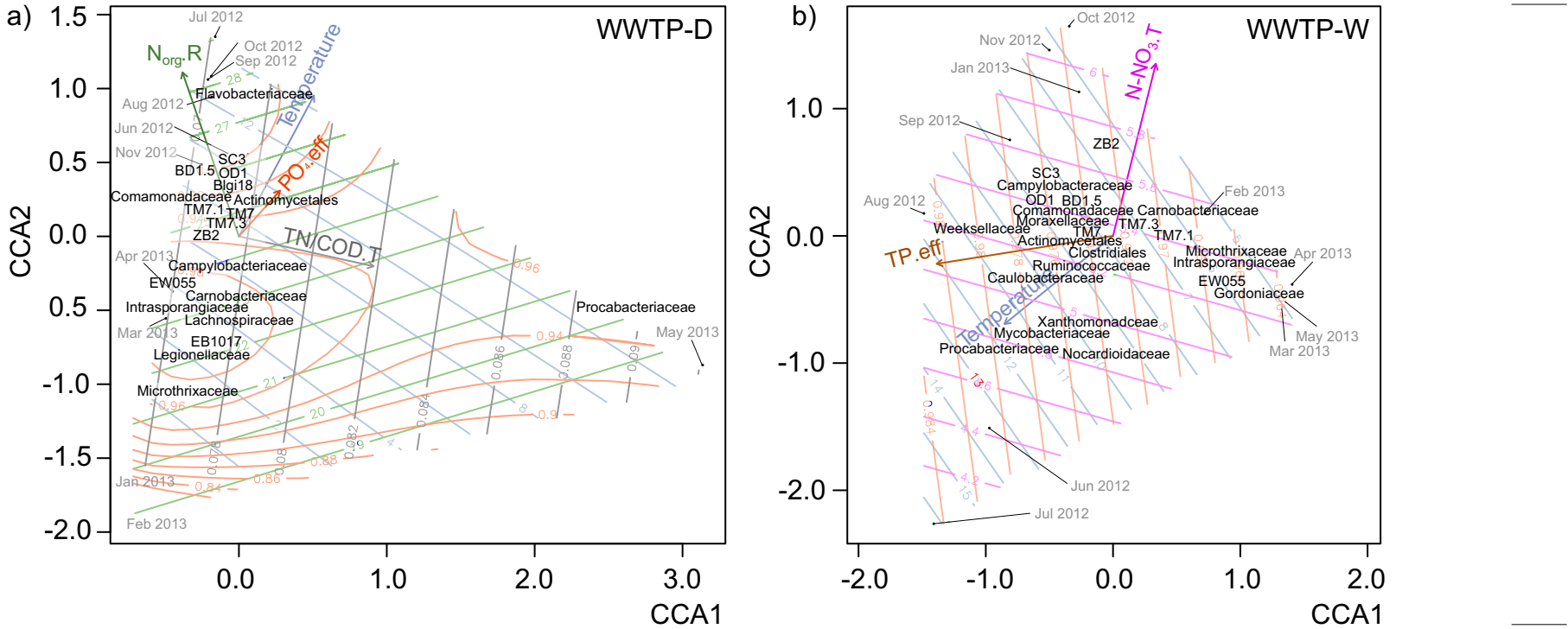

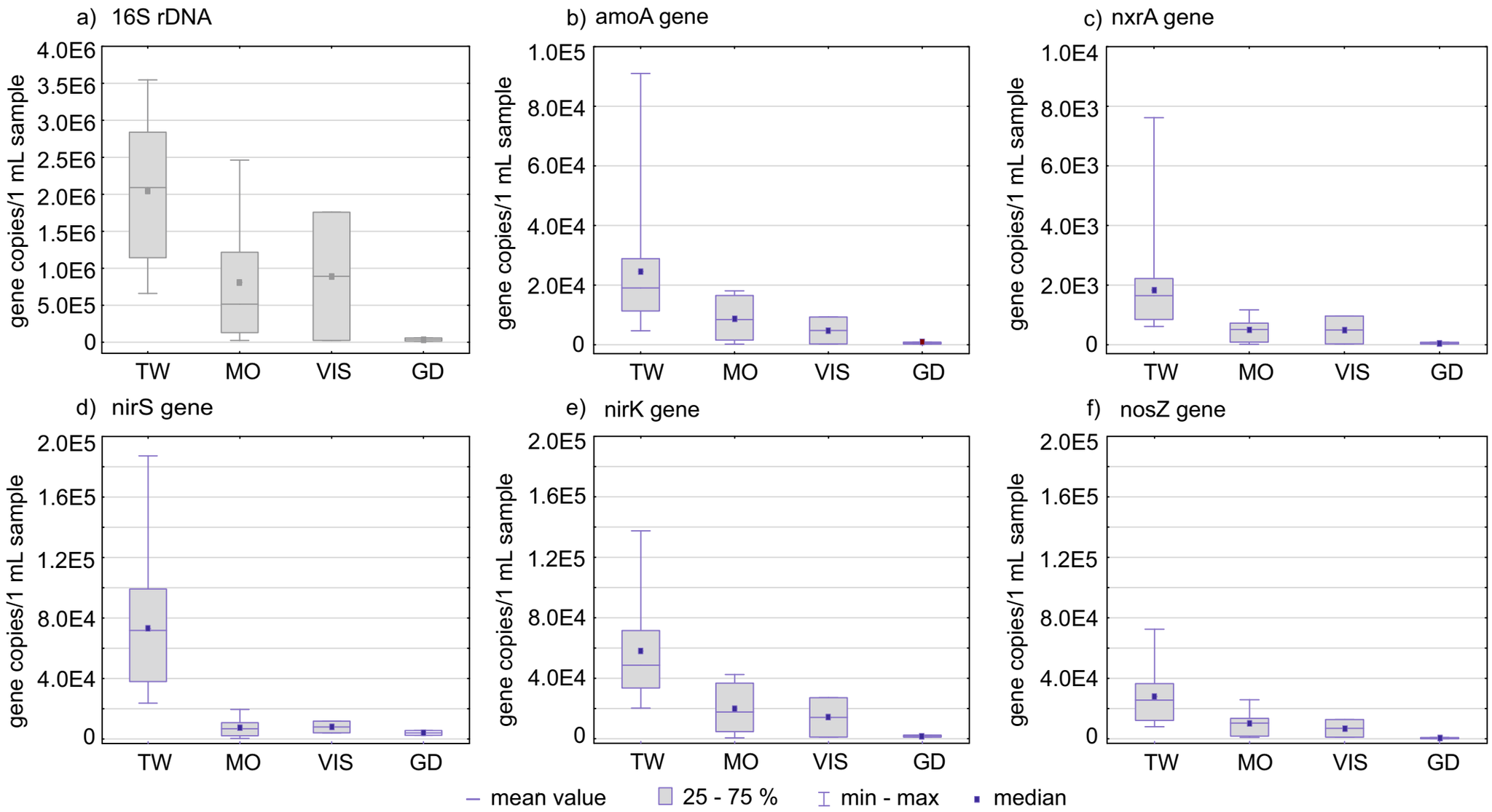

Figure3

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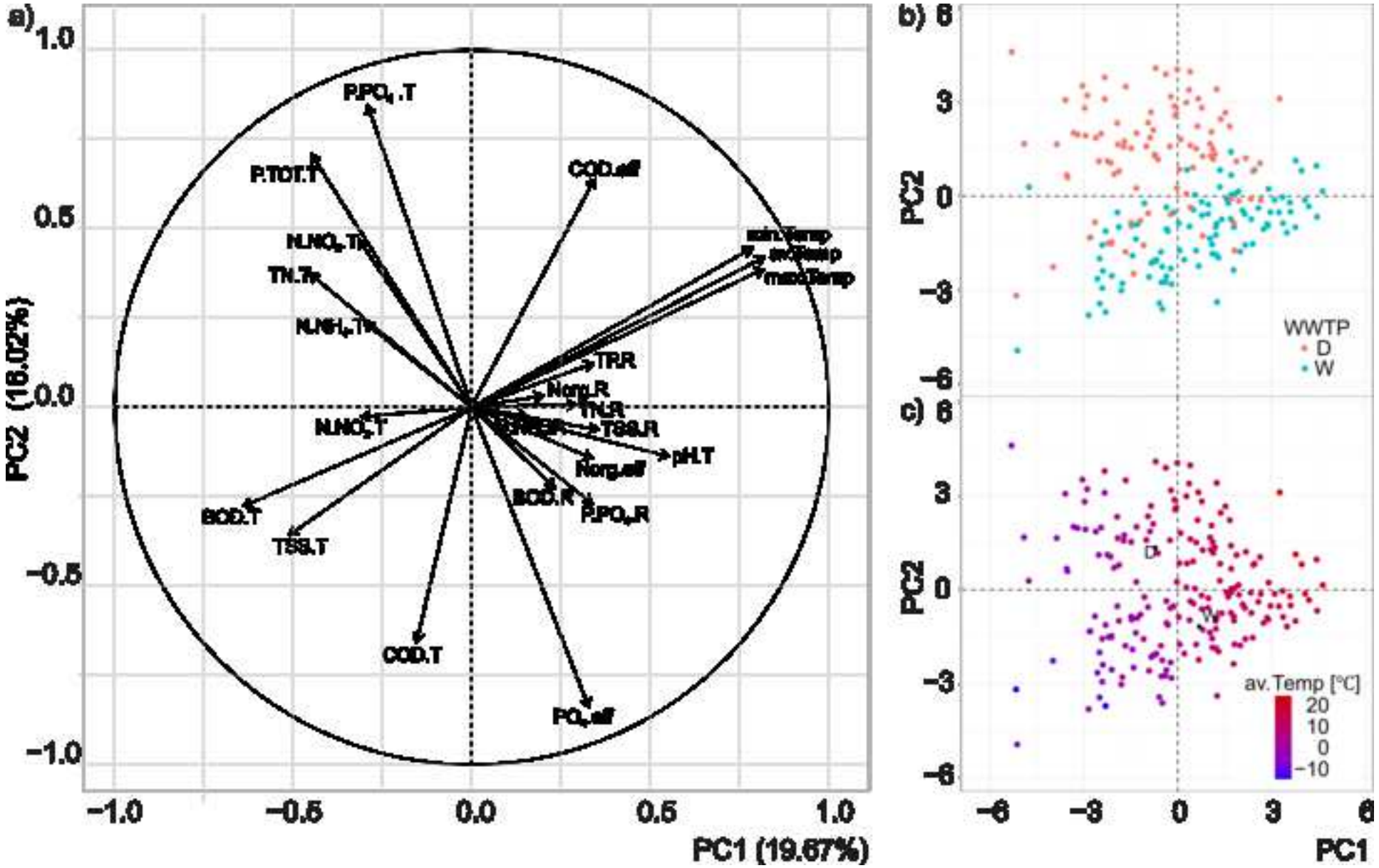


Figure4

Click here to access/download;Figure;Fig. 4 cultivation+microscope.png

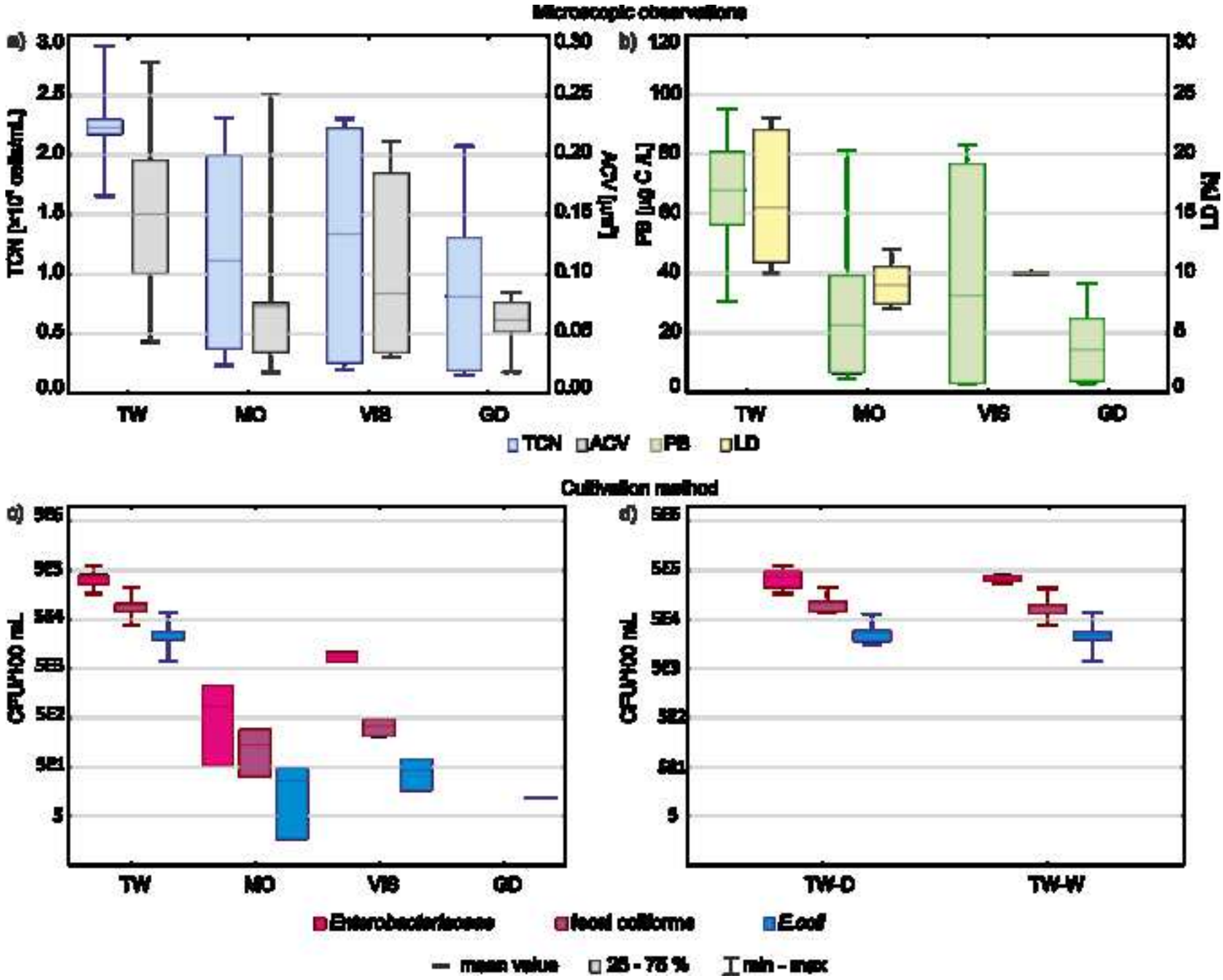
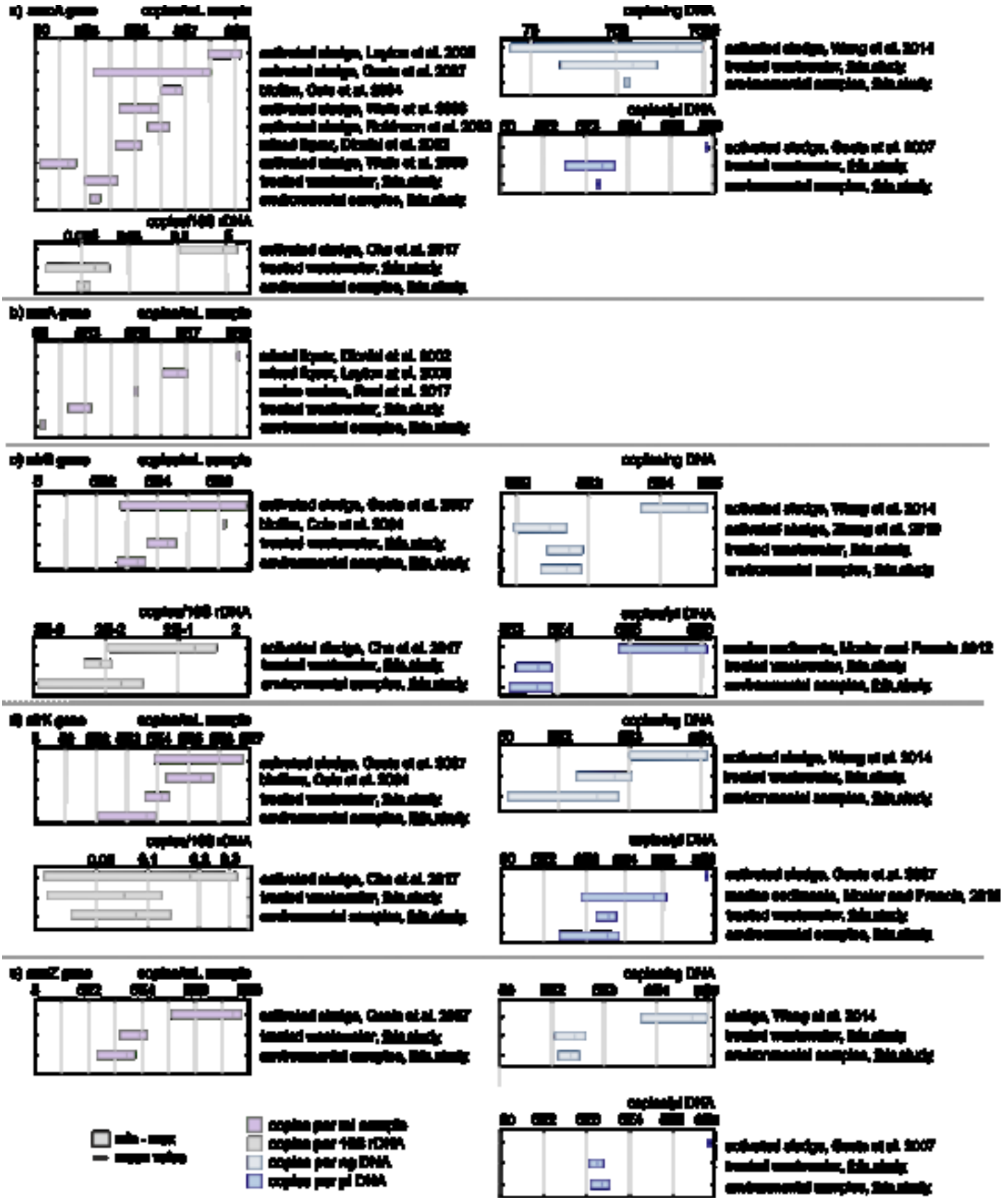


Figure8

Click here to access/download;Figure;Fig. 8 genes.png



Insights into the microbial community of treated wastewater, its year-round variability and impact on the receiver, using cultivation, microscopy and amplicon-based methods

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SUPPLEMENTARY MATERIALS

Table S1. Characteristics of the studied WWTPs: WWTP-W (Gdansk-Wschod) and WWTP-D (Gdynia-Debogorze). People Equivalent (PE) and average flow given as in 2015.

WWTP	Connected number of residents	PE [in BOD_5]	Designed capacity [m^3/d]	Average flow (min-max) [m^3/d]	Treatment technology			Influent Characteristic
					Mechanical	Biological	Chemical	
WWTP-W	570 000	742 500	120 000	92 958 (73 222-132 424)	screens, aerated grit chamber with a grease trap, and primary settling tanks	anaerobic/anoxic/oxic system (A2/O); advanced biological nutrient removal	PIX dosing system for occasional phosphorus removal	Industrial wastewater (11%) mostly from the food & chemical industry and shipyards. Hospital wastewater <1% of the total inflow; one infectious hospital effluent disinfected with UV
WWTP-D	360 000	476 000	73 000	55 294 (37 888-91 324)		Bardenpho system with simultaneous denitrification in Carroussel system; advanced biological nutrient removal		Industrial wastewater (10%) mostly from food, pharmaceutical & cosmetics industry, shipyards. Hospital wastewater (0,1%) discharged without disinfection

17 **Table S2** Characteristic of the cultivation media used in the study

Medium	Symbol	Incubation temperature	Incubation time	Cultivated bacteria	Characteristic of enumerated bacterial colonies
Chromocult® Coliform Agar	**C**	37°C	21-24 h	*Enterobacteriaceae*	All growing on the medium
				fecal coliforms	Blue to violet
Membrane Fecal Coliform Agar	**mFC**	44°C	22-24 h	*Escherichia coli*	Dark blue colonies

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19 **Table S3.** Primer sequences used to amplify fragments from *nirS*, *nirK*, *nosZ*, *amoA* and *nxrA*

20 genes in the denitrification pathway

Primer	Primer sequence (5'-3')	Reference
nirS1F	TACCACCCSGARCCGCGCGT	Kim et al. 2011
nirS 3r	GCCGCCGTCRTGVAGGAA	
nirK876	ATYGGCGGVCAYGGCGA	
nirK1040	GCCTCGATCAGRTTRTGGTT	
amoA-1-F	GGGGTTTCTACTGGTGGT	Li et al. 2012
amoA-2R	CCCCTCKGSAAAGCCTTCTTC	
nxrA-RT-F	GTGGTCATGCGCGTTGAGCA	Gerbl et al. 2014
nxrA-RT-R	TCGGGAGCGCCATCATCCAT	
nosZ-F	CGYTGTTCMTCGACAGCCAG	Throback et al. 2004
nosZ1622-R	CGSACCTTSTTGCCSTYGCG	

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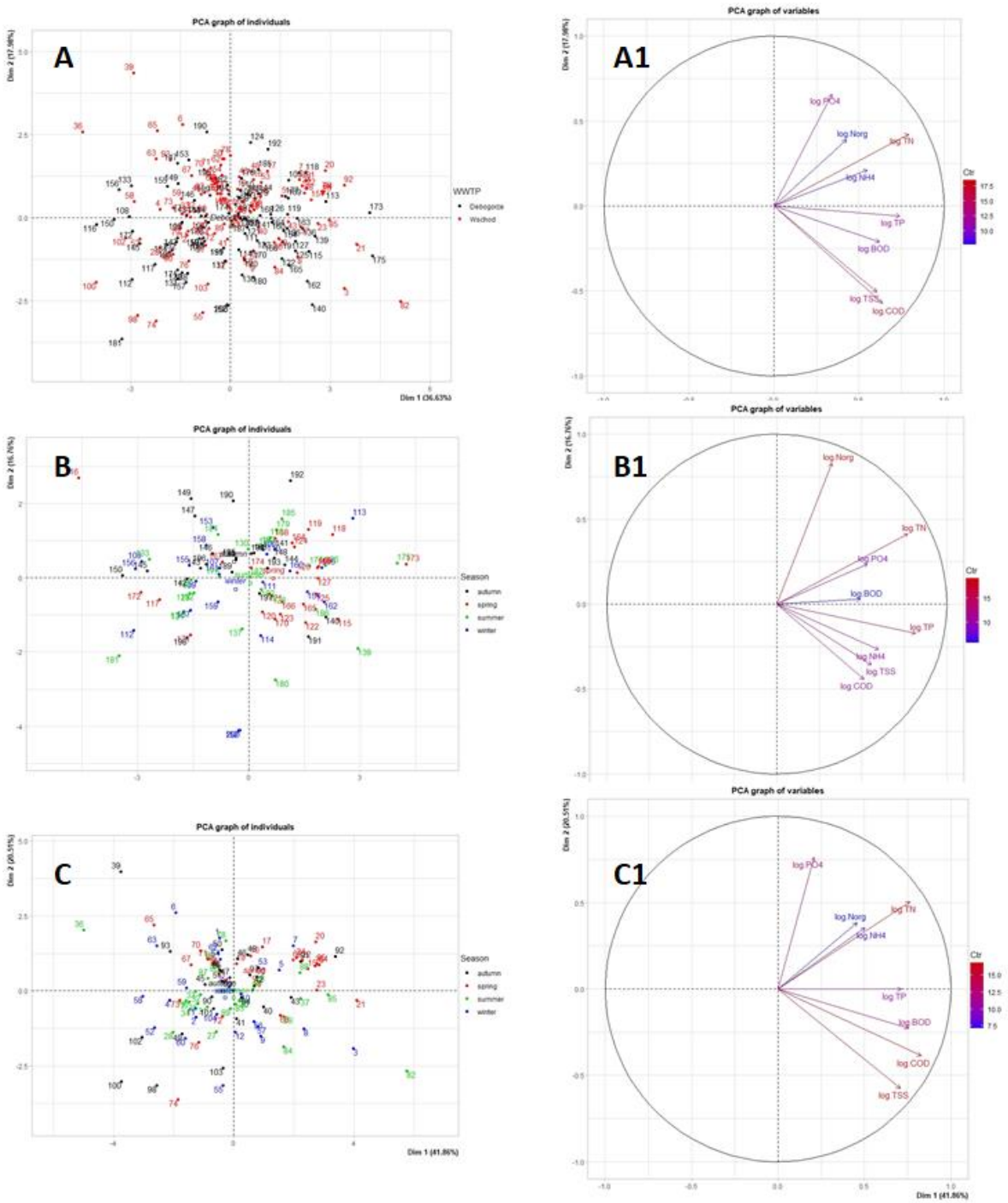

Figure S1. Principal Component Analysis (PCA) results for chemical parameters in raw wastewater of (A) both WWTPs, (B) WWTP-D and (C) WWTP-W. Numbers on the graph of individuals refer to the sample number. Additionally, the samples on Fig. B and C are coloured with respect to the season.

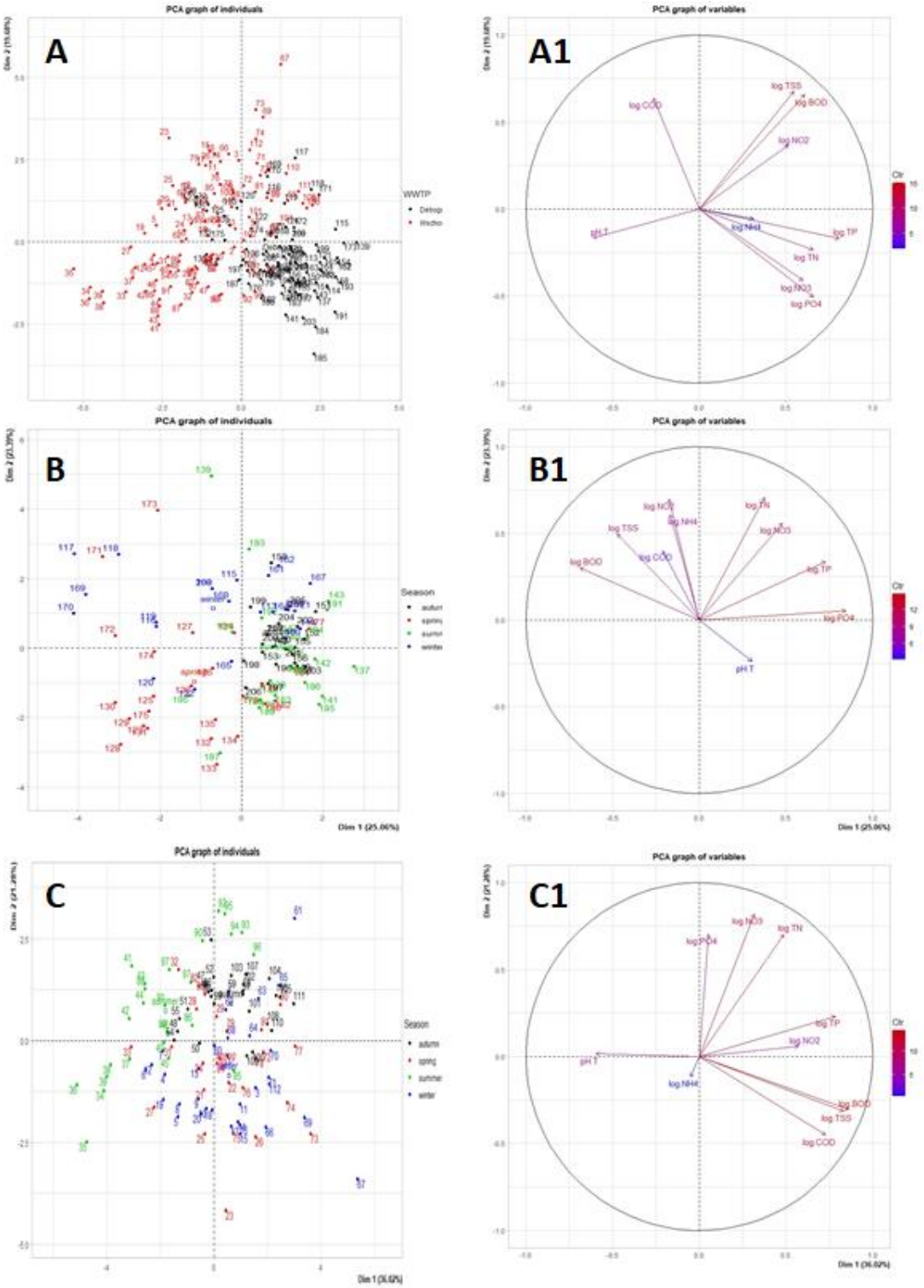

Figure S2. Principal Component Analysis results for chemical parameters in treated wastewater of (A) both WWTPs, (B) WWTP-D and (C) WWTP-W. Numbers on the graph of individuals refer to the sample number. Additionally, the samples on Fig. B and C are coloured with respect to the season.

Table S4. Basic chemical characteristics of the raw and treated wastewater for both WWTPs. Data given in format: av. / min.-max. / p = statistical significance of the difference between WWTPs

Sample		pH [–]	COD [mg O_2/dm^3]	BOD [mg O_2/dm^3]	TSS [mg/dm^3]	TN [mg N/dm^3]	N-NH_4 [mg N/dm^3]	N_{org} [mg N/dm^3]	N-NO_3 [mg N/dm^3]	N- NO_2 [mg N/dm^3]	TP [mg P/dm^3]	P-PO_4 [mg P/dm^3]
Influent	**Wschod**	7.73 7.33 – 8.01	1030 604 – 1710	458 290 – 880	516 190 – 1270	89 72 – 108	64 50 – 76	25.0 4.8 – 49.2	– –	– –	11.0 8.31 – 15.2	6.10 3.94 – 8.31
	Debogorze	– –	1150 744 – 1800	434 250 – 610	467 170 – 1200	89 68 – 106	64 49 – 81	24.2 9.5 – 38.4	– –	– –	11.4 6.15 – 17.2	5.52 3.71 – 8.34
	p	–	<0.001	0.038	0.0015	not significant	not significant	not significant	–	–	0.009	<0.001
Effluent	**Wschod**	7.92 7.44 – 8.20	33 24 – 50	2.93 1.10 – 8.00	5.23 2.00 – 18.00	7.80 4.80 – 10.8	0.57 0.08 – 1.70	1.68 0.43 – 3.63	5.55 3.41 – 8.30	0.07 0.01 – 0.47	0.32 0.14 – 0.91	0.06 0.01 – 0.50
	Debogorze	7.76 7.30 – 8.10	26 11 – 38	3.39 3.00 – 6.00	5.31 5.00 – 10.0	8.26 5.60 – 12.7	0.69 0.43 – 1.89	1.46 0.76 – 3.09	6.07 3.50 – 9.80	0.06 0.01 – 0.21	0.71 0.14 – 2.40	0.48 0.02 – 2.22
	p	<0.001	<0.001	0.0001	0.02	0.004	<0.001	0.038	<0.001	not significant	<0.001	<0.001
Removal efficiency [%]	**Wschod**	–	96.7 93.8 – 98.2	99.3 98.1 – 99.7	98.9 95.5 – 99.7	91.1 86.4 – 95.0	99.1 97.5 – 99.9	–	–	–	96.9 91.3 – 98.6	99.0 91.5 – 99.9
	Debogorze	–	97.5 82.3 – 99.1	99.2 98.5 – 99.5	98.7 97.0 – 99.5	90.7 86.6 – 93.9	98.9 97.2 – 99.3	–	–	–	93.6 78.6 – 98.8	91.1 54.9 – 99.7
marine outfalls (MO-W and MO-D)	**Summer**	–	30.22±12.96	2.45±0.96	2.6±0.89	0.51±0.16	0.03±0.01	–	–	–	0.07±0.02	0.02±0.01
	Winter	–	0.5±0.37	< LOD	3.2±1.92	0.40±0.07	0.03±0.01	–	–	–	0.12±0.03	0.08±0.01
Vistula River estuary (VIS)	**Summer**	–	32.8	3.6	2.6	0.09	0.04	–	–	–	<0.5	0.11
	Winter	–	0.40	< LOD	3.9	0.85	< 0.015	–	–	–	<0.5	< 0.05

Physicochemical data for Gdansk Deep (GD) were unavailable

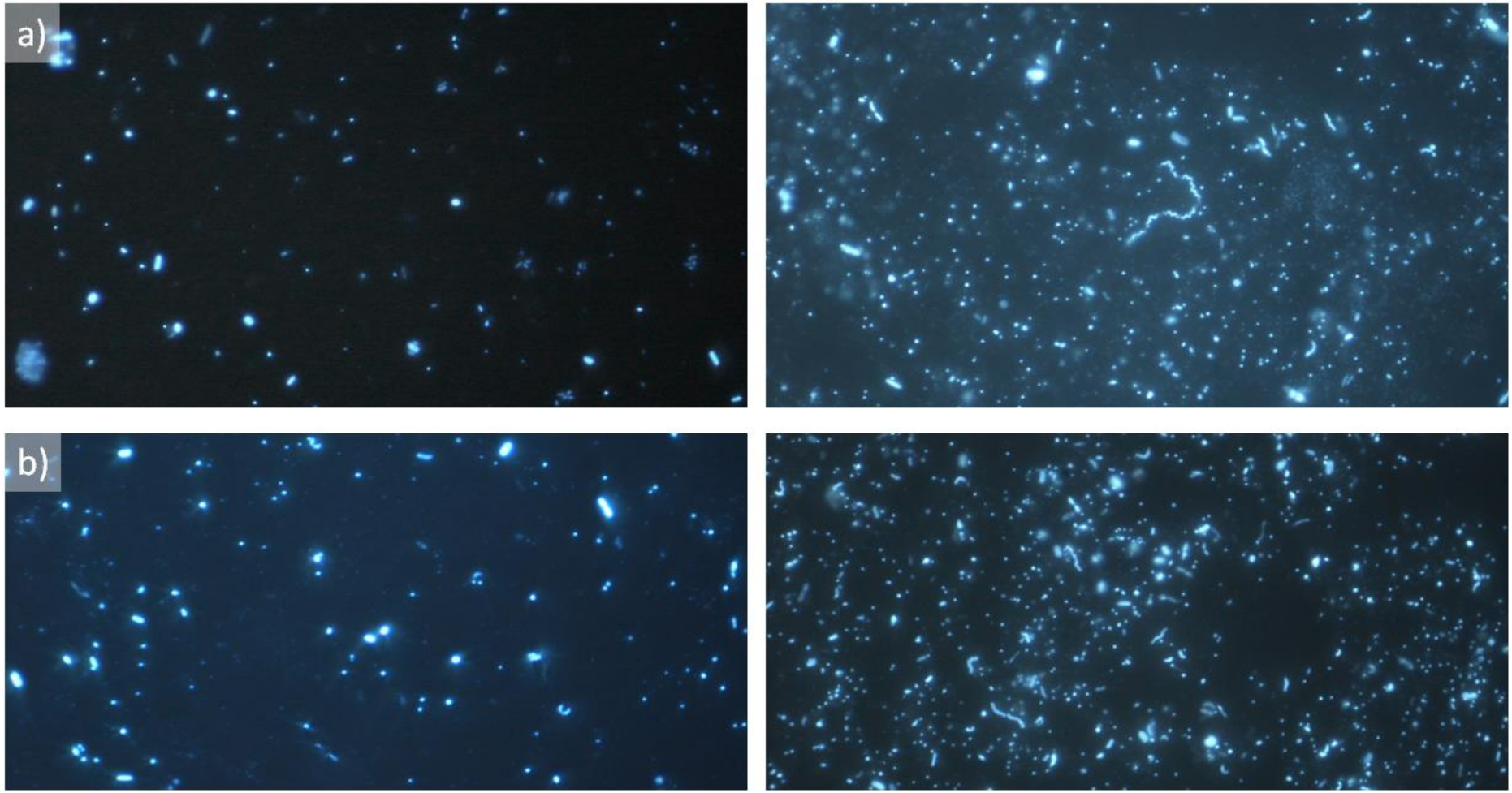

Figure S3. Photos of DAPI staining of treated wastewater from a) Gdynia-Debogorze WWTP and b) Gdańsk-Wschod WWTP. On the left samples from June, on the right from December

Table S5. Major taxa identified in the treated wastewater, marine outfalls, Vistula estuary and Gdansk Deep samples. All the number are given in % (nd - not detected).

					TW			MO			VIS			GD		
Phylum	**Class**	**Order**	**Family**	**Genus**	**min**	**max**	**av**	**min**	**max**	**av**	**min**	**max**	**av**	**min**	**max**	**av**
Acidobacteria	[Chloracidobacteria]	RB41	Ellin6075		<0,01	0,52	0,17	<0,01	0,03	0,01	<0,01	0,10	0,05	nd	nd	nd
Actinobacteria	Acidimicrobiia	Acidimicrobiales	C111		0,04	**1,79**	0,29	**4,26**	**14,13**	**10,31**	**8,40**	**8,80**	**8,60**	**20,91**	**24,49**	**22,70**
Actinobacteria	Acidimicrobiia	Acidimicrobiales	EB1017		0,07	**3,04**	0,70	0,04	0,62	0,33	0,32	0,32	0,32	0,17	0,17	0,17
Actinobacteria	Acidimicrobiia	Acidimicrobiales	Microthrixaceae	Candidatus Microthrix	0,09	**23,31**	**5,18**	<0,01	0,04	0,02	0,13	0,13	0,13	nd	nd	nd
Actinobacteria	Actinobacteria	Actinomycetales			0,93	**8,15**	**3,09**	**1,84**	**10,56**	**6,71**	**4,23**	**7,40**	**5,81**	**5,26**	**9,02**	**7,14**
Actinobacteria	Actinobacteria	Actinomycetales	ACK-M1		<0,01	0,03	0,02	0,74	**8,25**	**3,79**	**1,26**	**4,91**	**3,08**	**4,93**	**8,42**	**6,68**
Actinobacteria	Actinobacteria	Actinomycetales	Gordoniaceae	Gordonia	<0,01	**4,13**	0,50	0,11	**6,95**	**2,21**	0,17	**1,96**	**1,07**	0,96	**1,42**	**1,19**
Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Terracoccus	0,03	**2,31**	0,58	0,06	0,06	0,06	0,28	0,28	0,28	<0,01	<0,01	<0,01
Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae		0,07	**5,67**	**1,20**	<0,01	0,05	0,03	0,39	0,39	0,39	nd	nd	nd
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Clavibacter	<0,01	0,02	<0,01	0,19	**1,29**	0,58	0,10	**1,50**	0,80	0,07	0,08	0,07
Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium	0,04	**3,15**	0,65	0,04	**1,38**	0,54	0,07	0,73	0,40	0,09	0,64	0,37
Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Kribbella	<0,01	**1,74**	0,18	<0,01	0,02	0,01	nd	nd	nd	nd	nd	nd
Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides	0,01	**2,24**	0,17	nd	nd	nd	<0,01	<0,01	<0,01	nd	nd	nd
Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae		<0,01	**1,68**	0,38	<0,01	0,01	<0,01	0,02	0,02	0,02	nd	nd	nd
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	0,26	**1,66**	0,89	<0,01	<0,01	<0,01	0,02	0,02	0,02	nd	nd	nd
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Collinsella	0,26	**1,82**	0,86	<0,01	<0,01	<0,01	0,05	0,05	0,05	nd	nd	nd

Actinobacteria	Thermoleophilia	Gaiellales			<0,01	0,01	<0,01	<0,01	**1,12**	0,41	<0,01	0,81	0,41	0,10	0,24	0,17
Actinobacteria	Thermoleophilia	Solirubrobacterales			0,03	0,95	0,33	<0,01	**1,46**	0,64	0,03	0,83	0,43	0,37	0,57	0,47
Bacteroidetes	[Saprospirae]	[Saprospirales]	Saprospiraceae		0,04	**2,31**	0,62	<0,01	0,18	0,05	<0,01	0,07	0,04	<0,01	<0,01	<0,01
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	<0,01	**1,51**	0,58	<0,01	0,01	<0,01	0,01	0,01	0,01	nd	nd	nd
Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Chryseobacterium	<0,01	**5,33**	0,35	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	nd	nd	nd
Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Fluviicola	<0,01	0,15	0,03	0,44	**1,42**	0,80	0,38	0,80	0,59	0,81	**2,17**	**1,49**
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	<0,01	0,94	0,14	0,64	**2,44**	**1,39**	0,62	**4,83**	**2,73**	0,49	0,57	0,53
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Sediminicola	nd	nd	nd	0,13	**1,55**	0,91	0,04	0,38	0,21	0,89	0,90	0,89
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae		<0,01	**9,74**	**1,14**	0,28	0,87	0,55	0,45	0,75	0,60	0,45	0,78	0,61
Bacteroidetes	Sphingobacteriia	Sphingobacteriales			0,03	0,56	0,21	0,41	**2,05**	**1,14**	0,38	**1,30**	0,84	0,90	**1,27**	**1,08**
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	NS11-12		nd	nd	nd	0,01	**1,14**	0,30	0,03	0,18	0,10	0,16	0,21	0,18
Chlamydiae	Chlamydiia	Chlamydiales	Rhabdochlamydiaceae	Candidatus Rhabdochlamydia	<0,01	**2,03**	0,25	<0,01	0,13	0,03	<0,01	0,03	0,02	nd	nd	nd
Chlorobi	SJA-28				<0,01	**1,18**	0,13	<0,01	<0,01	<0,01	0,01	0,01	0,01	nd	nd	nd
Chloroflexi	Anaerolineae	Caldilineales	Caldilineaceae	Caldilinea	0,01	**1,93**	0,41	<0,01	<0,01	<0,01	0,02	0,02	0,02	nd	nd	nd
Chloroflexi	Anaerolineae	Caldilineales	Caldilineaceae		<0,01	0,78	0,21	<0,01	**2,21**	0,67	**1,90**	**1,90**	**1,90**	0,18	0,18	0,18
Cyanobacteria	Chloroplast				<0,01	0,54	0,06	0,01	**1,92**	**1,15**	**1,16**	**1,16**	**1,16**	0,08	0,16	0,12
Cyanobacteria	Chloroplast	Chlorophyta			<0,01	0,10	0,02	0,30	**1,05**	0,59	0,65	**1,33**	0,99	0,24	**1,76**	**1,00**
Cyanobacteria	Chloroplast	Chlorophyta	Chlamydomonadaceae		<0,01	0,01	<0,01	<0,01	0,03	0,02	<0,01	**1,92**	0,97	0,01	0,01	0,01
Cyanobacteria	Chloroplast	Cryptophyta			0,02	0,02	0,02	0,58	**4,05**	**1,83**	0,27	0,86	0,57	0,44	0,96	0,70

Cyanobacteria	Chloroplast	Stramenopiles			<0,01	**2,72**	0,48	**1,02**	**10,91**	**4,27**	**8,15**	**13,20**	**10,68**	0,91	**3,73**	**2,32**
Cyanobacteria	Chloroplast	Streptophyta			<0,01	**1,46**	0,38	nd	nd	nd	nd	nd	nd	nd	nd	nd
Cyanobacteria	Nostocophycideae	Nostocales	Nostocaceae	Dolichospermum	nd	nd	nd	0,13	**5,08**	**1,49**	0,09	0,09	0,09	0,14	0,14	0,14
Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae	Prochlorococcus	nd	nd	nd	0,43	**7,68**	**3,48**	0,46	**8,58**	**4,52**	0,43	**3,73**	**2,08**
Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae	Synechococcus	nd	nd	nd	**11,24**	**29,81**	**21,83**	**21,43**	**24,01**	**22,72**	**13,39**	**17,06**	**15,22**
Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae		0,34	**3,95**	**1,93**	<0,01	0,08	0,04	0,37	0,37	0,37	nd	nd	nd
Firmicutes	Clostridia	Clostridiales			0,85	**5,62**	**2,39**	<0,01	0,11	0,03	0,25	0,25	0,25	<0,01	<0,01	<0,01
Firmicutes	Clostridia	Clostridiales	Clostridiaceae		0,24	**2,23**	**1,19**	<0,01	0,06	0,02	0,14	0,14	0,14	nd	nd	nd
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	0,05	**1,78**	0,58	nd	nd	nd	<0,01	<0,01	<0,01	nd	nd	nd
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		0,19	**2,59**	0,77	0,01	0,01	0,01	0,02	0,02	0,02	nd	nd	nd
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		0,26	**3,11**	**1,01**	<0,01	0,03	0,02	<0,01	0,06	0,03	nd	nd	nd
Fusobacteria	Fusobacteriia	Fusobacteriales			0,01	**1,13**	0,33	nd	nd	nd	nd	nd	nd	nd	nd	nd
GN02	3BR-5F				0,10	**2,16**	0,48	<0,01	0,01	<0,01	0,03	0,03	0,03	nd	nd	nd
GN02	BD1-5				0,43	**4,71**	**2,01**	<0,01	0,04	0,01	<0,01	0,08	0,04	nd	nd	nd
OD1					0,47	**10,34**	**3,52**	<0,01	0,06	0,02	<0,01	0,25	0,13	nd	nd	nd
OD1	ABY1				0,10	**2,44**	0,87	<0,01	0,02	<0,01	<0,01	0,08	0,04	<0,01	<0,01	<0,01
OD1	ZB2				0,18	**6,62**	**2,70**	<0,01	0,05	0,01	0,02	0,12	0,07	nd	nd	nd
OP11	OP11-1				<0,01	**2,85**	0,48	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	nd	nd	nd
OP11	OP11-3				0,03	**1,78**	0,54	0,02	0,02	0,02	0,05	0,05	0,05	nd	nd	nd

OP11	OP11-4				0,01	**2,01**	0,59	<0,01	<0,01	<0,01	0,02	0,02	0,02	nd	nd	nd
OP11	WCHB1-64				0,01	**2,60**	0,56	<0,01	<0,01	<0,01	0,04	0,04	0,04	nd	nd	nd
OP11	WCHB1-64	d153			0,04	**2,52**	0,64	<0,01	0,34	0,12	0,03	0,03	0,03	<0,01	<0,01	<0,01
Planctomycetes	Planctomycetia	Gemmatales	Isosphaeraceae		<0,01	0,01	<0,01	<0,01	**1,08**	0,45	0,02	0,29	0,15	<0,01	<0,01	<0,01
Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae		0,02	0,25	0,08	0,39	**6,09**	**1,67**	**1,09**	**1,12**	**1,11**	**3,21**	**3,54**	**3,38**
Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces	<0,01	0,19	0,03	0,07	**1,71**	0,72	0,34	**1,68**	**1,01**	0,12	0,49	0,30
Proteobacteria	Alphaproteobacteria				0,04	0,54	0,15	0,48	**1,77**	**1,14**	0,52	**1,37**	0,94	**1,37**	**2,02**	**1,69**
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Mycoplana	<0,01	**2,66**	0,33	<0,01	0,10	0,03	0,13	0,13	0,13	<0,01	<0,01	<0,01
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacter	<0,01	0,62	0,08	0,02	**2,81**	0,71	0,02	0,74	0,38	0,14	0,33	0,23
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae		0,05	0,68	0,19	0,35	**2,81**	**1,35**	0,57	**1,94**	**1,26**	0,38	0,53	0,45
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae		<0,01	0,13	0,03	0,11	**1,05**	0,40	0,08	0,54	0,31	0,22	0,48	0,35
Proteobacteria	Alphaproteobacteria	Rickettsiales			0,04	**1,21**	0,41	0,02	**1,07**	0,35	0,03	0,11	0,07	0,06	0,08	0,07
Proteobacteria	Alphaproteobacteria	Rickettsiales	Pelagibacteraceae		nd	nd	nd	**3,56**	**14,34**	**10,10**	**1,56**	**7,68**	**4,62**	**7,73**	**13,59**	**10,66**
Proteobacteria	Alphaproteobacteria	Sphingomonadales			<0,01	<0,01	<0,01	<0,01	**1,50**	0,38	<0,01	0,25	0,12	<0,01	0,06	0,04
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingobium	<0,01	**1,28**	0,26	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	0,05	0,05	0,05
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	<0,01	**1,01**	0,15	<0,01	0,02	<0,01	0,05	0,05	0,05	nd	nd	nd
Proteobacteria	Betaproteobacteria				0,04	0,77	0,24	0,10	**3,39**	0,88	0,55	**1,07**	0,81	0,29	0,40	0,35
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Delftia	<0,01	**1,09**	0,13	nd	nd	nd	<0,01	<0,01	<0,01	nd	nd	nd
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rhodoferax	0,14	**3,48**	0,72	<0,01	0,60	0,22	0,93	0,93	0,93	nd	nd	nd

Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		0,79	**9,35**	**2,67**	0,57	**3,69**	**1,37**	**1,69**	**1,82**	**1,75**	0,10	0,29	0,20
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Polynucleobacter	<0,01	0,43	0,05	0,24	**1,60**	0,69	0,78	**3,09**	**1,94**	0,17	0,27	0,22
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae		<0,01	**1,44**	0,15	<0,01	0,10	0,04	0,07	0,09	0,08	<0,01	<0,01	<0,01
Proteobacteria	Betaproteobacteria	MWH-UniP1			nd	nd	nd	0,01	**1,22**	0,47	0,56	0,56	0,56	0,12	0,22	0,17
Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae		<0,01	**1,43**	0,19	<0,01	<0,01	<0,01	0,01	0,01	0,01	<0,01	<0,01	<0,01
Proteobacteria	Betaproteobacteria	Procabacteriales	Procabacteriaceae		<0,01	**38,97**	**2,91**	<0,01	0,01	<0,01	<0,01	<0,01	<0,01	nd	nd	nd
Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae		0,03	**1,67**	0,41	0,07	**1,58**	0,43	0,16	0,23	0,19	0,31	0,94	0,62
Proteobacteria	Deltaproteobacteria				0,01	**2,19**	0,46	<0,01	0,08	0,02	<0,01	<0,01	<0,01	nd	nd	nd
Proteobacteria	Deltaproteobacteria	Spirobacillales			<0,01	0,05	0,01	<0,01	**1,21**	0,30	<0,01	0,23	0,12	<0,01	0,03	0,01
Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae		0,26	**10,04**	**3,70**	<0,01	0,09	0,04	<0,01	0,07	0,03	nd	nd	nd
Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae		<0,01	**2,49**	0,48	<0,01	<0,01	<0,01	<0,01	0,01	0,01	0,19	0,19	0,19
Proteobacteria	Gammaproteobacteria	HOC36			0,05	**1,35**	0,27	<0,01	<0,01	<0,01	0,47	0,47	0,47	nd	nd	nd
Proteobacteria	Gammaproteobacteria	Legionellales			<0,01	**1,95**	0,22	<0,01	0,04	0,01	<0,01	0,04	0,02	0,05	0,05	0,05
Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Tatlockia	<0,01	**1,24**	0,16	<0,01	0,01	<0,01	<0,01	<0,01	<0,01	0,34	0,34	0,34
Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae		0,02	**7,55**	0,93	0,07	0,18	0,13	0,04	0,18	0,11	0,03	0,83	0,43
Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Candidatus Portiera	nd	nd	nd	0,24	**2,39**	**1,34**	0,09	**1,17**	0,63	**1,89**	**2,14**	**2,01**
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	0,02	**1,94**	0,49	nd	nd	nd	0,01	0,01	0,01	nd	nd	nd
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae		0,02	**2,84**	0,65	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	0,03	0,01
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae		<0,01	**1,92**	0,15	<0,01	0,02	0,01	<0,01	<0,01	<0,01	<0,01	0,07	0,04
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	<0,01	**1,00**	0,11	0,01	0,04	0,03	<0,01	0,01	0,01	nd	nd	nd

Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae		0,06	**5,15**	0,64	<0,01	0,09	0,03	<0,01	0,11	0,06	nd	nd	nd
SR1					0,03	**1,42**	0,33	<0,01	0,01	<0,01	<0,01	0,06	0,03	nd	nd	nd
TM6	SJA-4	S1198			<0,01	**2,44**	0,49	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	nd	nd	nd
TM7					**2,89**	**12,57**	**5,72**	<0,01	0,05	0,02	0,12	0,12	0,12	nd	nd	nd
TM7	SC3				0,19	**12,37**	**3,24**	<0,01	0,02	0,01	0,02	0,08	0,05	nd	nd	nd
TM7	TM7-1				**3,59**	**34,88**	**12,59**	<0,01	0,14	0,03	0,02	0,60	0,31	nd	nd	nd
TM7	TM7-3				**1,04**	**16,04**	**5,61**	<0,01	0,16	0,08	0,54	0,54	0,54	<0,01	0,24	0,12
TM7	TM7-3	Blgi18			<0,01	**4,69**	0,85	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	nd	nd	nd
TM7	TM7-3	EW055			<0,01	**5,34**	0,83	0,03	0,03	0,03	0,13	0,13	0,13	nd	nd	nd
TM7	TM7-3	I025			<0,01	**1,59**	0,19	0,02	0,02	0,02	nd	nd	nd	nd	nd	nd
Verrucomicrobia	[Methylacidiphilae]	Methylacidiphilales	LD19		<0,01	<0,01	<0,01	0,28	**2,24**	0,95	0,06	0,63	0,35	0,23	0,42	0,33
Verrucomicrobia	[Spartobacteria]	[Chthoniobacterales]	[Chthoniobacteraceae]	Candidatus Xiphinematobacter	<0,01	0,60	0,12	0,34	**5,35**	**2,35**	0,67	**5,23**	**2,95**	**2,10**	**7,51**	**4,80**
Verrucomicrobia	[Spartobacteria]	[Chthoniobacterales]	[Chthoniobacteraceae]	DA101	<0,01	<0,01	<0,01	0,02	0,56	0,28	0,13	0,48	0,30	<0,01	**1,12**	0,56
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae		<0,01	0,15	0,02	<0,01	**1,09**	0,29	0,04	**1,41**	0,73	0,10	0,10	0,10
WS6	B142				0,05	**1,46**	0,52	<0,01	0,01	<0,01	0,06	0,06	0,06	nd	nd	nd
Not assigned					0,05	**4,24**	0,51	<0,01	0,09	0,04	0,01	0,06	0,04	<0,01	0,05	0,03

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