

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

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

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

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

41 **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

42

43 **1. Introduction**

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

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

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

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

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

86 in reservoirs the WWTP effluents resulted in both an increase (García-Armisen et al., 2014;
87 Kalinowska et al., 2020; Price et al., 2018; Wakelin et al., 2008) and a decrease (Drury et al.,
88 2013; Lu and Lu, 2014) of bacterial communities diversity. Evaluation of the Urban Waste
89 Water Treatment Directive, prepared by the European Commission, indicated that load of the
90 targeted pollutants discharged via treated wastewater from urban point sources decreased
91 significantly between 1990 and 2014 (BOD by 61% nitrogen by 32% and phosphorus by 44%)
92 and improved the quality of bathing sites across the EU (SWD 700, 2019). Nonetheless, EU
93 waters fail to achieve good status under the Water Framework Directive and the inappropriately
94 treated urban wastewater has been still pointed as an area for improvement. Additionally, there
95 is growing evidence that contaminants of emerging concern are not targeted and are
96 continuously discharged to the environment, even via appropriately treated wastewater.
97 Especially pharmaceuticals, microplastics, human-related bacteria and antimicrobial resistance
98 are recognized as a global threat (Everaert et al., 2020; Marano et al., 2020; Roca et al., 2015),
99 however activated sludge-related bacteria are also disseminated. Their potential of participation
100 in C, P, and N cycling in the receiver requires attention. Nitrification and denitrification are one
101 of the most frequently investigated wastewater treatment processes. The abundance of bacteria
102 responsible for these processes can be quantified using specific genes as molecular markers:
103 *amoA* and *nxrA* genes for nitrifiers, and *nirK*, *nirS* and *nosZ* for denitrifying bacteria (Huang et
104 al., 2019; Tang et al., 2020; Wang et al., 2014; Zhang et al., 2019).

105 This study covered two largest municipal WWTPs (WWTP-W and WWTP-D) located upon
106 the Baltic Sea in northern Poland. They receive wastewater generated by a relatively large
107 metropolitan area of Tricity (over 1 mln inhabitants, area around 400 km²), with various
108 branches of industry. In this study, it has been hypothesized that treated wastewater disposal
109 can shape the microbial community of the recipient, especially by discharge of human related
110 bacteria, washout of activated sludge community and release of functional N-cycling genes,

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

120 **2. Materials and Methods**

121 **2.1. Study area, sampling, and WWTP characteristics**

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

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

134 Both WWTPs discharge treated wastewater into the Gulf of Gdansk (Natura 2000 area), approx.
135 2.3 km from the coastline at a depth of about 8 m via submarine collectors equipped with
136 diffusers (Fig. 1). Samples of the marine waters at the point of treated wastewater discharge
137 (marine outfalls: MO-W and MO-D, respectively to the name of each WWTP) were sampled
138 three times (August and September 2012, February 2013). Additionally, two reference points:
139 Vistula River estuary (VIS) and Gdansk Deep (GD) were sampled twice (summer 2012 and
140 winter 2012/2013). Vistula River was chosen due to the ecological importance of its flow on
141 the Gulf of Gdansk quality. It is the longest river in Poland (1047 km) as well as in the area of
142 the Baltic Sea, with its catchment equal to 194,424 km² (87% in Poland). The Vistula flows
143 directly into the Gulf of Gdansk through a straight, man-made outlet, with an average annual
144 flow of about 1×10^3 m³/s at the mouth. Gdansk Deep (GD) is located in the open sea and is
145 assumed to be isolated from the anthropogenic impact (Maksymowska et al., 2000). All
146 environmental samples (points MO-W, MO-D, VIS, and GD) were collected with a Niskin
147 bottle and transferred to sterile polyethylene bottles. The bottles were rinsed with the sampled
148 water three times before sample collection. Samples were immediately transported in the cooler
149 box at +4°C to the laboratory.

150 2.2. Chemical analysis

151 Chemical analyses were conducted in all influent and effluent samples (twice a week for two
152 years). Parameters were determined according to the Standard Methods (APHA, 2005): total
153 nitrogen (TN), ammonium nitrogen (N-NH₄), nitrate nitrogen (N-NO₃), total phosphorus (TP),
154 phosphate phosphorus (P-PO₄) and chemical oxygen demand (COD) were analyzed by a XION
155 500 spectrophotometer (Dr. Lange, GmbH, Germany); 5-day biochemical oxygen demand
156 (BOD₅) – by a manometric respirometric BOD OxiTop® method; total suspended solids (TSS)
157 – 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 µg 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),

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

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

194 **2.3.2. Cultivation methods**

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

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

204 **2.3.3. DNA extraction and bacterial 16S rRNA gene amplification**

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

220 **2.3.4. Sequencing, taxonomic assignment, and data analysis**

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

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

234 **2.3.5. Quantitative PCR of nitrogen-cycling-related genes**

235 Quantitative real-time PCR was used to validate the absolute abundance of 16S rRNA and some
236 functional genes, including *amoA*, *nxrA*, *nirS*, *nirK*, and *nosZ*. The fragments coding these
237 genes were amplified with specific primers listed in Table S3 (Supplementary Materials).
238 Amplification of real-time PCR products was carried out with Stratagene Mx3000P
239 thermocycler (Agilent Technologies, US) using SYBR Green as a detection system in a reaction
240 mixture of 20 μ L containing: 0.1 μ L of each *nirS* and *nirK* primers, 0.4 μ L for *nxrA* primers
241 and 1 μ L for *amoA* and *nosZ* primers; 10 μ L of Real-Time 2xRT-PCR Mix SYBR A mixture
242 (A&A Biotechnology, Poland), 1 μ L of DNA diluted template corresponding to 10 ng of total
243 DNA, and Rnase-free water to complete the 20 μ L volume. All primer pairs amplifying gene
244 fragments of *nirS* and *nirK* were run with an initial denaturation of the DNA at 95 $^{\circ}$ C for 3 min,
245 followed by 40 cycles of 15 s at 95 $^{\circ}$ C, 30 s at 58 $^{\circ}$ C, and 30 s at 72 $^{\circ}$ C. For *amoA*, *nxrA*, and
246 *nosZ* a similar procedure was applied, with the only difference in primer annealing temperature
247 (55 $^{\circ}$ C, 63 $^{\circ}$ C, and 65 $^{\circ}$ C, respectively).

248 **2.4. Statistical analysis**

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

262 **3. Results and discussion**

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

270 3.1. Chemical parameters

271 The wastewater profiles depend on many factors, e.g.: catchment size and type of sewer
272 network, number of people served, and industrial discharges (Deblonde et al., 2011). This study
273 focused on two WWTPs (WWTP-W and WWTP-D), which treat wastewater generated by a
274 metropolitan area of Tricity (Fig.1). Chemical parameters (COD, BOD₅, TSS, TN, N-NH₄ and
275 TP) in raw wastewater (Fig. 2a) were typical for the studied urban catchment (Krzeminski et
276 al., 2012; Pasztor et al., 2009) and indicated high similarity between WWTPs (Supplementary
277 Fig. S1A). Factors explaining the variability of raw wastewater were most importantly: TP, TN,
278 and N_{org} (organic nitrogen) for WWTP-D, and COD, TSS, and TN for WWTP-W
279 (Supplementary Fig. S1B and C).

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

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

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

298 Note that deterioration of treated wastewater quality is usually connected with an increase in
299 WWTP effluent turbidity, and inter-related parameters such as mainly COD and BOD (Fig. 2b,
300 3a) were mainly linked to the turbulence in the activated sludge process. The reason may be a
301 seasonal change of activated sludge community structure (see Section 3.3) due to the drop of
302 both wastewater and ambient temperature. As a consequence, sedimentation disturbance and
303 activated sludge biomass washout may occur and lead to deterioration of the treated wastewater
304 quality, causing the need to change the wastewater treatment strategy. A common practice is to
305 increase the density and age of activated sludge by prolonging biomass retention time
306 (necessary to maintain an appropriate rate of autotrophic processes such as nitrification), while
307 dosing of coagulants (e.g.: PIX/PAX) prevents biomass washout with treated wastewater
308 (Boguniewicz-Zablocka et al., 2020). Nevertheless, despite preventive measures undertaken at
309 both studied WWTPs, microscopic analysis indirectly confirmed dispersed biomass being
310 washed out during the winter season (Section 3.2.1 and Fig. S3), observed especially between
311 October 2012 and April 2013. This phenomenon was also confirmed by NGS analysis of TW-
312 D and TW-W (for details see Section 3.3.1). Despite the seasonal deterioration of the TW-D
313 and TW-W quality, all indicators of treated wastewater quality remained lower than current
314 discharge limits (COD < 125 mg O₂ L⁻¹, BOD < 15 mg O₂ L⁻¹, TN < 10 mg L⁻¹, TP < 1 mg L⁻¹,
315 TSS < 35 mg O₂ 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-NH₄ 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 m³/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

340 microscope (TCN, PB, ACV, and Live/Dead) were on average higher in the treated wastewater
341 (TW) than in the environmental samples: MO, VIS and GD (Fig. 4a, 4b). TCN and PB values
342 in the treated wastewater were similar for both WWTPs (average 2.22 mln cells/mL and 69.22
343 $\mu\text{g C/mL}$ for TW-D, 2.21 mln cells/mL and 68.27 $\mu\text{g C/mL}$ for TW-W). They ranged between
344 1.65 – 2.91 mln cells/mL and 30.6 – 80.5 $\mu\text{gC/L}$, what is the same magnitude as observed in
345 other WWTP effluents (Bray et al 2021, Kalinowska et al 2021).

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

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

364 Baltic Sea area (Piwosz et al., 2013, Kudryavtseva et al., 2012, Ameryk et al., 2014).
365 Prokaryotic biomass followed the same pattern and ranged between 3.7-81,5 µgC/L for MO,
366 2.5-82.8 µgC/L for VIS and 2.9-36.5 µgC/L for GD. Large fluctuations of microbial
367 microscopic parameters in the marine samples result from sampling in warm (August,
368 September) or cold period (November, February) and they correspond to the general seasonal
369 trend confirmed by other authors (Ameryk et al., 2021; Freese et al., 2006; Piwosz et al., 2013).
370 Gulf of Gdańsk is an eutrophicated water reservoir, rich in phyto- and bacterioplankton during
371 the vegetation period from April to October (Witek et al. 1997) and several studies (Danovaro
372 and Fabiano, 1997; La Ferla et al., 2014, 2010) indicate that the availability of nutrients affects
373 the bacterial parameters. Both terrigenous organic matter and autochthonous matter of
374 phytoplankton origin can also support the growth of heterotrophic bacteria (Ameryk et al.
375 2005). The lower ambient temperature and limited nutrient availability in the marine waters
376 during winter were followed by one magnitude lower bacterial cell abundance and biomass
377 (February) than in the summer season (August, September). Also the Vistula River introduces
378 nutrients to the Gulf of Gdansk, therefore it stimulates the bacterial production in its internal
379 part (Ameryk et al., 2005; Wielgat-Rychert et al., 2013) what is reflected by both high values
380 and large fluctuations in the microscopic parameters at VIS. Gdansk Deep (GD) was
381 characterized by the lowest values of all microbial parameters (Fig. 4a, 4b, 4c), which supports
382 its choice as the reference point, being under the limited impact of anthropogenic and riverine
383 origin.

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



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

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

408 3.2.2. Bacteria cultivation

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

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

419 An average number of *Enterobacteriaceae* was similar in both WWTPs effluents (3.6×10^5
420 CFU/100 mL for TW-D and 3.3×10^5 CFU/100 mL for TW-W, Fig. 4d), but values in TW-W
421 were more uniform throughout the year (from 2.6 to 3.8×10^5 CFU/100 mL versus from 1.7 to
422 6.1×10^5 CFU/100 mL in TW-D, Fig. 4d). No clear seasonal pattern was observed in terms of
423 their variability. In environmental samples both *Enterobacteriaceae* and *E. coli* were on average
424 three orders of magnitude lower than in WWTP effluents. Interestingly, *Enterobacteriaceae*
425 were ten times less abundant at MO than at VIS (8.4×10^2 CFU/100 mL versus 8.7×10^3 CFU/100
426 mL) and not detected at GD (Fig. 4c).

427 Fecal coliforms, as well as their representative - *E. coli*, were detected also in environmental
428 samples and presented a similar trend as the *Enterobacteriaceae* family: their average values
429 were higher for the Vistula River estuary than the marine outfalls of the treated wastewater, but
430 Gdansk Deep presented the lowest abundance of these bacteria. The number of *E. coli* varied
431 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
432 TW-W, respectively (Fig. 4d), which is rather typical for treated wastewater (Łuczkiwicz et
433 al., 2010; Marano et al., 2020). It was also reported by others that fecal bacteria, even if removed
434 with high efficiency of over 90%, are still released to the recipient with WWTP effluent as a
435 result of their high initial number (Lucena et al., 2004; Marano et al., 2020). However, in none

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

443 **3.3. Metagenomic analysis of microbial community**

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

458 For an open-sea sampling station GD, the alpha diversity was the lowest, with the exception of
459 Chao1, which is highly influenced by the presence of rare taxa. Samples being under treated
460 wastewater impact (MO) presented higher microbial diversity, what has been also noted in other
461 studies (García-Armisen et al., 2014; Kalinowska et al., 2020; Price et al., 2018; Wakelin et al.,
462 2008). This supports the hypothesis of increasing biochemical potential of the natural waters
463 due to WWTP effluent discharge. Nevertheless, these changes may be temporary and may
464 depend highly on the water mixing (Price et al., 2018). The share of treated wastewater in
465 recipients may also play a significant role. Increased biodiversity indices for VIS may reflect
466 the massive amounts of river waters introduced by the Vistula River to the Gulf of Gdansk
467 (approx. 30 km³ annually) and therefore the intensive mixing of marine and freshwater
468 communities in the river estuary.

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

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

482 minor shares (less than 1% in each sample). Fig. 5 shows the relative abundances of the most
483 abundant phyla and 11 of them were common for all the samples analyzed, however their
484 abundance varied significantly. They belonged to *Actinobacteria* (4.5-41.0%), *Proteobacteria*
485 (5.5-56.9%), *Bacteroidetes* (0.5-12.4%), *Firmicutes* (0.03-16.6%), *Verrucomicrobia* (0.1-
486 9.7%), and *Planctomycetes* (0.04-8.5%), with smaller shares of *Acidobacteria* (<0.01–1.3%),
487 *Chlamydiae* (<0.01-2.3%), (0.01-3.9%), *Cyanobacteria* (0.05-49.4%) and
488 *Saccharibacteria/TM7* (<0.01-60.9%). Heatmap with dendrogram (Fig. 5e) confirms the higher
489 similarity among the environmental samples, clearly separated from TW samples.

490 3.3.1. Microbial community of WWTP effluents (TW-D and TW-W)

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

506 were noted in the outflows of WWTP located in Belgium (García-Armisen et al., 2014):
507 *Proteobacteria* (up to 74%), followed by *Bacteroidetes* (up to 37%) and *Actinobacteria* (up to
508 18%). In WWTP from Hong Kong (Cai et al., 2014) the following share of phyla were found:
509 *Proteobacteria* (up to 60%), *Saccharibacteria/TM7* (up to 25%), *Bacteroidetes* and
510 *Acidobacteria* (up to 20%), followed by *Firmicutes* (up to 14%). The microbial community of
511 treated wastewater has been rarely studied, especially in terms of seasonal variations in
512 composition and diversity (Wang et al., 2016), therefore this issue is not fully recognized and
513 understood. However, worldwide similarities observed until now at high taxonomic levels may
514 suggest that the microbiome composition of WWTP effluents is to some extent consistent
515 among WWTPs (Adrados et al., 2014; Cai et al., 2014; Silva-Bedoya et al., 2016). Wastewater
516 treatment is intensively adjusted by operators in winter as a response to the current effectiveness
517 of microbiological processes, particularly those linked to nitrogen and phosphorus removal.
518 Thus, the metagenomic approach indirectly confirmed the seasonal disruptions of wastewater
519 processes, already confirmed by elevated chemical parameters (see Section 3.1) and presence
520 of numerous, small-structured flocks in TW-D and TW-W during the cold season (see Section
521 3.2.1, and Fig. S3).

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

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

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

542 The total variance for WWTP-W samples explained by CCA was lower than for WWTP-D and
543 involved less variables: total phosphorus removal efficiency (TP.eff), nitrate nitrogen
544 concentration in the effluent ($N-NO_3.T$) and ambient temperature (Fig. 6b). It showed the
545 dominance of similar taxa in the WWTP-W effluent all over the year. Winter/spring samples
546 were associated with presence of bacteria that imply treatment efficiency deterioration. In April
547 and May, particularly high shares of *Gordonia* (an opportunistic human pathogen widely
548 distributed in aquatic and terrestrial environments) were found. Summer/autumn samples,
549 characterized by higher $N-NO_3$ concentrations contained microorganisms commonly found in
550 activated sludge reactors (representatives of *TM7/Saccharibacteria* phylum, eg. *EW055*,
551 Gómez-Acata et al., 2017) or in environmental samples: anoxic (*ZB2* and other representatives
552 of *Parcubacteria/OD1*, Harris et al., 2004), or potentially associated with mammal presence
553 (*BD1.5* - representatives of *Gracilibacteria/GN02*, Dudek et al., 2017). It is worth noting that
554 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-NO₃ 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 to 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

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

593 In addition to nitrogen removal, both WWTPs perform enhanced biological phosphorus
594 removal, which is carried out by polyphosphate accumulating organisms (PAOs) that can
595 accumulate P in amounts exceeding their growth requirement. *Candidatus Accumulibacter* and
596 *Tetrasphaera* are most frequently identified PAOs in full-scale wastewater plants even
597 geographically distinct (Nielsen et al., 2019; Onnis-Hayden et al., 2020). Among other bacterial
598 PAOs connected with activated sludge *Actinobacteria* (*Friedmaniella*, *Candidatus Microthrix*,
599 *Microthrix*, *Tessaracoccus*), *Proteobacteria* (*Dechloromonas*, *Pseudomonas*, *Ca.*
600 *Accumulimonas*, *Quatrionicoccus*, *Malikia*, *Lampropedia*), and *Gemmatimonadetes*
601 (*Gemmatimonas*) are also mentioned (Akbari et al., 2021). In this study, only *Candidatus*
602 *Microthrix* (up to 23.31%), *Dechloromonas* (up to 0.6%), *Pseudomonas* (up to 0.5%) and
603 *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

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

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



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

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



676 3.4. Functional gene detection and quantification

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

683 Obtained data indicated that WWTPs effluent contained 16S rRNA target molecules ranging
684 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
685 mL of the sample), while in environmental samples they varied in wider range from 4.2×10^4 to
686 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
687 sample), Fig. 7. These values, together with the abundance of N-functional genes, were
688 compared with the data available in the literature (Fig. 8). In all samples of treated wastewater
689 (TW-D, TW-W) the studied genes were present, with the highest abundance of *nirS* gene
690 (encoding a haem-containing nitrite reductase) and *nirK* gene (encoding Cu-containing nitrite
691 reductase), which are responsible for the reduction of nitrite to nitrogen oxide. Thus, both
692 unrelated *nir*-genes, which never occur together in the same organism, are used as markers of
693 denitrifiers. As expected, the *nirS* and *nirK* washed out from WWTPs with treated wastewater
694 were noted on a similar level and ranged from 10^5 to 10^6 gene copies mL^{-1} , which was much
695 lower than in activated sludge samples (Fig. 8c,d). In marine outfalls (MO-D and MO-W) and
696 Vistula river estuary (VIS) both genes did not exceed 10^5 gc mL^{-1} , while in the Gulf of Gdansk
697 (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 N₂O 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 10⁴ to 10⁵ gc mL⁻¹, except GD point, where they were less prevalent: 10² to
703 10³ 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 overcompeted for
712 NO₂⁻, 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 NO₂⁻ 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

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

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

738 From the sanitary point of view, the abundance of fecal indicators in WWTP effluent did not
739 present a clear seasonal pattern, neither it exceeded the current standard for bathing sites in the
740 coastal waters impacted by treated wastewater. However, despite high treatment efficiency
741 (<95%) in terms of chemical and microbiological parameters, the treated wastewater can still
742 be a source of both nutrients and bacteria (also human-related ones) to the receiving waters.
743 Treated wastewater discharge can also increase the biochemical potential of the receiving

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

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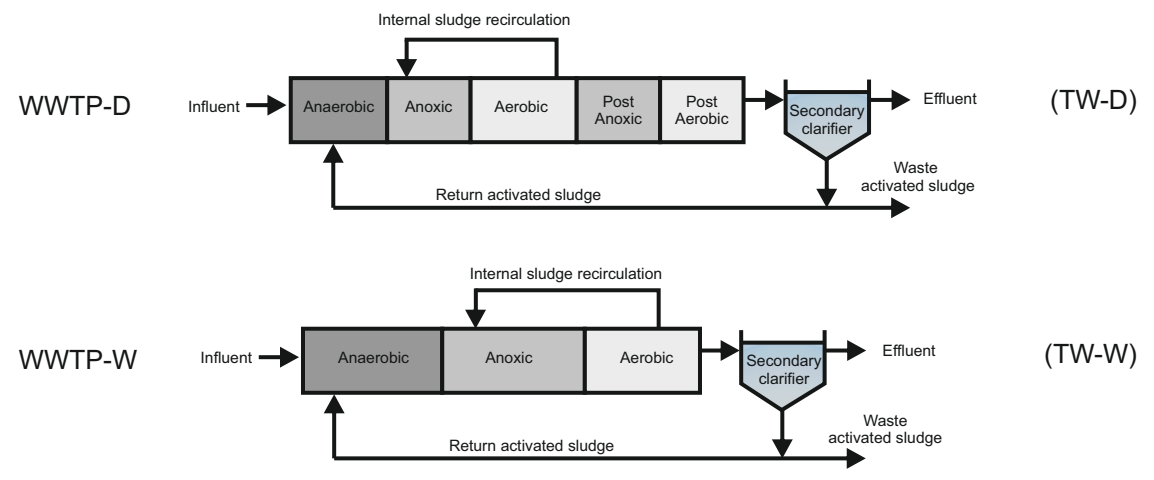
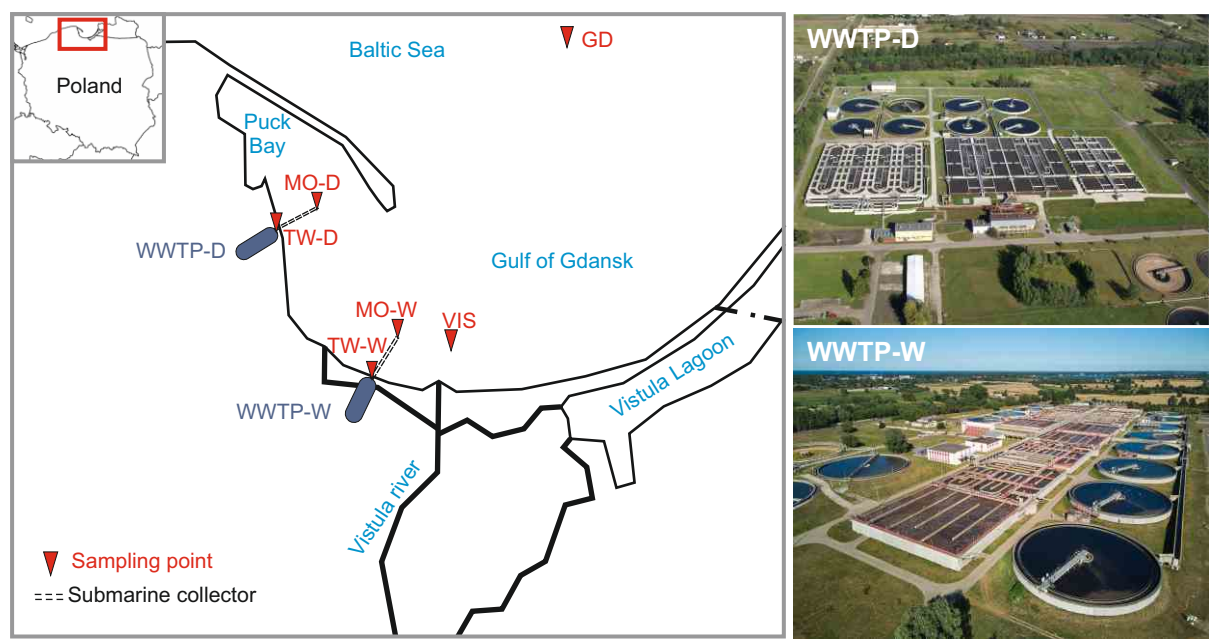
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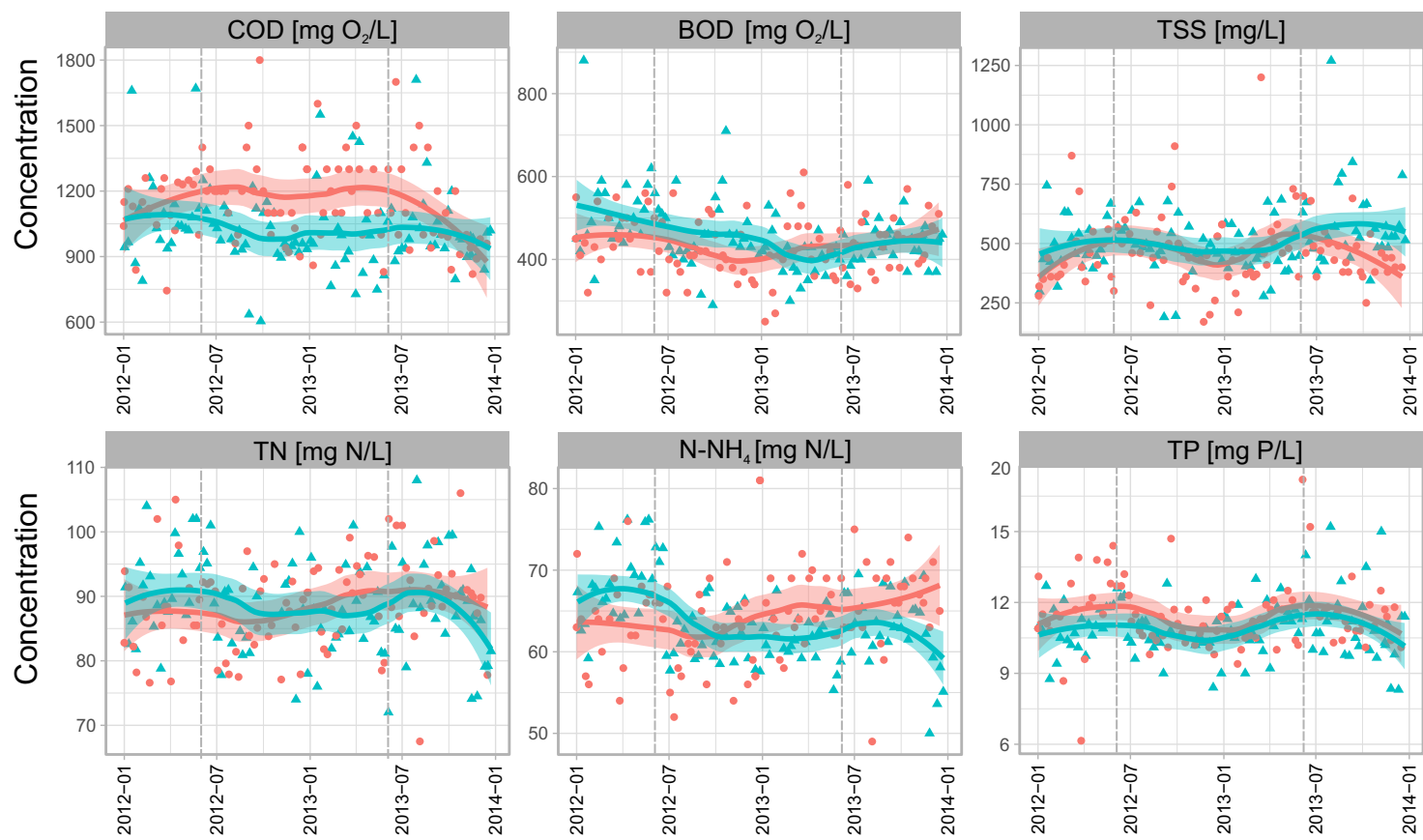
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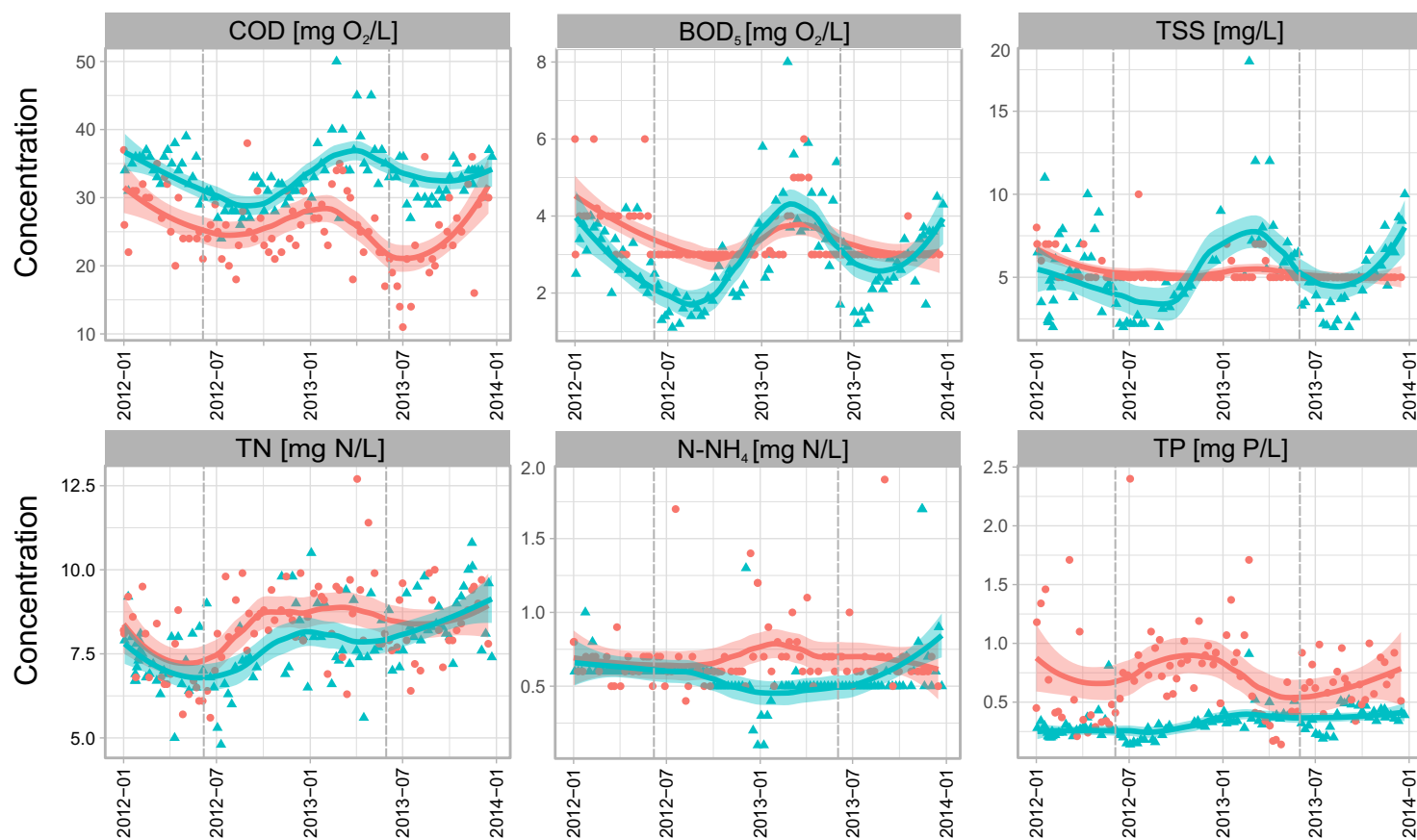
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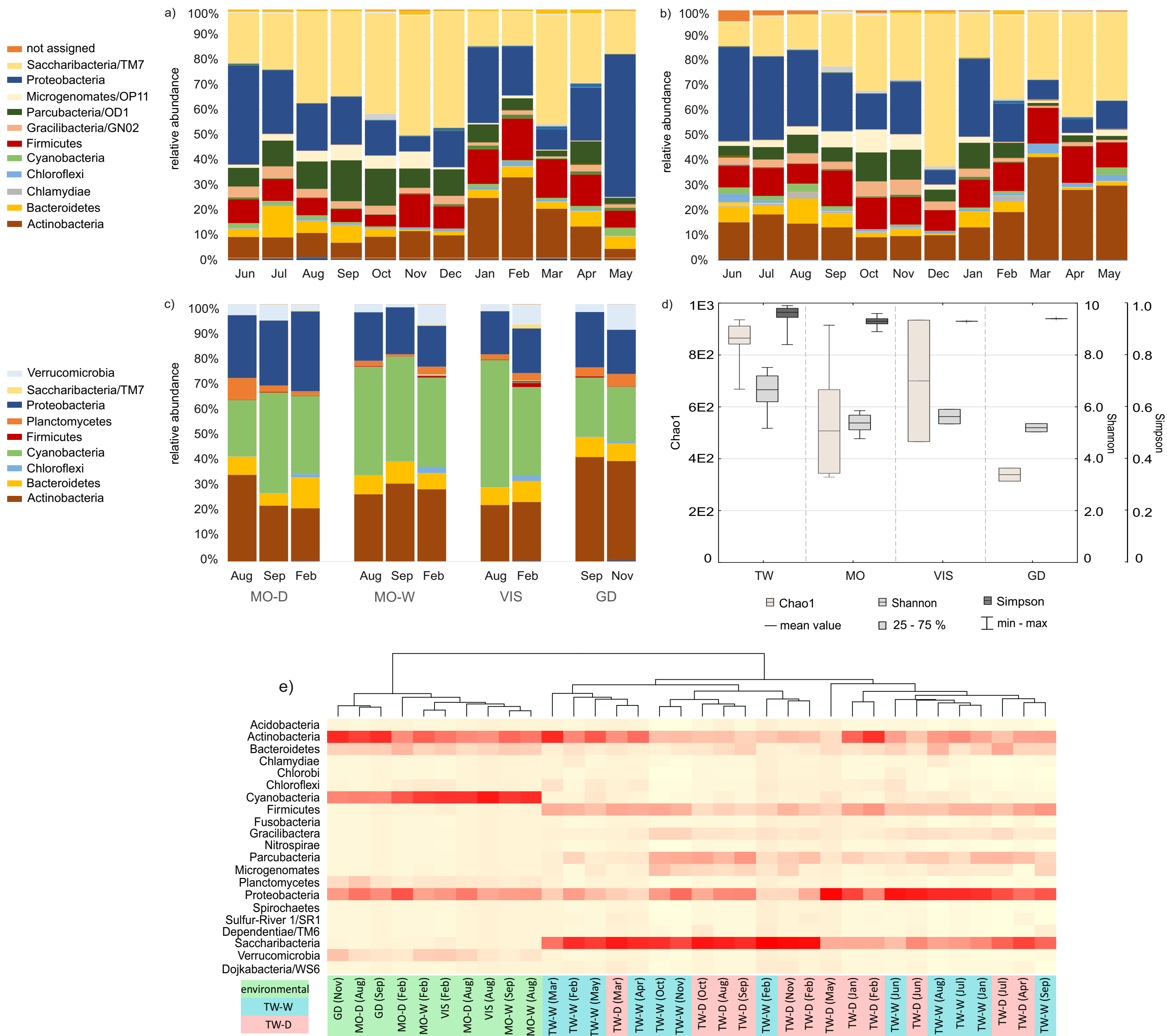


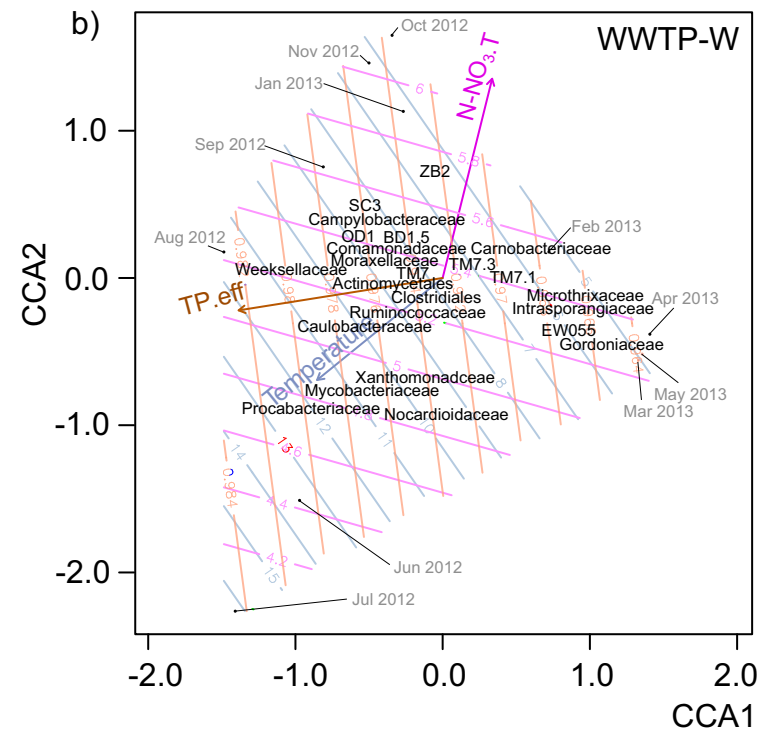
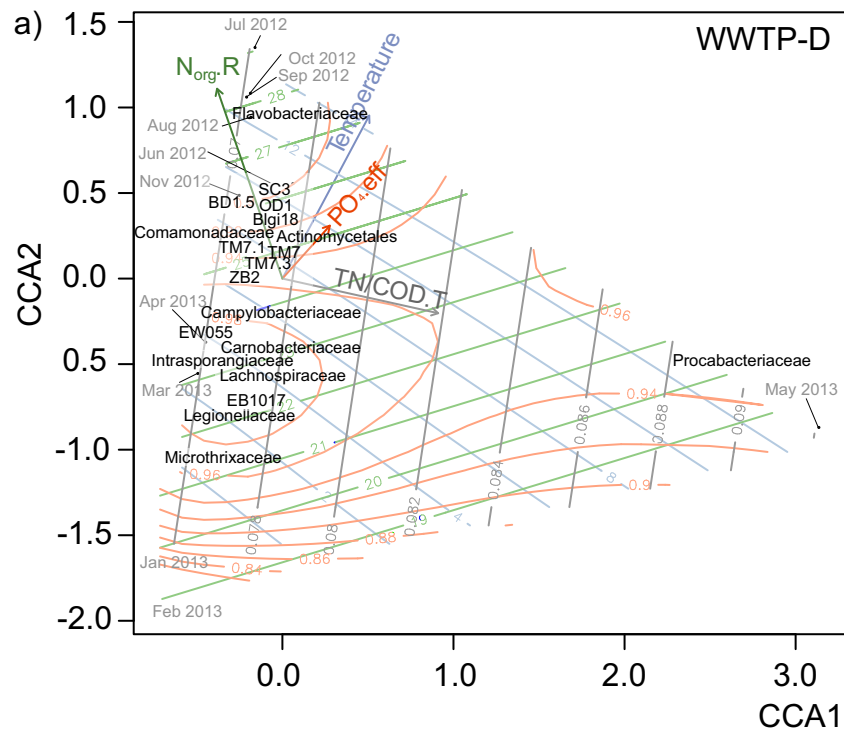
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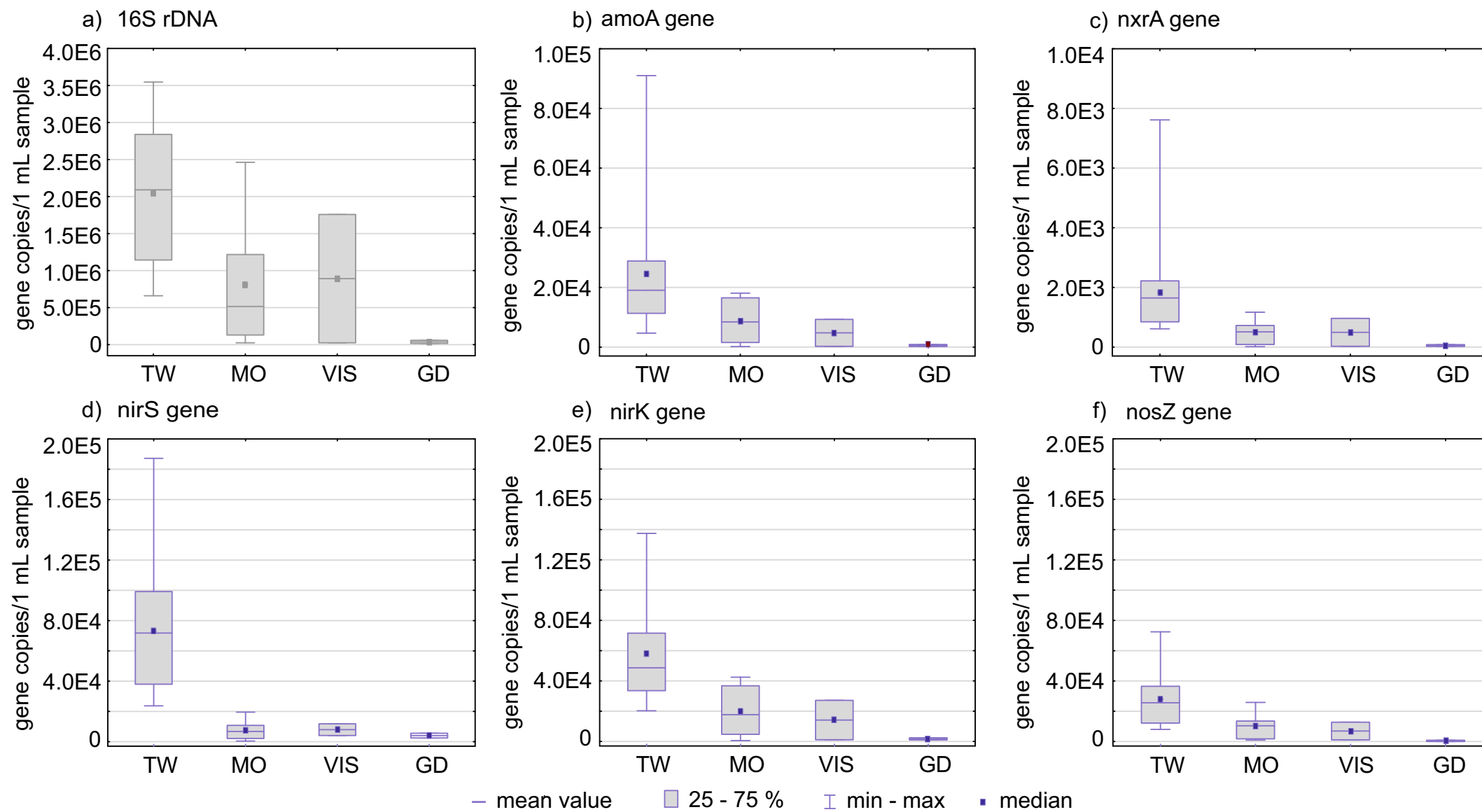


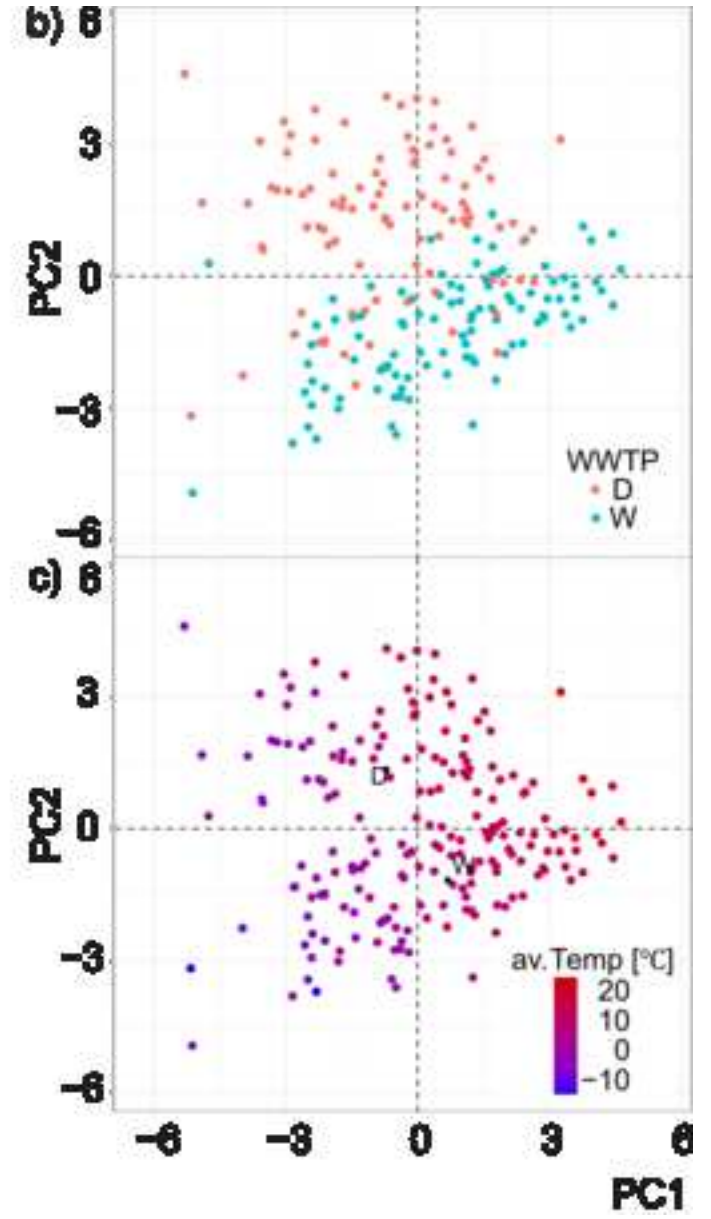
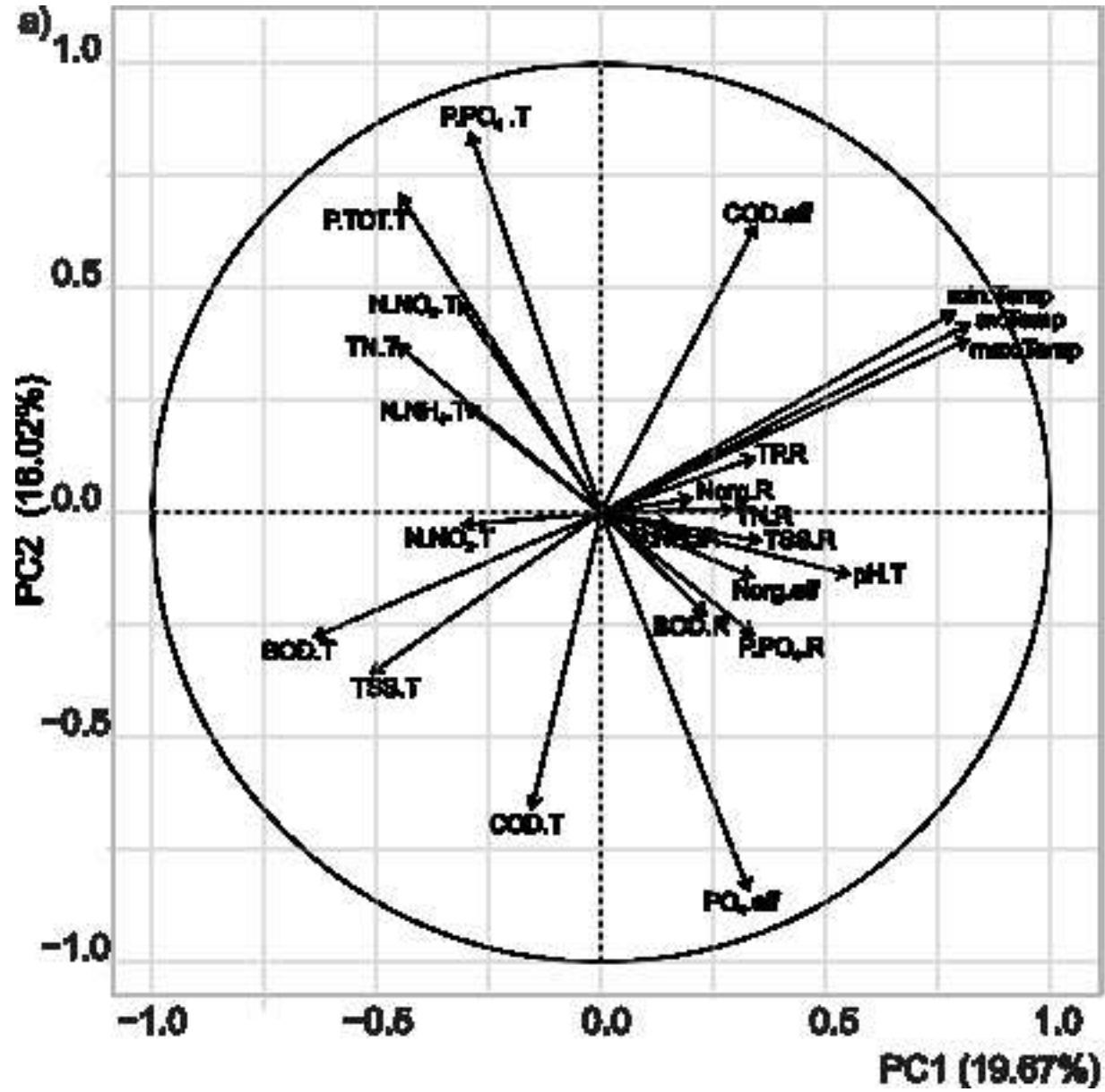
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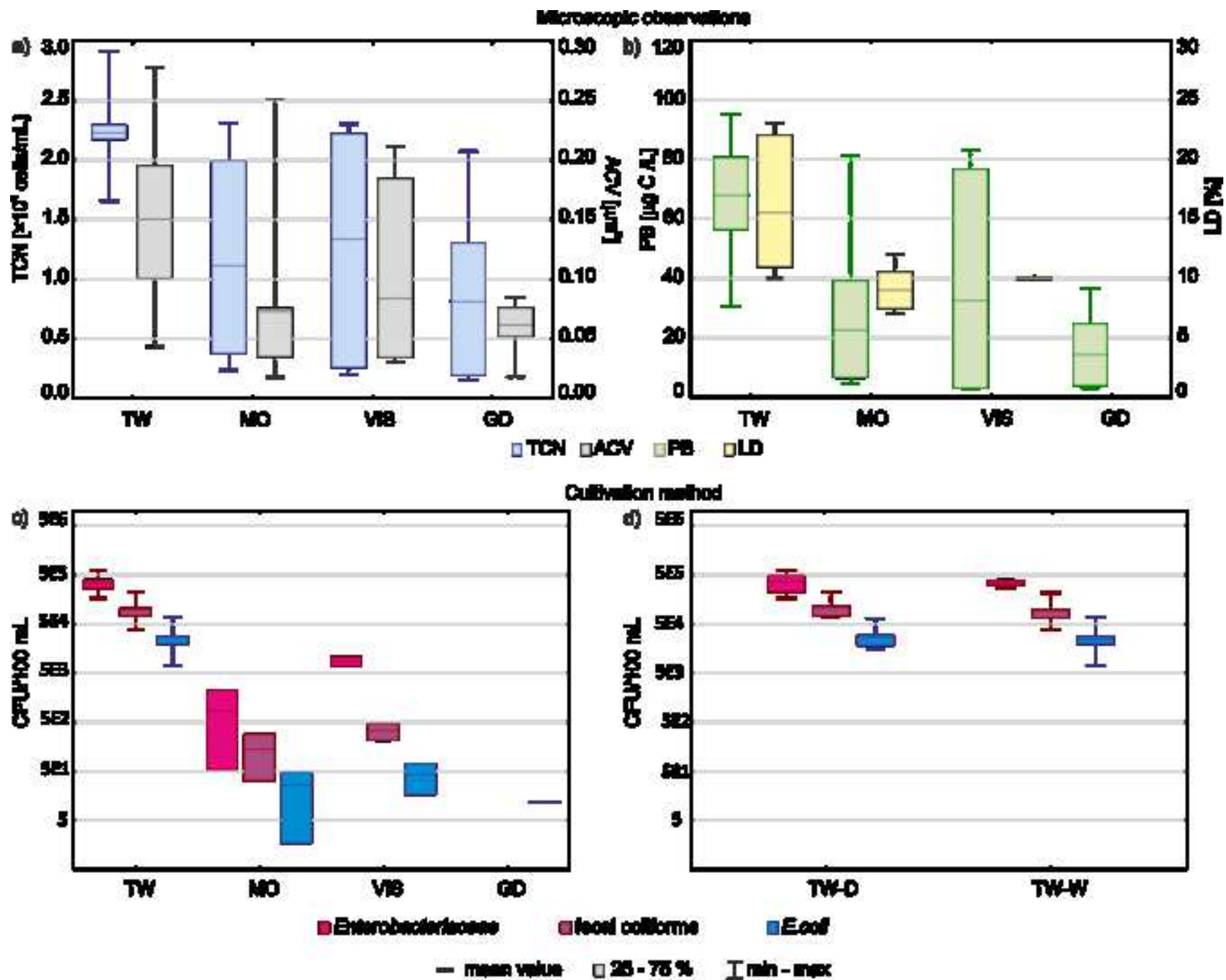


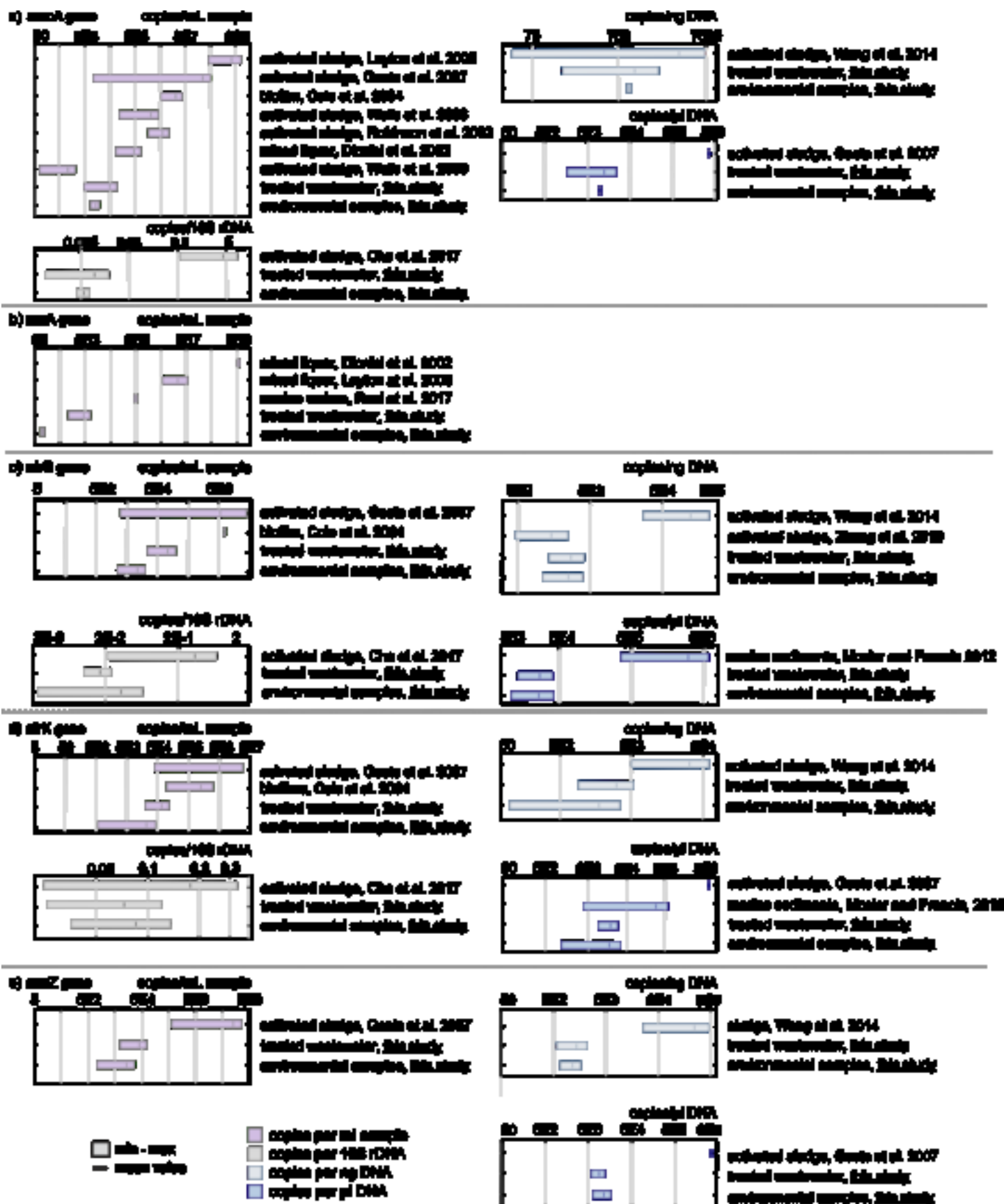












1 **Insights into the microbial community of treated wastewater, its year-round variability and impact on the receiver, using cultivation, microscopy and**
2 **amplicon-based methods**

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11 **SUPPLEMENTARY MATERIALS**
12
13

14 **Table S1.** Characteristics of the studied WWTPs: WWTP-W (Gdansk-Wschod) and WWTP-D (Gdynia-Debogorze). People Equivalent (PE) and average flow
 15 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

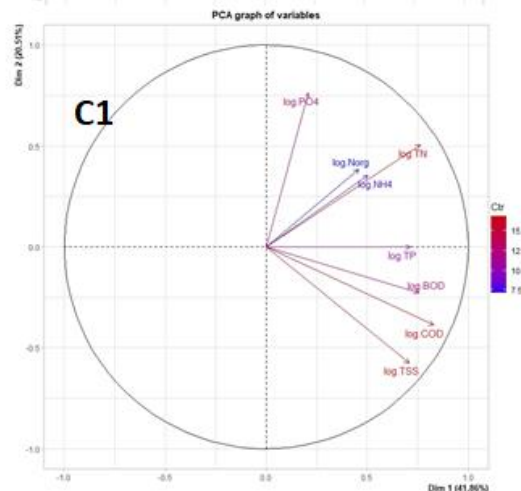
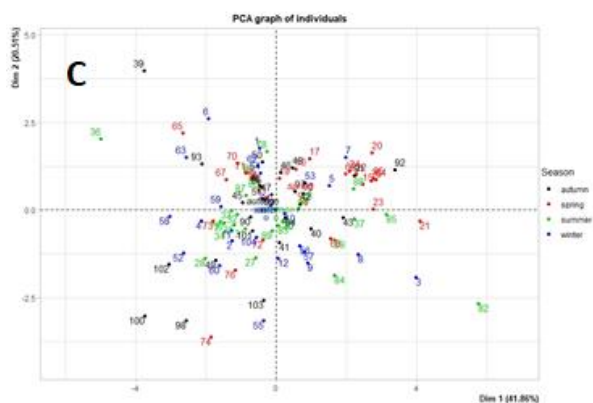
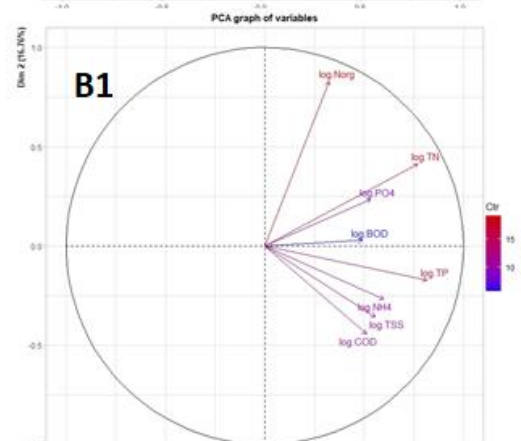
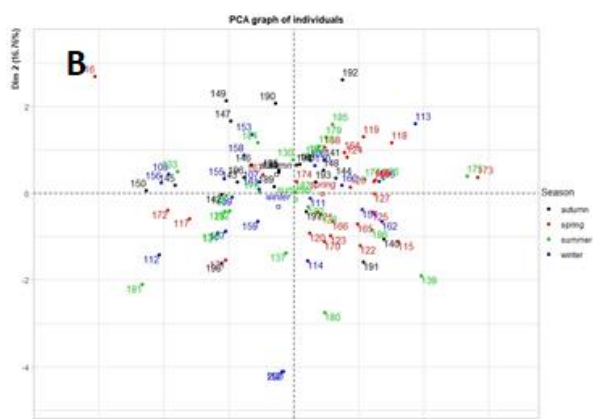
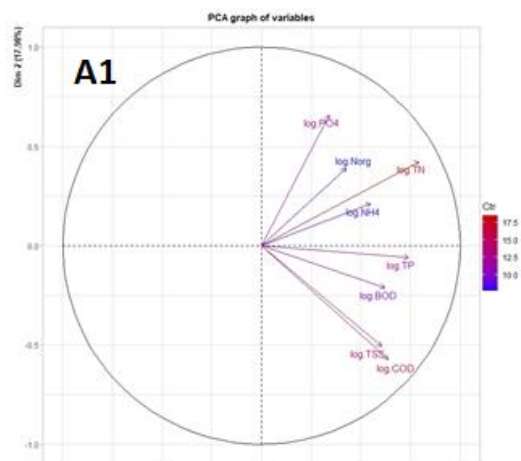
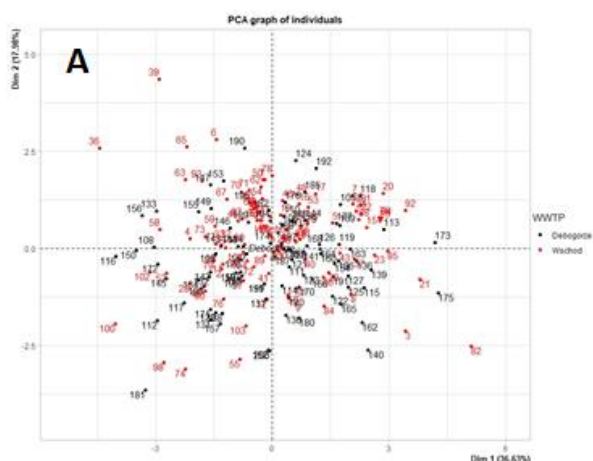
Medium	Symbol	Incubation temperature	Incubation time	Cultivated bacteria	Characteristic of enumerated bacterial colonies
Chromocult® Coliform Agar	C	37°C	21-24 h	<i>Enterobacteriaceae</i>	All growing on the medium
				fecal coliforms	Blue to violet
Membrane Fecal Coliform Agar	mFC	44°C	22-24 h	<i>Escherichia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

18

19 **Table S3.** Primer sequences used to amplify fragments from *nirS*, *nirK*, *nosZ*, *amoA* and *nxrA*
 20 genes in the denitrification pathway

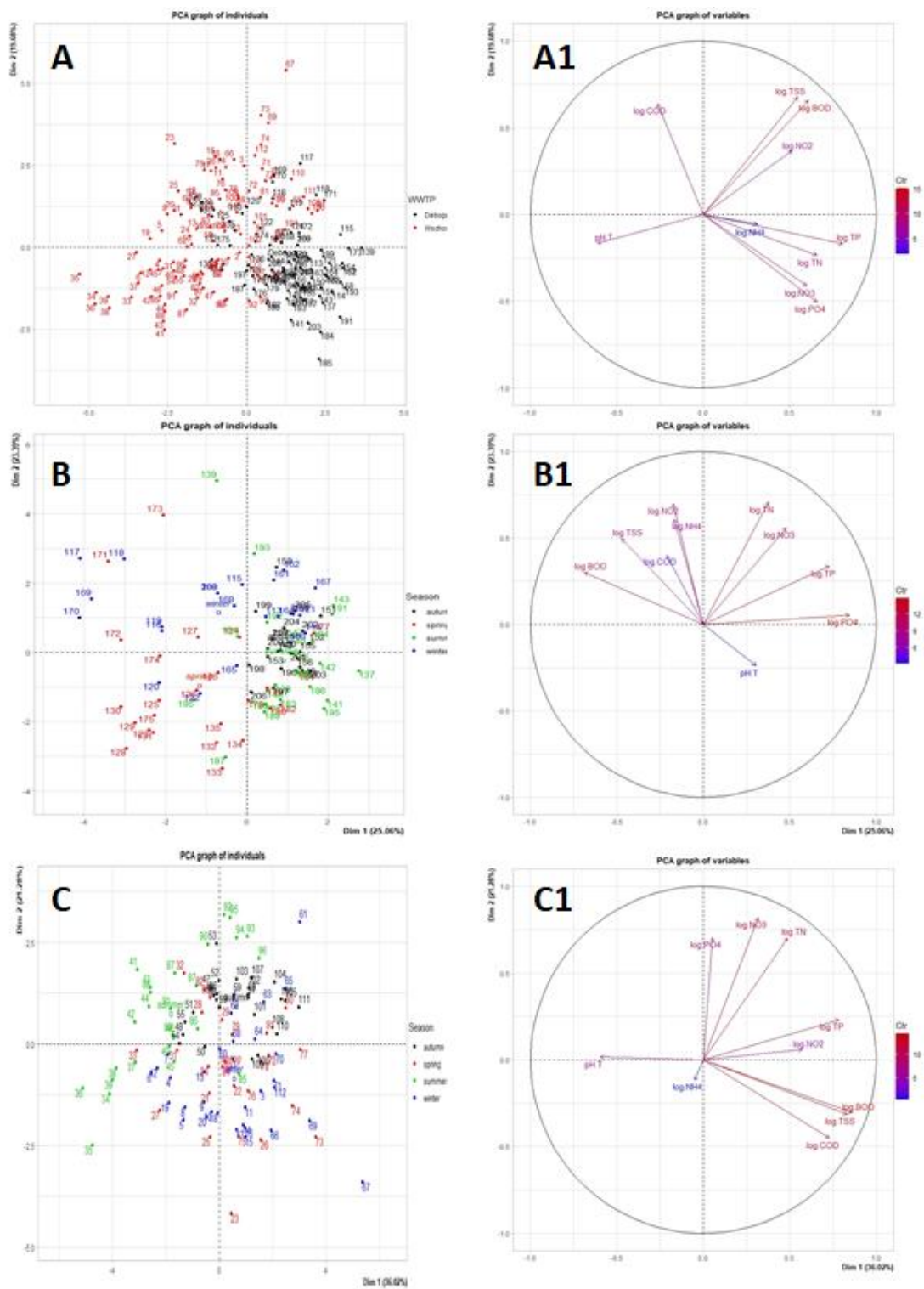
Primer	Primer sequence (5'-3')	Reference
nirS1F	TACCACCCSGARCCGCGCGT	Kim et al. 2011
nirS 3r	GCCGCCGTCRTGVAGGAA	
nirK876	ATYGGCGGVCA YGGCGA	
nirK1040	GCCTCGATCAGRTRTRTGTT	
amoA-1-F	GGGGTTTCTACTGGTGGT	Li et al. 2012
amoA-2R	CCCCTCKGSAAAGCCTTCTTC	
nxrA-RT-F	GTGGTCATGCGCGTTGAGCA	Gerbl et al. 2014
nxrA-RT-R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

21



22

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



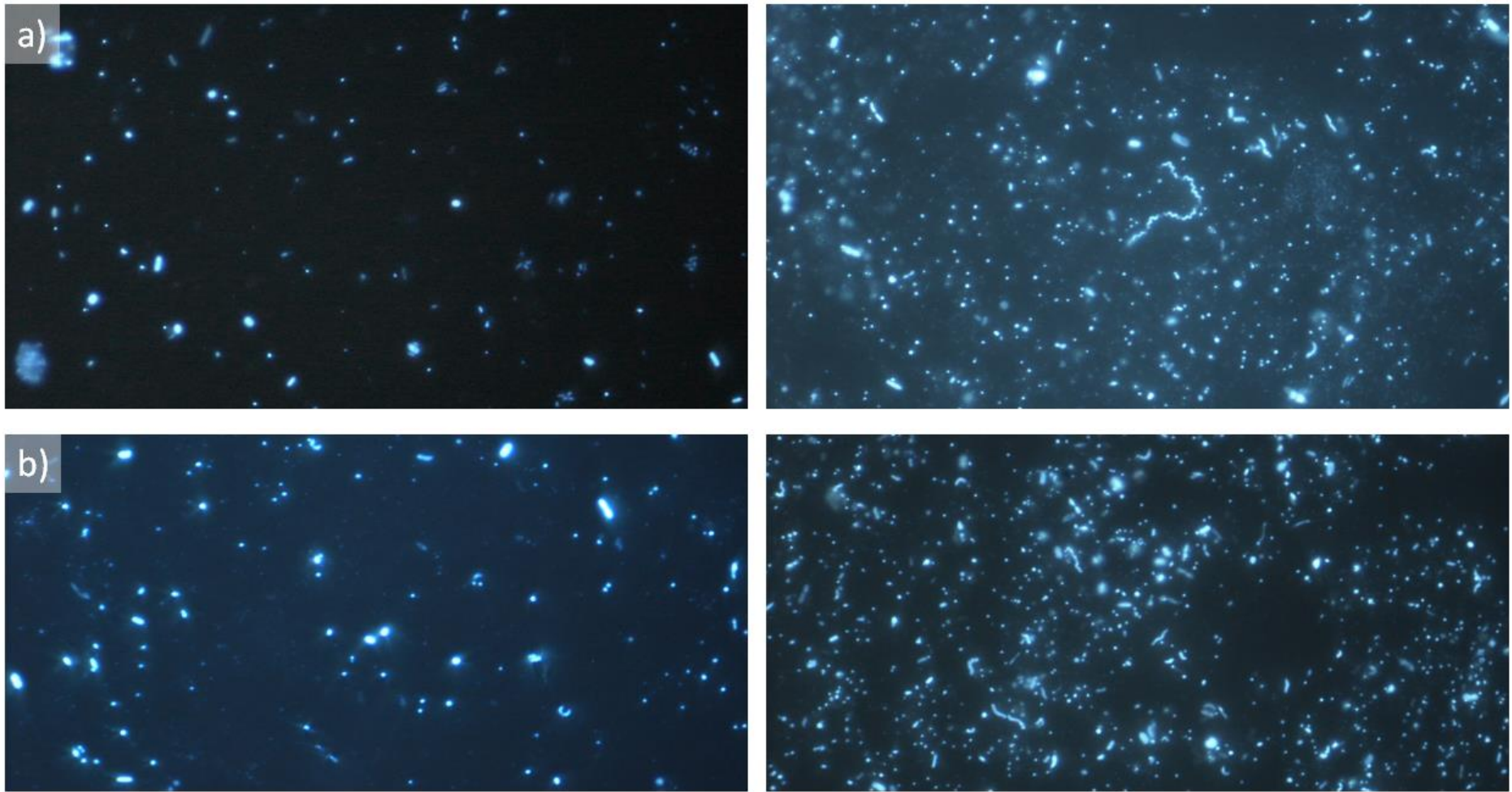
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28 **Figure S2.** Principal Component Analysis results for chemical parameters in treated wastewater
 29 of (A) both WWTPs, (B) WWTP-D and (C) WWTP-W. Numbers on the graph of individuals
 30 refer to the sample number. Additionally, the samples on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

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

Sample	pH [-]	COD [mg O ₂ /dm ³]	BOD	TSS [mg/dm ³]	TN	N-NH ₄	N _{org} [mg N/dm ³]	N-NO ₃	N- NO ₂	TP [mg P/dm ³]	P-PO ₄	
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±12.9 6	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

34 Physicochemical data for Gdansk Deep (GD) were unavailable



35

36 **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,
37 on the right from December

38

39 **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
 40 detected).

Phylum	Class	Order	Family	Genus	TW			MO			VIS			GD		
					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	Nocardioideaceae	Kribbella	<0,01	1,74	0,18	<0,01	0,02	0,01	nd	nd	nd	nd	nd	nd
Actinobacteria	Actinobacteria	Actinomycetales	Nocardioideaceae	Nocardioides	0,01	2,24	0,17	nd	nd	nd	<0,01	<0,01	<0,01	nd	nd	nd
Actinobacteria	Actinobacteria	Actinomycetales	Nocardioideaceae		<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	[Saprosirae]	[Saprosirales]	Saprosiraceae		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	Mycoplasma	<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-UniPI			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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