Performance of full-scale rural wastewater treatment plants in the reduction of antibiotic-resistant bacteria and antibiotic resistance genes from small-city effluents

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Abstract

The main objective of this study was to evaluate the performance of full-scale rural wastewater treatment plants (WWTPs) in the reduction of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) from small-city effluents. Twenty full-scale WWTPs in rural Chile with different biological treatment technologies (vermifiltration (VF), activated sludge (AS) and biodisc (BD)) and disinfection treatments (chlorination (Cl) and UV irradiation (UV)) were monitored and studied in two campaigns: Campaign 1 (20 WWTPs) and Campaign 2 (6 WWTPs). In both campaigns, the rural WWTPs improved the water quality of the effluents very significantly (90%, 70%, and 40% reductions in TU, COD, and NH_4^+-N, respectively) and reduced ARB and ARG loads by 2 to 4 log units. All three biological treatments contributed to the final quality of the effluents, especially in terms of microbiological parameters, with statistically indistinguishable efficiencies between them. These results show the importance of rural WWTPs in improving the water quality of urban effluents while reducing microbiological risk and the spread of antibiotic resistance into the environment. The study demonstrates the utility of a non-centralized, self-managed wastewater treatment scheme that can be implemented in many sparsely populated areas around the world.

Keywords: antibiotic-resistant bacteria; antibiotic resistance genes; biological treatment; water quality parameters, principal component analysis, disinfection

1. Introduction

Wastewater treatment plants (WWTPs) have a significant role in the spread of resistance among the bacteria into the environment [1–2]. On the one hand, WWTPs contribute greatly to the protection of public health and the aquatic environment by reducing the transmission of waterborne diseases and aquatic pollution [3]. On the other hand, the presence of diverse selection pressures, such as high antibiotic concentrations, and microbial growth conditions in WWTPs create a favorable environment for the transfer of antibiotic resistance genes (ARGs) and the proliferation of antibiotic-resistant bacteria (ARB) [4].

The performance of urban WWTPs has been widely investigated, with reported ARB and ARG reductions varying between 1.0 – 4.0 log units relative to the untreated inputs [5–7]; however, fewer studies have focused on evaluating the performance of rural WWTPs. In general, the chemical oxygen demand (COD) and ammonium nitrogen (NH_4^+-N) removal efficiencies in those systems varied between 30 – 860 g/m^3·d and between 10 – 140 g/ m^3·d, respectively [3]. In the case of ARB and ARGs, the reported reductions lie within the 1 – 3.0 log units range [8]. This information is significant for developing countries like Chile, where 12% of the population resides in rural areas, which have only 20% wastewater treatment coverage [9,10]. In this context, access to water and sanitation in Chilean rural areas is facilitated through small committees, in which residents manage and operate small drinking water and wastewater treatment facilities. These organizations, known as “Rural Sanitary Services,” aim to provide water and treatment to concentrated and semi-concentrated rural populations ranging from 80 to 3000 inhabitants [11]. Among the available biological wastewater treatment technologies, activated sludge treatment (AS) is the most employed for rural WWTPs, although vermifiltration (VF) and biodisc (BD) systems are also commonly used [10]. Both AS and BD involve aerobic treatment, with the difference being that BD uses rotating discs to develop a biofilm capable of degrading organic matter, while AS consists of an aeration tank where a flocculent culture of microorganisms is developed [12,13]. In the case of VF, this promising treatment is composed of earthworms and compost bedding, in which earthworms are responsible for degrading organic

matter and nutrients [14]. Although these technologies were not designed to remove ARB and ARGs, it has been proposed that treatment processes can reduce ARB and ARGs loads, avoiding microbiological risks to the environment and human health [4].

The influence of different wastewater technologies on ARG and ARB removal has been studied to a limited extent. Many studies have revealed that the removal of ARGs and ARB in wastewater is influenced by technology type, operational and design parameters, and disinfection treatment [1,4,15,16]. The efficiency of these technologies in reducing ARB and ARG loads has been extensively reported for AS, but there are few comparable studies for BD and VF. The efficiencies of the three treatments appear to be very similar, with reduction between 1.8 – 2.6 log units for ARBs and 1.1 – 4.1 log units for ARGs in the case of AS [6], and between 2.0 – 4.0 log units for BD and VF [17,18]. Regarding disinfection processes, Zhuang et al. [19] and Zheng et al. [20] investigated the efficiency on ARG removal using chlorination (Cl), ultraviolet irradiation (UV), and ozonation (O_3) disinfection in WWTPs. In both cases, disinfection by Cl presented better performances, with reduction values above 3.0 log units.

Against this backdrop, the present study is among the first to analyze the contribution of rural WWTPs to the spread of antimicrobial resistance (AMR), examining the difference between biological and disinfection technologies. Thus, the main objective of this study was to evaluate the performance of full-scale rural wastewater treatment plants in the reduction of ARB and ARGs, analyzing twenty WWTPs in rural Chile that use various biological wastewater treatment technologies (VF, AS, BD) and disinfection processes (Cl and UV).

2. Material and Methods

2.1 Sample collection from rural wastewater treatment plants

This study was conducted in two sample-collection campaigns (Campaign 1 and Campaign 2) at twenty full-scale rural WWTPs located in the Biobio Region, Chile (36°46′22″S 73°03′47″W, Figure S1 in the

supplementary information). Figure 1 shows the sampling map, displaying the locations of the WWTPs and their corresponding biological treatments. The selected WWTPs serve areas with populations that vary between 88 and 2533 inhabitants (Table S1 in the supplementary information). During Campaign 1, the twenty full-scale rural WWTPs were studied and monitored, with physicochemical and microbiological parameters and ARB abundances analyzed. After the initial screening in Campaign 1, two plants per biological treatment technology (VF, AS, BD) were resampled, resulting in a total of six WWTPs monitored during Campaign 2. The criteria used to select these WWTPs were geographic proximity to the laboratories and operating efficiencies (data not shown). In Figure 1, these WWTPs were highlighted in red. In addition to the analysis carried out in campaign 2, ARG abundances were determined.

Figure 1

2.1.1. Campaign 1

Figure 2 shows a schematic diagram of different biological and disinfection treatment technologies. In Campaign 1, four, nine, and seven rural WWTPs with AS, BD, and VF biological treatment technologies, respectively, were analyzed. Each plant used a primary treatment of grids and sieves, followed by a secondary treatment involving a biological technology (VF, BD, or AS), and finally a Cl or UV disinfection treatment process. For each WWTP, samples were collected at three monitoring points: influent (after the primary treatment) (I), secondary effluent (SE), and final effluent (FE) (Fig 2). The sampling was conducted at a single point per plant from September 2021 to November 2021. Samples were stored in amber bottles at 4 °C and processed within 48 h.

Figure 2

2.1.2. Campaign 2

Six full-scale rural WWTPs were selected from Campaign 1 using the specific biological treatment as a selection criterion. For VF technology, the selected WWTPs were Copiulemu (VF1, 36º 53' 25.474" S and

72º 48' 26.64" W) and Villa Las Almendras (VF3, 37º 16' 51.715" S and 72º 38' 25.221" W). Both plants use a primary treatment of grids and sieves followed by a secondary treatment using VF technologies. Regarding disinfection, VF1 employed UV irradiation, while VF3 used a Cl process. For AS technologies, both plants use a primary treatment with grids and sieves followed by a secondary AS treatment. These plants employed Cl as a disinfection process. The WWTPs selected were Rere (AS1, 37º 7' 52.19" S and 72º 43' 37.653" W) and Río Claro (AS2, 37º 12' 17.029" S and 72º 37' 9.583" W). The selected BD systems have the same treatment train as the VF systems, using different disinfection processes. In the case of Villa Laja (BD1, 37º 15' 23.321" S and 72º 41' 30.14" W), the disinfection process used was UV irradiation, whereas Santa Fe (BD2, 37º 28' 22.733" S and 72º 34' 35.696" W) used Cl as a disinfection technology. Water samples were collected as in Campaign 1 (I, SE, and FE), with the sampling performed at fixed points in each plant between May 2022 and June 2022 (Fig.2). Water samples were stored in amber bottles at 4 °C and processed within 48 h after sampling.

Figure 2

2.2. Physicochemical characterization of water samples

Physicochemical characterization of water samples was carried out during both Campaign 1 and Campaign 2. The I, SE, and FE samples from each WWTP were filtered using a 0.45-µm-pore-size Whatman membrane. The in-situ parameters such as pH, temperature (°C), electrical conductivity (EC), and oxidation-reduction potential (redox) were measured using OAKTON portable equipment (PC650-480485, OAKTON, USA). Dissolved oxygen (DO) concentration and turbidity (TU) were determined using a portable oxygen sensor (HANNA OXI 330i/set HI 9146-04, HANNA Instruments Inc., USA) and a portable waterproof turbidimeter (OAKTON T-100, OAKTON, USA), respectively. The concentrations of COD (colorimetric method), NH_4^+-N (colorimetric method at 640 nm), nitrate nitrogen (NO_3^--N, colorimetric method at 220 nm and 275 nm), nitrite nitrogen (NO_2^--N, colorimetric method at 543 nm), and phosphate phosphorus (PO_4^{-3}-P, colorimetric method at 890 nm) were measured based on the protocols described in

Standard Methods [21]. In the case of microbiological parameters, total coliforms (TC) and fecal coliforms (FC) were counted by serial filtration of water samples (I, SE, EF) in nitrocellulose with a 0.45 µm pore size. The membrane filters were placed on chromogenic selective agar (Chromocult ® Coliform Agar ES, Merck) and incubated at 36 °C for 24 h. The filters with countable salmon-red colonies (TC) and dark blue to violet colonies (FC) were selected.

2.3. Determination of antibiotic-resistant bacteria (ARB) in water samples

ARB abundances were determined in water samples (I, SE, EF) during Campaign 1 and Campaign 2. The plate count technique based on the ability of bacteria to grow in the presence of an antibiotic was used to measure the ARB abundances, expressed in colony-forming units (CFU)/100 mL. The I, SE, and FE water samples from each WWTP were incubated at 30 °C for 24 h using MacConckey agar as a culture medium; this is a selective medium for Gram-negative bacteria. The plates were supplemented with commonly used antibiotics in Chile, such as ciprofloxacin (CIP), amoxicillin (AMX), and ceftriaxone (CTX) (OXOID, Thermo Scientific), at concentrations of 2 mg/L, 32 mg/L, and 4 mg/L. These concentrations represent the breakpoint values for defining resistance to these antimicrobial agents [22]. For the spread of plate method, different water dilutions were used depending on the water samples. In the case of I, SE and FE, the water dilutions were from 10^{-5} to 10^{-2}, from 10^{-4} to 10^{-1} and from 10^{-3} to samples without dilutions, respectively. Only plates with 30 to 300 colonies were used for enumeration.

2.4. Quantification of ARGs

2.4.1. DNA extraction from water samples

DNA extraction and ARG quantification were carried out only for water samples (I, SE, FE) from the WWTPs selected for Campaign 2 analysis (VF1, VF3, AS1, AS2, BD1, and BD2). Water volumes of 0.5 L, 0.5 L, and 1 L for I, SE, and FE samples, respectively, were filtered through a membrane with 3.0-µm (Polycarbonate membrane filter, Millipore, Merck, USA) and 0.2-µm pore sizes (Polytetrafluoroethylene

(PTFE) membrane filter, Omnipore, Merck, USA) and stored at -20°C until further use. DNA was extracted using Qiagen DNeasy Power Soil kit (Qiagen, Inc., Valencia, CA) to a final elution volume of 50 µL from the different membrane used (3.0-µm and 0.2-µm pore sizes). The concentration and the quality of the DNA were tested using a Nanodrop Spectrophotometer 8000 (Thermofisher Scientific, Inc., Waltham, MA). The extracted DNA from the water samples from each WWTP was stored at -20°C until further use.

2.4.2. qPCR

Five ARGs of interest were selected and analyzed in water samples from each WWTP during Campaign 2: *sul*1, bla_{TEM}, *qnr*S, $bla_{\text{CTX-M-32}}$, and *tet*M, as well as 16S rDNA and *intI*1. ARGs were quantified using SYBR green (A F. Hoffmann–La Roche AG, Inc, Switzerland), except for *tet*M, for which Dynamo ColorFlash SYBR green was used (Thermofisher Scientific, Inc.), in a Lightcycler 480 (A F. Hoffmann–La Roche AG, Inc, Switzerland). The thermal cycling conditions were 95 °C for 10 min, 45 cycles at 95 °C for 15 s, and Ta for 1 min. The quantitative PCR (qPCR) reactions were performed in duplicate using fixed dilution of raw DNA extract. The quality criterion was $R^2 > 0.99$. The ARG quantification was based on the protocols described by Cerqueira et al. [23].

2.5. Statistical analyses

In this study, the performance of full-scale rural WWTPs in ARB, ARG, TC, and FC reductions and physicochemical parameters among different biological treatments and disinfection treatments during Campaign 1 and Campaign 2 was analyzed using RStudio version 4.3.1, with a significance level of $p = 0.05$. The data were tested for normality and homogeneity of variance using the Shapiro Wills test and Fligner-Killen test, respectively. Then, an ANOVA test for the data with a normal distribution and a Kruskal-Wallis test for the data without a normal distribution were performed. In this case, comparison between I, SE, and FE and the effect of WWTP characteristics was carried out by means of the Kruskall-Wallis test and Dunnet post-hoc test using the Dunn.test R package (https://cran.r-

177 project.org/web/packages/dunn.test/index.html). The effects of biological technologies and disinfection
178 processes were studied by means of a two-way Permanova test using the vegan R package (https://cran.r-
179 project.org/web/packages/vegan/index.html). Finally, PCA was performed using the principal function from
180 the psych R package (https://cran.r-project.org/web/packages/psych/index.html).

181 **3. Results and discussion**

182 **3.1. Influence of full-scale rural WWTPs on physicochemical and microbiological parameters**
183 **(Campaign 1)**

184 The efficiency of the studied WWTPs in removing nutrients and microbiological (including ARB) loads is
185 presented in Table 1. The physicochemical analysis of influents (I, Table 1) allows them to be classified as
186 concentrated, according to Henze et al. [24] (COD > 300 mg/L, NH_4^+-N > 50 mg/L and FC > 8.0 log
187 (CFU/100 mL) (Table 1). WWTP treatment reduced TU, COD, and NH_4^+-N loads by 92%, 79%, and 53%,
188 respectively (Table 1, all WWTP combined). Primary and secondary treatments accounted for essentially
189 all (COD and NH_4^+-N) or most (TU) of the observed load reduction, suggesting that the tertiary disinfection
190 treatments made a moderate contribution to the quality of the final effluent, at least in terms of
191 physicochemical parameters (Table 1). These performance levels were in the ranges reported in previous
192 analyses of WWTPs equipped with similar biological tertiary treatments [17,25,26].

193 ***Table 1***

194 WWTP treatment also very significantly reduced the overall microbiological loads (Table 1). TC and FC
195 counts were reduced by 3.1 – 3.8 log units and 2.4 – 2.7 log units, respectively, with no significant
196 differences among the three biological treatments ($p > 0.05$, Table 5S). A similar tendency was observed
197 for the reduction of CIP-, AMX- and CTX-resistant bacterial loads (ARB^{CIP}, ARB^{AMX} and ARB^{CTX},
198 respectively, Table 1). In this case, the tertiary treatment reduced TC, FC, and ARB^{CIP} loads relative to the
199 secondary effluent, demonstrating its utility for removing potentially pathogenic microorganisms and
200 reducing AMR spread into the environment (Table 1). These findings are consistent with those of Zhuang

et al. [19], who studied the effects of Cl, UV, and O_3 on ARB and ARG reductions, reporting an ARB reduction of 3 – 4 log units from SE to FE; however, no significant differences in overall performance were observed among the three biological (VF, AS, and BD, Table 5S) and disinfection treatments. These performances in coliform and ARB load reduction are similar to those found in the literature, which ranged between 2 and 4 log units [27,28]. They also compared well with those obtained using more sophisticated, more energy- and resource-intensive, and potentially more aggressive secondary treatments, like the oxidation ditch (OD) or a membrane biological reactor (MBR) [18]. These results show that the full-scale rural WWTPs of this study decreased the environmental risks of the FE in terms of eutrophication regardless of the type of biological technology.

The mutual correlations among the 60 water samples analyzed in this work (three samples (I, SE, and FE) each from each 20 WWTPs) according to their physicochemical and microbiological characteristics were analyzed using PCA (two components, 47% of total variance explained, Figure 3). The score plot (Figure 3a) shows input (I) samples neatly separated from the treated (SE and FE) ones, consistent with the characteristics of the influent and the observed effect of the treatment on the studied parameters. The same plot shows much less separation between secondary and final effluent samples, also in agreement with the results of the global analyses (see Table 1). It also shows no differences among the three biological and disinfection treatments, as FE samples from WWTPs equipped with all three methods are clustered together in the graph (Figure 3a, see green circles, diamonds, and triangles). Comparison of the loading plot with the score plot (Figure 3b) indicates that influent samples are associated with high loads COD, TU, NH_4^+-N, microbial parameters (TC and FC), and ARB abundances (ARB^{CIP}, ARB^{AMX} and ARB^{CTX}), which is expected due to the characteristics of the input wastewater and the reduction of these loads due to the secondary and tertiary treatments (Table 1). Therefore, this multivariate parametric analysis reflected the global results, indicating that most of the depuration effects corresponded to the primary and secondary WWTP treatments (SE), whereas the tertiary disinfection treatment contributed a minor refinement,

225 although it was likely significant for the improvement of the microbiological parameters. The relative
226 performance of the different steps of the water treatment may be attributed to the fact that the biological
227 treatments used in this study were aerobic.

228 ***Figure 3***

229 **3.2. ARG removal and its relationship to physicochemical and microbiological water quality**
230 **parameters (Campaign 2)**

231 Physicochemical and microbiological parameters of the water samples collected in the six selected WWTPs
232 for resampling in Campaign 2 presented TU, COD, NH_4^+-N, TC, and ARB removal efficiencies similar to
233 the results from Campaign 1 (Table 2, compared with Table 1) despite the smaller sample size, the six
234 months between the two campaigns, and the subsequent change in season (spring for Campaign 1, fall for
235 Campaign 2). In addition, analysis by qPCR of absolute abundances of 16S rDNA, *intI*1, and the ARGs
236 *sul*1, bla_{TEM}, *qnr*S, $bla_{CTX\text{-}M\text{-}32}$, and *tet*M showed a very effective removal of these DNA sequences from the
237 final effluent relative to the input, with levels in some cases falling to below quantitation limits (Table 2).
238 As in Campaign 1, most of the reduction in nutrient and microbiological loads occurred during secondary
239 treatment, although the tertiary treatment provided a further reduction in several parameters, particularly
240 TC, *intI*1, $bla_{CTX\text{-}M\text{-}32}$, and *tet*M (Table 2). Munir et al. [18] observed little change in ARG and ARB
241 abundances between pre- and post-disinfected effluents. As in the case of Campaign 1, no differences were
242 observed among the three biological treatments and disinfection processes (Cl vs. UV) (Figure 5, see also
243 supplementary Table 7S), although the statistical tests may be hampered by the relatively small amount of
244 samples (only two samples per biological treatment class). Figure 4 shows the distribution of samples taken
245 during Campaign 2 (PCA, two components, 67% of total variance explained). The plots show a strong
246 similarity to their counterparts for Campaign 1 (Figure 3), although the separation between SE and FE
247 samples in the score plot (Figure 4a) is clearly better defined in the Campaign 2 analysis. The loading plot
248 in Fig. 4b suggests a strong co-correlation between microbiological parameters (TC, FC), ARB (ARB^{CIP},

249 ARB^{AMX}, ARB^{CTX}) and ARGs. This tendency was also reported by Kumar et al. [29] who evaluated the
250 prevalence of ARB and ARGs in two municipal WWTPs and one hospital WWTP and found a strong
251 association between coliform prevalence and ARB abundance. In the same line, Narciso-da-Rocha et al.
252 [30] reported that ARGs such as bla_{TEM} and van*A* presented a strong correlation with culturable-bacteria,
253 antimicrobial residues, and some bacterial populations. These associations between microbiological (TC
254 and FC) and AMR parameters (ARB and ARGs) are relevant for monitoring the risks of AMR dissemination
255 into the environment in rural WWTPs. While a molecular technique like quantitative PCR (qPCR) is a useful
256 and easy-to-use tool for the direct determination of ARGs, the application of such techniques in rural
257 WWTPs is difficult in terms of the need for sophisticated equipment and highly trained personnel. For this
258 reason, TC and FC could be a useful indicator of reductions in microbiological and AMR parameters in
259 rural WWTPs [6].

260 ***Figure 4***

261 In general, the most abundant ARGs in the input samples were *sul*1 and *intI*1, with values that fluctuated
262 between 5.0 – 5.5 log copies/mL (Table 2). A relationship between *sul*1 and *intl*1 abundances was observed
263 in the literature; this tendency is expected, as *sul*1 is located in the 3' conserved region of Class 1 integrons
264 [31]. This finding is consistent with Shen et al. [32] and Zhang et al. [33], who reported a correlation between
265 *sul* genes and *int*l1 in WWTPs in China. The reduction of over 4 log units in ARB and most ARGs loads
266 (Table 2) is similar to those found in studies on WWTPs in the literature, with values that varied between
267 2.0 – 4.5 log units [6,26,33]. Regarding the overall effect of disinfection in rural WWTPs, the results confirm
268 that the biological treatment and subsequent disinfection could play an important role in removing ARGs
269 and serve as a barrier that limits ARB and ARGs dissemination into the environment [20,34–37].

270 ***Figure 4***

271 **4. Conclusions**

The overall results regarding the performance of rural WWTPs in both campaigns demonstrated that the combination of secondary and tertiary treatments is a good design for reducing the risks of microbiological and AMR dissemination and improving FE quality in rural areas where the WWTP management and operation have economic and technical limitations. It is also revealing that no differences between the types of biological and disinfection technologies were reported, indicating a similar efficiency for all of them, at least within the limitations of our study. The overall reductions in TU, COD, and NH_4^+-N loads were above 90%, 70%, and 40% in both campaigns, whereas coliform loads were reduced by between 2.0 and 4.0 log units, ARB loads between 2.0 and 4.6 log units, and ARG loads between and 2.3 and 4.0 log units. These results underscore the importance of these rural WWTPs in improving the water quality of effluents, decreasing eutrophication, and reducing microbiological risks and the spread of AMR. The fact that these results were obtained in essentially self-managed WWTPs only adds interest to a non-centralized scheme of wastewater treatment that has proved very useful for sparsely populated areas around the world.

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Statement & Declarations

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Authors Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ana María Leiva and Gloria Gómez. The first draft of the manuscript was written by Ana María Leiva and Gerardo González-Rocha, Benjamín Piña and Gladys Vidal corrected and commented on previous versions of the manuscript. All authors read and approved the final manuscripts.

Ethical approval

The manuscript does not contain data which requires ethical approval.

Consent to participate

All the authors have consent to participate.

Consent for publication

All the authors have consent to publish.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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433

434 **Table 1.** Overall physicochemical and microbiological characterization of I, SE, and FE from WWTPs monitored during Campaign 1

Parameter	Unit	Overall			
		I	SE	FE	Final Reduction* or Removal Efficiencies** (%)
pH	-	7.5 ± 0.9	6.9 ± 0.8	6.9 ± 0.7	n/s
T	°C	19.0 ± 1.7	19.1 ± 1.9	19.2 ± 1.9	n/s
Redox	mV	54.8 ± 170.9	85.1 ± 140.2	129.5 ± 175.0	n/s
EC	µS/cm	1.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	n/s
DO	mg/L	2.7 ± 1.8	3.2 ± 1.8	3.6 ± 2.2	n/s
TU	NTU	176.2 ± 58.5^{A}	36.5 ± 24.3^{B}	13.9 ± 12.9^{C}	92.3 ± 6.0%**
COD	mg/L	365.5 ± 213.5^{A}	88.1 ± 60.8^{B}	63.0 ± 42.0^{B}	77.2 ± 21.9%**
NH_4^+-N	mg/L	57.2 ± 35.1^{A}	27.0 ± 15.8^{B}	24.1 ± 18.7^{B}	52.9 ± 25.2%**
NO_3^--N	mg/L	4.0 ± 3.0	3.4 ± 3.1	3.1 ± 3.2	n/s
NO_2^--N	mg/L	0.2 ± 0.3	0.2 ± 0.3	0.3 ± 0.3	n/s
PO_4^{2-}-P	mg/L	10.0 ± 6.9	8.1 ± 4.0	8.1 ± 3.5	n/s

TC	log (CFU/100 mL)	8.7 ± 8.5 ^{A}	7.2 ± 7.5 ^{B}	5.8 ± 6.2 ^{C}	2.9 ± 1.2 logs*
FC	log CFU/100 mL	7.9 ± 8.2 ^{A}	6.7 ± 7.0^{B}	5.6 ± 5.9 ^{C}	2.4 ± 1.2 logs*
ARB^{CIP}	log (CFU/100 mL)	5.6 ± 0.5 ^{A}	2.9 ± 2.0^{B}	0.9 ± 1.8^{B}	2.7 ± 2.2 logs*
ARB^{AMX}	log (CFU/100 mL)	6.1 ± 0.4 ^{A}	4.3 ± 1.0^{B}	1.7 ± 2.5^{B}	2.4 ± 2.5 logs*
ARB^{CTX}	log (CFU/100 mL)	5.8 ± 0.6 ^{A}	4.2 ± 1.1^{B}	1.4 ± 2.2^{B}	2.0 ± 2.2 logs*

Note: I: influent; SE: secondary effluent; FE: final effluent; T: temperature; redox: oxidation-reduction potential; EC: electrical conductivity; DO: dissolved oxygen; TU: turbidity; COD: chemical oxygen demand; NH_4^+-N: ammonium nitrogen; NO_3^--N: nitrate as nitrogen; NO_2^--N: nitrite as nitrogen; PO_4^{-3}-P: phosphate as phosphorus, TC: total coliform; FC: fecal coliform; ARB^{CIP}: ciprofloxacin-resistant bacteria; ARB^{AMX}: amoxicillin-resistant bacteria; ARB^{CTX}: ceftriaxone-resistant bacteria. (VF: n = 7, AS: n = 4, BD: n = 9, total = 20). Superscripts "A","B", and "C" denote statistically different distributions (Kruskal-Wallis/Dunnet tests). n/s, non-significant differences. *) The reductions of microbiological and antibiotic resistance parameters were reported in log units. These values were calculated considering the average between the different reductions obtained in the 20 WWTPs. **) For these parameters, removal efficiencies were calculated considering percentage calculated by ((I-FE)/I) x 100%. These performances were calculated considering the average between the different removal efficiencies obtained in the 20 WWTPs.

444 **Table 2.** Overall physicochemical and microbiological characterization of I, SE, and FE from the WWTPs monitored during Campaign 2.

445

Parameter	Unit	Overall			
		I	**SE**	**FE**	**Final Reduction* or Removal Efficiencies** (%)**
pH	-	7.0 ± 1.1	6.7 ± 0.4	6.6 ± 0.2	n/s
T	°C	14.0 ± 2.9	15.1 ± 2.8	15.1 ± 3.7	n/s
Redox	mV	54.1 ± 1.2	67.7 ± 172.6	157.7 ± 261.3	n/s
EC	µS/cm	1.2 ± 0.4	0.8 ± 0.4	0.9 ± 0.2	n/s
DO	mg/L	2.3 ± 1.8	3.3 ± 1.4	4.4 ± 1.8	n/s
TU	NTU	167.1 ± 48.2 ^{A}	26.3 ± 29.9 ^{B}	29.4 ± 21.4 ^{B}	82.5 ± 12.9 %**
COD	mg/L	314.0 ± 236.3 ^{A}	104.7 ± 96.0 ^{B}	52.1 ± 41.1 ^{B}	79.3 ± 20.7 %**
NH_4^+-N	mg/L	58.9 ± 18.9 ^{A}	20.3 ± 11.1 ^{B}	12.3 ± 8.3 ^{B}	79.3 ± 12.4 %**
NO_3^--N	mg/L	2.3 ± 1.3	3.2 ± 3.5	3.6 ± 3.8	n/s
NO_2^--N	mg/L	0.2 ± 0.3	0.2 ± 0.1	0.3 ± 0.3	n/s
PO_4^{2-}-P	mg/L	10.6 ± 8.4	5.9 ± 4.2	6.0 ± 2.7	n/s
TC	log (CFU/100 mL)	8.4 ± 0.7 ^{A}	6.1 ± 1.7 ^{A}	3.8 ± 1.7 ^{C}	4.5 ± 1.4 logs*

FC	log (CFU/100 mL	7.3 ± 0.9 [A]	5.4 ± 1.2 [A]	3.2 ± 1.5 [B]	4.1 ± 1.4 logs*
ARB^{CIP}	log (CFU/100 mL)	5.6 ± 0.4 [A]	3.5 ± 1.4 [B]	2.7 ± 2.6 [B]	4.6 ± 1.1 logs*
ARB^{AMX}	log (CFU/100 mL)	6.1 ± 0.4 [A]	4.3 ± 0.9 [B]	5.0 ± 0.9 [B]	2.6 ± 0.4 logs*
ARB^{CTX}	log (CFU/100 mL)	5.8 ± 0.5 [A]	4.0 ± 1.1 [B]	3.5 ± 1.9 [B]	3.9 ± 0.4 logs*
16S	log (copies/100 mL)	8.3 ± 0.3 [A]	6.2 ± 1.2 [B]	5.2 ± 0.9 [B]	3.1 ± 0.8 logs*
***sul*1**	log (copies/100 mL)	5.3 ± 0.3 [A]	4.3 ± 0.4 [B]	3.0 ± 1.2 [B]	2.3 ± 0.9 logs*
***intI*1**	log (copies/100 mL)	5.6 ± 0.3 [A]	4.6 ± 0.8 [A]	2.7 ± 1.1 [B]	2.9 ± 1.0 logs*
***bla*$_{TEM}$**	log (copies/100 mL)	3.6 ± 0.8 [A]	1.6 ± 0.5 [B]	1.1 ± 0.4 [B]	2.6 ± 0.5 logs*
***qnr*S**	log (copies/100 mL)	3.7 ± 1.2 [A]	2.3 ± 1.5 [B]	1.9 ± 1.3 [B]	2.4 ± 0.5 logs*
***bla*$_{CTX-M-32}$**	log (copies/100 mL)	3.1 ± 1.5 [A]	1.1 ± 0.5 [B]	< LOD [C]	3.4 ± 0.2 logs*
***tet*M**	log (copies/100 mL)	5.0 ± 0.6 [A]	2.2 ± 0.5 [B]	< LOD [C]	4.0 ± 1.0 logs*

Notes: I: influent; SE: secondary effluent; FE: final effluent; T: temperature; redox: oxidation-reduction potential; EC: electrical conductivity; DO: dissolved oxygen; TU: turbidity; COD: chemical oxygen demand; NH_4^+-N: ammonium nitrogen; NO_3^--N: nitrate as nitrogen; NO_2^--N: nitrite as nitrogen; PO_4^{-3}-P: phosphate as phosphorus, TC: total coliform; FC: fecal coliform; ARB^{CIP}: ciprofloxacinresistant bacteria; ARB^{AMX}: amoxicillin-resistant bacteria; ARB^{CTX}: ceftriaxone-resistant bacteria, LOD: limit of detection, [a]: negative removal efficiencies (n = 6). Superscripts "A","B", and "C" denote statistically different distributions (Kruskal-Wallis/Dunnet tests). . *) The reductions of microbiological and antibiotic resistance parameters were reported in log units. These values were calculated considering the average between the different reductions obtained in the 6 WWTPs. **) For these parameters, removal efficiencies were calculated considering percentage calculated by ((I-FE)/I) x 100%. These performances were calculated considering the average between the different removal efficiencies obtained in the 6 WWTPs.

Figure Captions

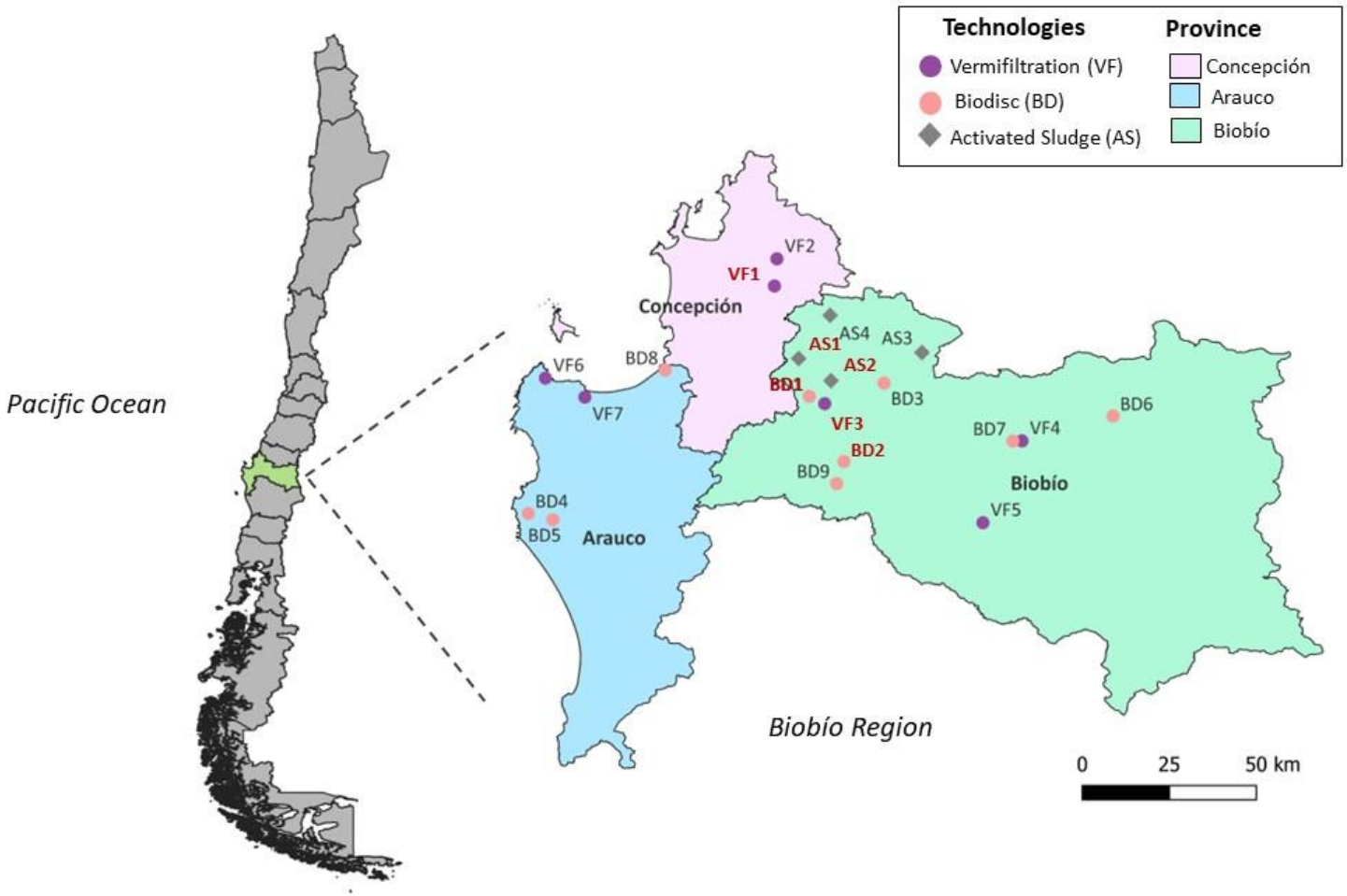
Figure 1. Map of the sampled WWTPs, displaying their locations and corresponding biological treatments. The following were sampled during Campaign 1: VF1: vermifilter in Copielemu, VF2: vermifilter in Villa Cristo Redentor, VF3: vermifilter in Villa Las Almendras, VF4: vermifilter in Villa Las Mercedes; VF5: vermifilter in Campamento; VF6: vermifilter in Llico; VF7: vermifilter in Las Peñas; AS1: activated sludge in Rere, AS2: activated sludge in Río Claro, AS3: activated sludge in Charrua, AS4: activated sludge in Tomeco; BD1: biodisc in Villa Laja; BD2: biodisc in Santa Fe; BD3: biodisc in La Aguada; BD4: biodisc in Santa Rosa; BD5: biodisc in Pehuén; BD6: biodisc in Antuco; BD7: biodisc in Canteras Sur; BD8: biodisc in Laraquete; BD9: biodisc in Coihue. The resampled WWTPs during Campaign 2 – VF1, VF3, AS1, AS2, BD1, BD2 – are highlighted in red.

Figure 2. Schematic diagram of different biological and disinfection treatment technologies: a) vermifiltration (VF); b) activated sludge (AS) and c) biodisc (BD).

Figure 3. Principal component analyses during Campaign 1: a) score plot and b) loading plot

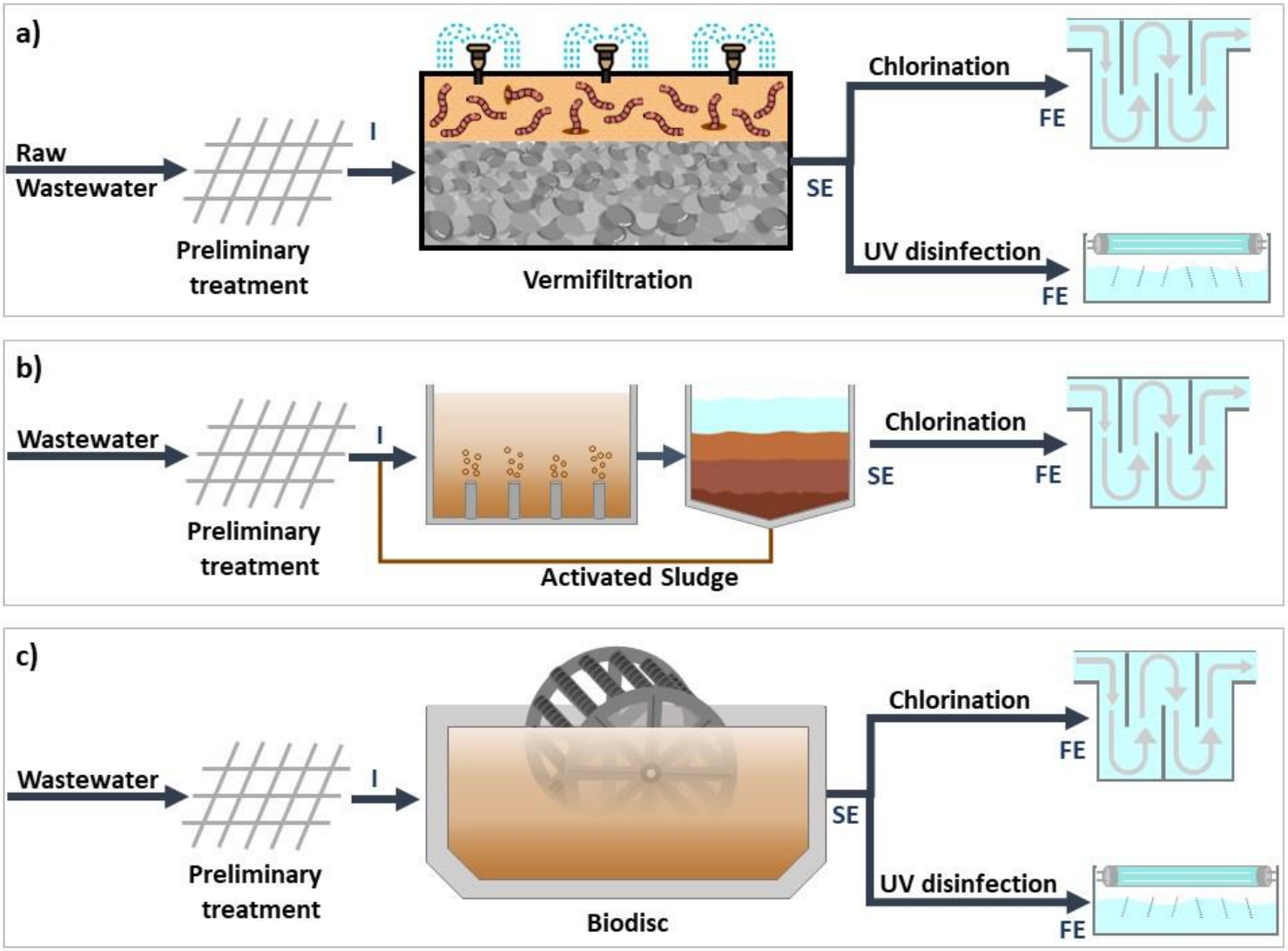
Figure 4. Principal component analyses during Campaign 2: a) biplot; b) score plot and c) loading plot

Figure 5. Reductions (relative to I) in microbiological parameters and ARGs in SE and FE from VF, AS and BD during Campaign 2: a) TC, b) FC, c) *intI*1, d) *sul*1, and e) *tet*M. Boxes cover the interval between the first and third quartles of the distribution and the thick line indicates the median. See the logarithmic scale on the Y-axis. Included -values relate to the comparison between SE and EF samples (Kruskal-Wallis test).



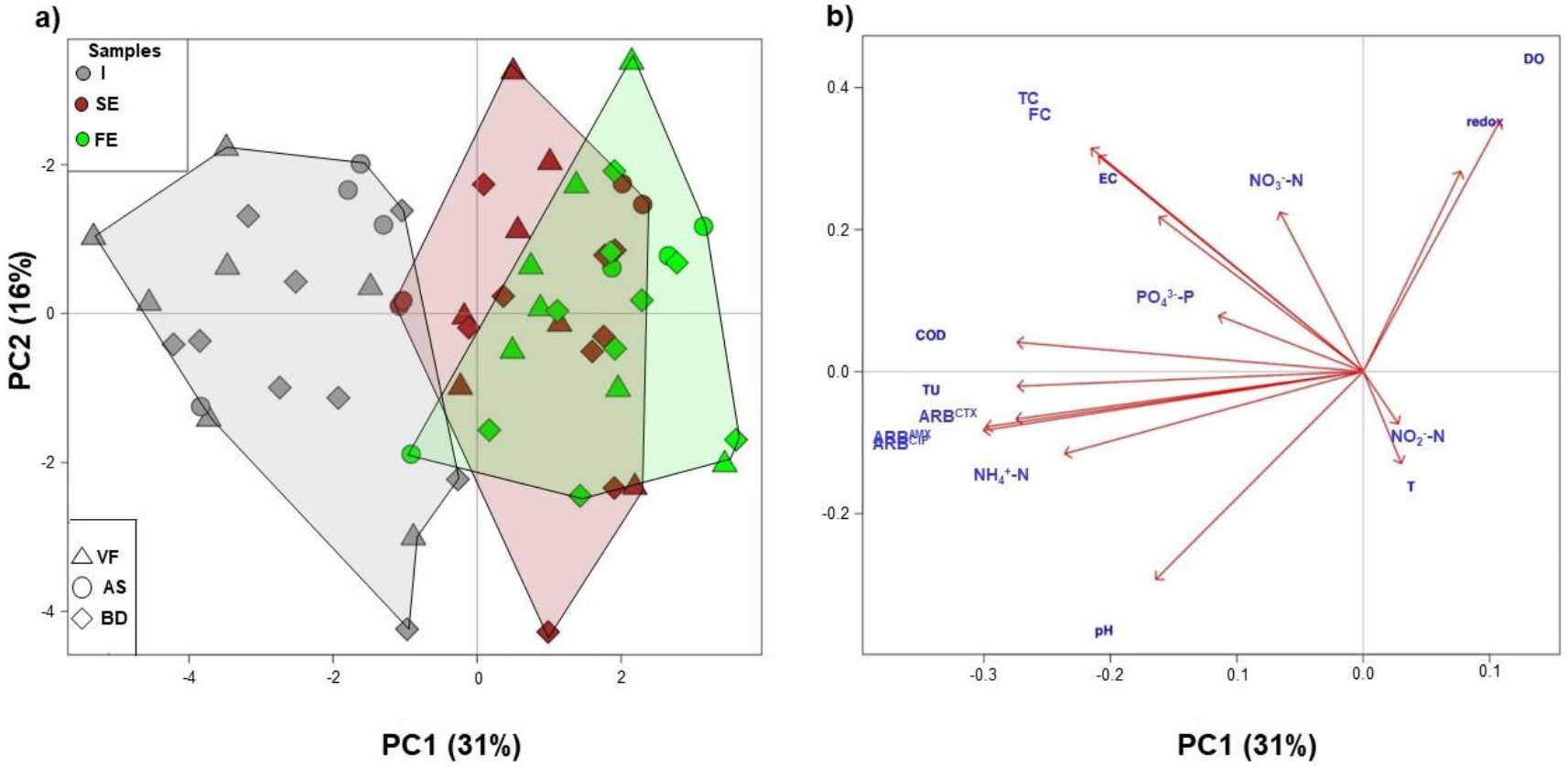
475

476 ***Figure 1***



477

478 ***Figure 2***



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482 ***Figure 3***

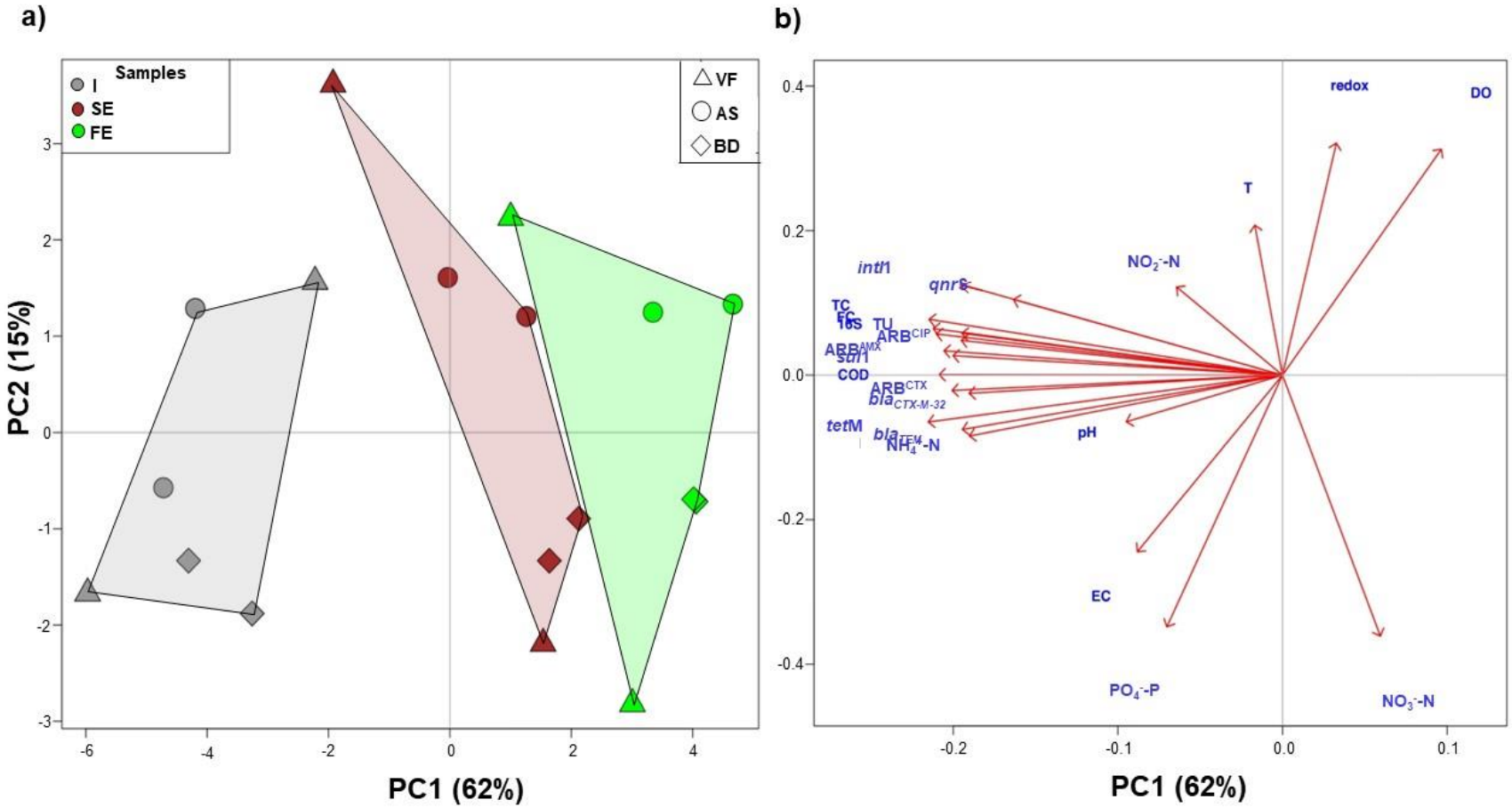
a)
Samples
I
SE
FE
VF
AS
BD
PC2 (15%)
PC1 (62%)
b)
redox
DO
T
NO_2^--N
intI1
qnrS
TC
FC
16S
TU
ARB^{CIP}
ARB^{AMX}
Sul1
COD
ARB^{CTX}
$bla_{CTX-M-32}$
tetM
bla_{TEM}
NH_4^+-N
pH
EC
PO_4^{3-}-P
NO_3^--N
PC1 (62%)

Figure 4

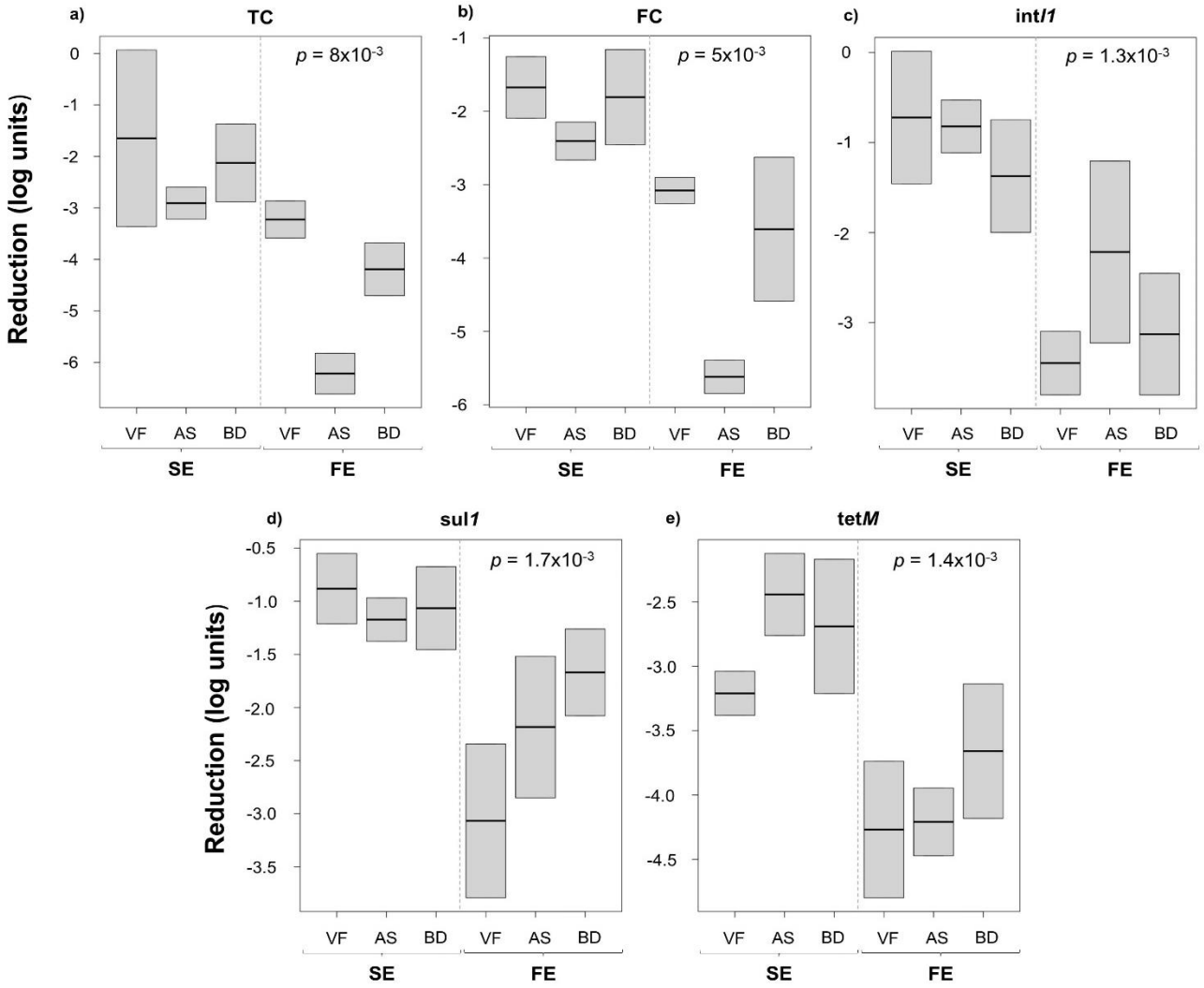
a)
TC
p = 8x10-3
b)
FC
p = 5x10-3
c)
int1
p = 1.3x10-3
d)
sul1
p = 1.7x10-3
e)
tetM
p = 1.4x10-3
Reduction (log units)
VF AS BD VF AS BD
SE
FE

Figure 5