Assessment of dead-end ultrafiltration for the detection and quantification of microbial indicators and pathogens in the drinking water treatment processes

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ABSTRACT

A safe water supply requires different treatments and monitoring to guarantee the absence of pathogens and substances potentially hazardous for human health. In this study we assessed the efficiency of the dead-end ultrafiltration (DEUF) method to concentrate faecal indicator organisms (FIO) and pathogens in water samples with different physicochemical characteristics. Water samples were collected at the different treatment stages of two drinking water treatment plants to analyse the concentration of a variety of 7 FIO and 4 reference pathogens: *Campylobacter* spp., enteroviruses, *Cryptosporidium* spp. and *Giardia* spp. The samples were analysed before and after concentration by DEUF. Percent recoveries were highly variable with a mean of 43.8 ± 17.5 %, depending on the FIO and inherent sample characteristics. DEUF enabled FIO concentration in high volumes of water (100 - 500 l), allowing a reduction in the detection limit compared to the non-concentrated samples due to the high volume processing capabilities of the method. As a consequence, the detection of FIO removal was 1.0 to 1.5 logarithms greater in DEUF-treated water compared to unfiltered samples.

The DEUF method improved the detection of target indicators and allowed for the detection of pathogens in low concentrations in water after the different treatment stages, confirming the suitability of DEUF to concentrate high volumes of different types of water. This method could be useful for microbial analysis in water treatment monitoring and risk assessment, allowing the identification of potential hazards in water destined for different uses. And critical points during the water treatment process.

Keywords: water quality, drinking water, *Campylobacter*, enteroviruses, *Cryptosporidium*, *Giardia*

INTRODUCTION

The accessibility of safe water is a major global concern: in 2016, 1,870,998 deaths are estimated to have been caused by inadequate water, sanitation and hygiene (WHO, 2019). To guarantee its safety, water is submitted to different treatments that ensure the absence of pathogens and harmful substances hazardous for consumer health. The expected quality of water depends on its final use, the highest being required for drinking water.

Pathogen detection and quantification by culture methods is costly and time-consuming, and it is unfeasible to analyse all waterborne pathogens. The development of molecular techniques has improved monitoring speed but does not provide information about pathogen viability and/or infective capacity. Consequently, the assessment of faecal indicator organisms (FIO) remains the main strategy for water quality monitoring.

Faecal indicator bacteria, such as *Escherichia coli* (EC) or intestinal enterococci (IE), have been used for many years in water supply management (Anderson et al., 2005; Hijnen et al., 2000; Tallon et al., 2005; Van Donsel et al., 1967). However, the efficiency of bacteria as indicators of viruses and protozoa has been questioned (Gerba et al., 1979; Keswick et al., 1984), because of differences in their structure, life cycle, persistence and survival in water. Bacteriophages and spores of sulphite-reducing clostridia (SSRC) have been recommended as more effective indicators of viruses and protozoa, respectively (Agulló-Barceló et al., 2013; IAWPRC Study Group on Health Related Water Microbiology, 1991; Payment and Franco, 1993), and some drinking water regulations now require their monitoring to guarantee water quality (Health Canada, 2019; NHMRC, 2011), besides pathogen analysis and removal.

Since 2004, the World Health Organization has promoted the implementation of water safety plans, which consist of risk assessment and management at all steps of the multibarrier treatment of drinking water, from the catchment to the end-user (WHO, 2011). In Spain, where the current study was performed, the implementation of water safety plans is obligatory in zones supplying water to 50,000 inhabitants or more. To perform the microbial risk assessment, in addition to the analysis of FIO, it is necessary to monitor different reference pathogens.

77 The infectious dose for some pathogens is as little as 1-10 cfu, as in the case of *E. coli* O157:H7
78 or *Shigella* spp.(Kothary and Babu, 2001) and pathogen analysis and removal requires the
79 analysis of high volumes of water, as pathogens are frequently present in lower numbers
80 compared to FIO. Although standardized protocols are available for the detection and
81 quantification of each pathogen, new approaches are needed that allow the simultaneous
82 concentration of multiple kinds of pathogens. With this aim, different concentration methods have
83 been extensively tested, including glass wool filtration, monolithic affinity techniques, and
84 ultrafiltration, the latter allowing the concentration of the highest volumes of water and different
85 microorganisms (Bridle, 2014; Polaczyk et al., 2008).
86 The ultrafiltration procedure is based on size exclusion and can be carried out by tangential
87 flow or dead-end concentration. Compared to other techniques, dead-end ultrafiltration (DEUF)
88 has the advantage of being able to handle higher volumes of water. Previous studies have tested
89 DEUF in drinking water samples, reclaimed water or spiked water samples, with the
90 physicochemical parameters and microorganism concentration set under controlled conditions
91 (Liu et al., 2012; Mull and Hill, 2012; Smith and Hill, 2009). However, little is known about its
92 performance in environmental samples with different characteristics or samples under natural
93 conditions.
94 The aim of this study was to assess the ability of DEUF to concentrate high volumes of water
95 samples from the different stages of two drinking water treatment plants (DWTPs) for the analysis
96 of FIO and bacterial, viral and protozoan pathogens. In addition, we studied the ability of the
97 method to better assess the FIO and pathogen removal efficiency of the different treatment steps
98 in both DWTPs compared to traditional sample processing. To perform this research, we analysed
99 four different faecal indicator bacteria: total coliforms (TC), EC and IE as non-conservative
100 parameters, and SSRC as a conservative parameter indicator of resistance forms. Three
101 bacteriophages were analysed as viral indicators: somatic coliphages (SOMCPH), F-specific
102 RNA coliphages (FRNAPH) and total coliphages (CB390PH). In this study, we also analysed
103 four different reference pathogens that follow the faecal-oral transmission route and are crucial
104 in assessing the microbial risk of drinking water. We chose *Campylobacter* spp. as a bacterial

105 pathogen, as it is the major source of bacterial gastroenteritis globally (European Centre for
106 Disease Prevention and Control, 2019; Kaakoush et al., 2015; WHO, 2012) and has a low
107 infective dose of 360 MPN (Hara-Kudo and Takatori, 2011). Enteroviruses (EV) were chosen as
108 viral pathogens because they have a low minimum infective dose and can cause serious diseases,
109 not only gastroenteritis, but also meningitis and myocarditis; for this reason, they are included in
110 some regulations as a reference pathogen (Health Canada, 2019; NHMRC, 2011; USEPA, 1998).
111 Finally, we analysed *Cryptosporidium* spp. and *Giardia* spp., two protozoa that are another major
112 cause of gastroenteritis worldwide (Fletcher et al., 2012). Moreover, the importance of
113 *Cryptosporidium* and *Giardia* lies in their ability to form oocysts and cysts, respectively,
114 resistance forms that can persist after different water treatments. In order to perform this study,
115 we analysed: i) the concentration of different FIO in the catchment of the DWTPs; ii) the
116 recoveries of the DEUF method through FIO concentration; iii) the removal of FIO in both
117 DWTPs, comparing the direct and concentration methods; iv) the pathogen concentrations and
118 their removal in the DWTPs.

119 **MATERIAL AND METHODS**

120 Samples and sampling site

121 This research was performed in two DWTPs located in Catalonia (Northeast of Spain) that
122 supply drinking water to more than 4.5 million inhabitants in the Barcelona Metropolitan Area.
123 DWTP A treats the surface water of a river in its lower course, and DWTP B treats the water of
124 a river in its middle course after a reservoir system. The two types of raw water therefore have
125 very different characteristics: pollution and values of turbidity and conductivity are much higher
126 in the water of the DWTP A catchment area compared to DWTP B, and consequently the
127 treatment required is also different. DWTP A has a maximum capacity of 3.2 m^3/s with 5 different
128 stages: catchment, clarification, sand filtration, active carbon filtration and chlorination with
129 NaClO. Before the chlorination, part of the water is treated by reversed electrodialysis to reduce
130 its high conductivity. DWTP B has a maximum capacity of 8 m^3/s and consists of 4 stages:
131 catchment, clarification, active carbon filtration and chlorination with NaClO.

A total of 12 sampling campaigns were performed in DWTP A and 9 in DWTP B from January 2018 to February 2019. Samples were taken at catchment intake and after each stage of the drinking water treatment. Sample volumes ranged between 150 and 506 l for the DEUF method and between 0,01 and 100 ml for the direct analysis of the different FIO using conventional methods as stated below.

Physicochemical parameters

Different physicochemical parameters were measured *in situ* to characterise each water sample: turbidity, temperature, total organic carbon (TOC) and conductivity.

Turbidity was measured with a 2100-N and 2100-P Turbidimeter (Hach, USA) in DWTP A and DWTP B, respectively. Temperature was measured with a Thermometer 0560 1113 (Testo, Australia) and TOC was registered with a TOC-V CSN and TOC-L CSH (Shimazdu, Japan). Finally, the conductivity was measured with a multiparametric analyser Crison Multimeter MM41 (Danaher, United States) in DWTP A and with a Crison GLP-32 analyser (Danaher, United States) in DWTP B.

Assessment of the DEUF method

For the assessment of the DEUF method samples were concentrated using Rexeed-25A™ hollow fiber filters (Asahi Kasei Medical America Inc, Japan) following a previously described method (Hill et al., 2007). The Rexeed-25A™ filters were pre-treated by recirculating 400 ml of 6.25% of sterile foetal bovine serum (FBS) blocking solution for 5 minutes to avoid bacterial adsorption to the filters. Filters were stored at 4°C for 72 hours until use. Samples were concentrated at 2 l/min by connecting the filter directly to a faucet that was available for sampling after each water treatment step, except for the raw and clarified water from DWTP A, which was concentrated using a peristaltic pump at 2.9 l/min. Filters were eluted using 500 ml of phosphate-buffered saline supplemented with 0.5 ml of 1% Antifoam A (Sigma-Aldrich, USA)/10% Tween 80 (Scharlab, Spain) and 0.5 ml of 10% NaPP (Sigma-Aldrich, USA). A back-flush elution was performed using a peristaltic pump at 0.65 l/min and the eluate was recovered obtaining a final volume between 480 ml and 640 ml.

159 In addition, we also collected 1 l of each sample to be analysed without DEUF concentration.
160 Direct and concentrated samples were analysed to detect and quantify the FIO and pathogens as
161 stated below.

162 Enumeration of faecal indicator organisms

163 *Escherichia coli* (EC) and total coliform bacteria (TC) were analysed following ISO 9308-
164 1:2014 (ISO, 2014). Samples were filtered through 0.45 µm diameter pore size nitrocellulose
165 membranes and the filters were then incubated on Chromocult® agar (Merck, Germany) at 37°C
166 for 20 hours. Dark blue and purple colonies were enumerated as *E. coli.* The sum of pink colonies
167 plus dark blue and purple colonies were enumerated as TC.

168 Intestinal enterococci (IE) were enumerated following ISO 7899-2:2000 (ISO, 2000a).
169 Samples were filtered through 0.45 µm diameter pore size nitrocellulose membranes and the
170 filters were then incubated on BD Difco Enterococcus agar (Thermo Scientific®, USA) at 37°C
171 for 48 hours. In order to confirm the positive colonies, filters were transferred to Bile Esculin
172 Azide Agar (Scharlab, Spain) and incubated at 44°C for 4 hours.

173 Spores of sulphite-reducing clostridia (SSRC) were enumerated as described previously (Ruiz-
174 Hernando et al., 2014). Samples were subjected to a thermal shock at 80°C for 10 minutes and
175 anaerobically cultured in *Clostridium perfringens* selective agar (Scharlab, Spain) at 44 °C for 24
176 hours.

177 For bacterial indicator analyses, the maximum volume analysed per sample was 100 ml in
178 direct samples and 2 ml in samples concentrated by DEUF. For volume samples less than 10 ml
179 sample volume was increased up to 10 ml by adding sterile PBS. Therefore, the theoretical
180 detection limit of the used method to analyse direct samples was 1 cfu/100 ml, and in the DEUF
181 samples it was about 0.05 cfu/100 ml, according to the volume concentrated and the volume
182 obtained in the elution process. The DEUF estimated detection limit was calculated by taking into
183 consideration the theoretical detection limit and the efficiency in the recovery of FIO by the
184 DEUF method compared to the non-concentrated samples, which was about 0.02 cfu/100 ml.

185 Somatic coliphages (SOMCPH), F-specific RNA coliphages (FRNAPH) and total coliphages
186 (CB390PH) were enumerated by the double agar layer technique following the protocols

187 described in ISO 10705-2:2000, ISO 10705-1:1995 and Agulló-Barceló et al (2016), respectively
188 (Agulló-Barceló et al., 2016; ISO, 2000b, 1995). A maximum of 10 ml of each sample was
189 analysed in direct samples and 2 ml of the eluate in concentrated samples. Therefore, the
190 theoretical detection limit was 10 pfu/100 ml and about 0.05 pfu/100 ml in direct and concentrated
191 samples, respectively. The estimated detection limit was calculated as for the FIO, resulting in
192 0.01 pfu/100 ml.

193 Enumeration of pathogens

194 The pathogen concentrations were only analysed in DEUF samples due to their low values
195 that made necessary to concentrate high volumes of water. To quantify *Campylobacter* spp., 150
196 ml of eluate (equivalent to 50-125 l of the original sample) was further concentrated by
197 centrifugation at 7,700 g for 20 min at 20 °C and the pellet was resuspended in 5 ml of the
198 discarded eluate. The enumeration of *Campylobacter* spp. was performed following ISO
199 17995:2005 with some modifications in order to adapt the protocol to a Most Probable Number
200 (MPN) method, as previously described (Rodríguez and Araujo, 2012). Briefly, this method
201 consisted in 3 tenfold serial dilutions of the samples and selective enrichment in Preston
202 *Campylobacter* Selective Enrichment Broth (Oxoid, United Kingdom) at 42 °C for 48 h in
203 microaerobic conditions. Samples were inoculated in *Campylobacter* Agar Base Blood Free
204 (Oxoid, United Kingdom) and incubated at 42 °C for 48 h in microaerobic conditions. Grey
205 colonies were considered presumptive colonies of *Campylobacter* spp. and were confirmed by
206 Gram stain. The theoretical detection limit of *Campylobacter* spp. was about 0.0001 MPN/100
207 ml.

208 The detection and quantification of infective enteroviruses also required eluate concentration.
209 Different volumes of eluate, ranging from 70 to 192 ml (equivalent to 50-160 l), were
210 concentrated using Centricon® Plus-70 Centrifugal Filter Units (Merck Millipore, Germany) to
211 obtain a final volume of 1 ml. 780 µl of the concentrate was resuspended in 20 ml of Eagle's
212 Minimum Essential Medium (Sigma-Aldrich, USA) and filtered through 0.22 µm pore size
213 hydrophilic polyethersulfone membrane to remove non-viral microorganisms. Infective
214 enteroviruses were quantified by a double agar layer plaque assay in Buffalo green monkey

kidney cells as described (Mocé-Llivina et al., 2004). The theoretical detection limit of EV was about 0.001 pfu/100 ml.

In order to detect and quantify *Cryptosporidium* spp. and *Giardia* spp a volume of 50 ml of eluate (equivalent to 15-50 l) was further concentrated by centrifugation at 3,000 xg for 10 min and the pellet was recovered and resuspended in 5 ml of phosphate buffered saline to detect and quantify *Cryptosporidium* spp. and *Giardia* spp., as previously described (USEPA, 2012). Samples were subjected to Ziehl-Neelsen staining (Henriksen and Pohlenz, 1981) and merthiolate-iodine-formaldehyde staining (Sapero et al., 1951) to detect and quantify *Cryptosporidium* spp. and *Giardia* spp., respectively, after their observation by optical microscope. The volumes analysed resulted in a detection limit of about 0.2 oocysts/100ml of *Cryptosporidium* spp. and 0.1 cyst/100 ml of *Giardia* spp.

Statistical analysis

In order to analyse the results, FIO and pathogen concentrations were $\log_{10}$-transformed. We used the value corresponding to the detection limit as a result for negative results.

The recovery was calculated as the result of the fraction of the values obtained by the DEUF method and the values obtained in the direct samples.

The normality distribution of the data was checked by Shapiro-Wilk's test and data analysis and plots were performed using R Studio software v. 1.2.5001. Finally, we analysed the correlations among different parameters using Spearman's correlation test. Spearman's coefficient, r, with *P* values lower than 0.05 were considered statistically significant.

RESULTS

Concentration of samples by DEUF

Different volumes of each sample were concentrated by DEUF which varied according to their physicochemical parameters (Table 1). For most samples, the filtered volume was approximately 500 l, which was eluted in 560 ml and consequently, 1 ml of the eluate was equivalent to 0.9 l of the direct sample. The exception was the raw and clarified water samples of DWTP A, for which the equivalents were 0.3 l and 0.4 l, respectively.

242 The physicochemical parameters observed confirmed the differences between the catchment
243 of DWTP A and DWTP B, referred to henceforth as catchment A and catchment B, respectively.
244 As well as high turbidity, water from catchment A presented high conductivity values. A
245 statistically significant correlation between the streamflow and the turbidity was observed
246 (r=0.46; P<0.01) (Fig. A.1) using the values registered in the DWTP during the studied period.
247 The conductivity values were statistically negatively correlated with the streamflow (r= -0.75;
248 P<0.01), meaning that high streamflow produced a dilution of the electrolyte concentration and
249 reduced the conductivity of the raw water.
250 The water samples of catchment B showed low values of turbidity, conductivity and TOC,
251 making it possible to concentrate 500 l at each stage.
252 FIO concentration in the DWTP catchments by conventional analysis
253 DWTPs are designed to remove particles, microorganisms and substances that could affect
254 consumer health, incorporating different stages to optimise the treatment. In order to assess the
255 DWTP performance, it was necessary to characterise the raw water at the point of intake.
256 Considerable differences were found in FIO concentrations between the two catchments (Fig.
257 A.2). In direct samples of catchment A, concentrations ranged from 2 to 4 $\log_{10}$(cfu/100ml) or
258 $\log_{10}$(pfu/100ml); the exceptions were for TC, 3 to 5 $\log_{10}$(cfu/100ml), and FRNAPH, 1 to 3
259 $\log_{10}$(pfu/100ml). In direct samples of catchment B, FIO concentrations were roughly at the
260 detection limit, ranging from 0 to 1.5 $\log_{10}$(cfu/100ml) or $\log_{10}$(pfu/100ml); the exception was for
261 TC, 1.5 to 2.5 $\log_{10}$(cfu/100ml).
262 The concentration of faecal bacterial indicators in samples of catchment A was about 2-2.5
263 $\log_{10}$ higher than in catchment B. There was a similar difference in concentration of bacteriophage
264 viral indicators (2 $\log_{10}$), with the exception of FRNAPH, which was only 1 $\log_{10}$ higher in
265 catchment A. Viral indicators were present at lower concentrations than bacterial indicators; in
266 catchment B, bacteriophages were detected at roughly the detection limit, or in some samples not
267 at all.

FIO concentration in the DWTP catchments by DEUF-method

We observed lower FIO values in DEUF-concentrated samples compared to the direct samples, with differences of about 1 log_{10} in both catchments, suggesting a loss of microorganisms during the concentration process and a recovery lower than 100%. These differences were statistically significant for TC, SSRC, SOMCPH and CB390PH in catchment A (P<0.05) and for IE, SOMCPH, CB390PH and FRNAPH in catchment B (P<0.05).

FIO recoveries after concentration by DEUF

The percentage of microorganism recovery is key in the assessment of a concentration method. In order to analyse the effectivity of concentration by DEUF, we enumerated the concentrations of 7 FIO in direct and DEUF concentrated samples and we calculated the recovery of each FIO at every sampling campaign (Fig. 1). We obtained 1 to 3 outlier values for each FIO, which were removed for a better understanding of the results. Taking into account all the FIO and both DWTPs, the mean recovery was 43.8 ± 17.5 %, while the recovery of bacterial indicators was 45.5 ± 24.0 and the bacteriophages recovery was 22.4 ± 9.3.

The recoveries of each FIO differed considerably between samples and DWTPs. This could be explained by the variable physicochemical characteristics of environmental water, which can affect the efficiency of the concentration method. In our case, more variable recovery was obtained in bacterial than viral indicators in both DWTPs, but especially in DWTP A, where bacterial concentration was higher. Although recovery percentages were slightly higher in DWTP B for faecal bacterial indicators and in DWTP A for viral indicators, the differences were not statistically significant (P > 0.05).

To better understand how the environmental conditions of the water influence microorganism recovery in the DEUF method, we correlated FIO recoveries with the main physicochemical parameters (Fig. 2), finding no statistical significance in the correlations overall. However, the results showed that some parameters were highly relevant for the concentration method, including conductivity (r=-0.8; P<0.01), TOC (r=-0.61; P<0.01) and turbidity (r=-0.59; P<0.01), which presented strongly and moderately significant negative correlations with the filtered volume.

295 FIO removal in the DWTPs

296 In this research, we analysed the presence of FIO after each treatment stage (Table 2). In
297 catchment A, FIO were detected in all direct and concentrated samples. In catchment B, faecal
298 bacterial indicators were also detected in almost all the samples; however, bacteriophages were
299 found in only a few direct samples and FRNAPH not at all, whereas in concentrated samples,
300 SOMCPH and CB390PH were detected in 88.9% and 100%, respectively, and FRNAPH only in
301 44% of the samples.

302 The clarification stage removed part of the FIO in both DWTPs; in DWTP B removal was
303 almost total, with only TC still detected in clarified water. In the clarified water of DTWP A, a
304 high percentage of the direct samples still showed faecal bacterial indicators (83.3% for TC, EC
305 and IE and 91.7% for SSRC), whereas fewer were positive for bacteriophages (66.7% for
306 SOMCPH and CB390PH, and 33% for FRNAPH); a high percentage of concentrated samples
307 were also positive for faecal bacterial indicators (66.7% - 83.3%) and viral indicators (33.3% to
308 75.0%).

309 After the sand filtration in DWTP A, the target microorganisms were almost all absent, except
310 TC, which was detected in 1 direct and 3 concentrated samples (8.3% and 25% of positive
311 samples, respectively). Additionally, 3 concentrated samples (25%) were also positive for *E. coli*.

312 After the active carbon filtration in DWTP A, TC was detected in 41.7% of the concentrated
313 samples and SSRC in one sample (8.3%). In DWTP B, TC was also found in direct (11.1%) and
314 concentrated (2.2%) samples.

315 The final samples from both DWTPs did not show the presence of the tested FIO, achieving
316 the quality expected for drinking water. In general, a lower number of positive results were
317 obtained in direct than in concentrated samples when the concentrations were roughly the
318 detection limit. This was noticeable in the advanced stages of the treatment process of both
319 DWTPs and in catchment B.

320 To assess the operation of both DWTPs, FIO removal was compared in direct samples and
321 samples concentrated by DEUF. FIO removal (Fig. 3) was calculated as the difference between
322 the FIO $\log_{10}$ concentrations in the catchment and treated water. In samples without detectable

323 microorganisms, the detection limit was used. FIO removal was approximately 2 $\log_{10}$ higher in
324 DWTP A than in DWTP B, a reflection of the difference in FIO concentrations in raw water,
325 which was about 2 $\log_{10}$ higher in catchment A. The lower detection limit of the DEUF method
326 meant that most FIO values were higher in the filtered than direct samples (by 0.4 - 1.8 $\log_{10}$),
327 which facilitated the monitoring of FIO removal throughout the water treatment process. Removal
328 was statistically significantly higher in DEUF samples for all the FIO in both DWTPs (P<0.05),
329 except for SSRC in DWTP A and FRNAPH in DWTP B (P>0.05).

330 Pathogen concentrations in the catchments and their removal in DWTPs

331 In order to evaluate DEUF as a method for pathogen monitoring in environmental samples,
332 we assessed the concentration and the presence of four different kinds of pathogens in all the
333 stages of both DWTPs: *Campylobacter* spp. as a bacterial pathogen, enteroviruses as viral
334 pathogens and *Cryptosporidium* spp. and *Giardia* spp. as parasites (Table 3). We detected EV in
335 83% of the samples and the mean concentration of EV in catchment A was 0.01 pfu/100 ml. while
336 the concentrations of *Campylobacter* spp. in catchment A, ranging from 0.3 to 5.2 MPN/100 ml.
337 Concentrations of *Cryptosporidium* spp. in catchment A ranged from 3.3 oocysts/l to 52
338 oocysts/l and a mean concentration of 18.4 oocysts/l while we detected *Giardia* spp. in 11 out of
339 12 (91%) catchment A samples, with a mean concentration of 4.6 cysts/l.
340 We studied the correlations between the FIO and pathogen concentrations in the catchments
341 of the both DWTPs. In the DWTP A we found a statistically significant correlation between IE
342 and *Campylobacter* spp (r=0.69; P<0.05), SSRC and *Cryptosporidium* spp. (r=0.89; P<0.01) and
343 between SSRC and *Giardia* spp. (r=0.94; P<0.01) while in DWTP B we did not find statistically
344 significant correlations.
345 In DWTP A, the clarification stage removed the highest percentage of pathogens, after which
346 41.7% of samples were positive for *Campylobacter* spp., 16.7% for EV, 33.3% for
347 *Cryptosporidium* spp. and 8.3% for *Giardia* spp. However, after the sand filtration, all pathogens
348 had been removed with the exception of *Cryptosporidium* spp. detected in one sample of treated
349 water in DWTP A at a concentration of 1.02 oocysts/l.

In DWTP B, pathogens were observed in concentrations of roughly the detection limit, with the exception of EV, which was not detected. The raw water samples for catchment B were 85.7% positive for *Campylobacter* spp. with concentrations ranging from 0.003 MPN/100 ml to 0.05 MPN/100 ml; 62.5% for *Giardia* spp. with a mean concentration of 0.78 cysts/l and *Cryptosporidium* spp. were detected in 100% with concentrations ranging from 2.3 oocyst/l to 6.9 oocysts/l. The clarification stage of DWTP B removed almost all the pathogens, resulting in only 1 out of 8 samples (12.5%) positive for *Cryptosporidium* spp. After the active carbon filtration, *Cryptosporidium* was also detected in 1 out of 8 samples (12.5%).

DISCUSSION

In order to ensure the absence of pathogens and harmful substances in tap water, DWTPs provide a multibarrier system where each stage is optimised to achieve the best water quality. The detection and quantification of faecal indicators and reference pathogens in each stage of the drinking water treatment process is crucial for the water quality management.

The DEUF method performed in this study allowed the concentration of high volumes from the different stages of the both DWTPs. The lower volumes of filtered raw and clarified water from DWTP A can be explained by the procedure, as they were concentrated by a peristaltic pump. Moreover, another relevant factor in the catchment water was high turbidity, which resulted in an earlier saturation of the filters by particles. The higher conductivity of water at catchment A can be explained by its passing through an area of salt mines. The low values of turbidity, conductivity and TOC at catchment B allowed for the concentration of 500 l. In this case, the reservoir system works as a huge sedimentation tank, clarifying the water.

In both DWTPs water presented low turbidity and TOC values after the clarification stage. However, the conductivity in DWTP A was not reduced until the end of the treatment process, where part of the water was subjected to reversed electrodialysis.

The levels of faecal pollution were also different in both catchments. The concentrations of faecal indicator bacteria in catchment A were similar to those reported in previous studies, whereas SOMCPH concentrations were slightly higher (by 0.5 $\log_{10}$) (Montemayor et al., 2005;

377 Muniesa et al., 2012). The lower FIO concentrations in catchment B were due to the reservoir
378 system, located above the intake point, which removed part of the faecal pollution. These results
379 agree with previous studies performed at the same waterbody (Araujo et al., 1997). The
380 bacteriophage concentrations detected in this study agree with (Lucena et al., 2003), who reported
381 that concentrations of SOMCPH were also about 1 $\log_{10}$ higher than FRNAPH in rivers in South
382 America, France and Spain.

383 In this study we analysed the recovery of FIO using DEUF method, considering the values
384 obtained by the direct analysis of 100 ml as reference for the calculations. The recovery of the
385 DEUF method presented high variability in bacterial and viral indicators that can be caused by
386 their attachment to particles (LeChevallier et al., 1988; Templeton et al., 2008). This trend was
387 also described by Liu and collaborators, who reported high variability in the recoveries of
388 microorganisms, primarily bacteria, after DEUF of reclaimed water (Liu et al., 2012). The outlier
389 values, which ranged from 124% to 900%, could be attributed to different factors, such as the
390 variation in the filtration process. The concentration of catchment samples took 3.7 ± 1.3 hours
391 in DWTP A and 4.6 ± 0.7 hours in DWTP B, and FIO concentrations can change during this time.
392 Another factor could be the disaggregation of flocs containing microorganisms during the elution
393 process, which would increase the FIO concentration in the eluate compared to the direct sample
394 (Hill et al., 2007).

395 The concentration method tested in this study was developed by CDC and USEPA in order to
396 concentrate and detect biothreat agents in drinking water with recoveries higher than 50%
397 (USEPA and CDC, 2011). Several filtration methods such as glass wool, nanoCeram, continuous
398 flow centrifugation or electropositive cartridge have been developed to concentrate pathogens,
399 but they are optimised for detecting one type of microorganism (Francy et al., 2013; Karim et al.,
400 2009). The DEUF method has been used to concentrate bacterial, viral and protozoan pathogens,
401 leading to recoveries of 60 - 80% in drinking water (Gunnarsdottir et al., 2020). The recoveries
402 obtained here, ranging from 9% to 121% and with a mean recovery of 43.8 %, agree with the
403 results of (Bosch et al., 2016), who reported recoveries of 9% - 102%, depending on the
404 microorganism and the sample characteristics. However, it is necessary to take into account that

405 the assays in previous studies were performed in a laboratory, using spiked samples under
406 controlled conditions, which could improve microorganism recoveries compared to the
407 environmental samples and natural conditions analysed here.

408 The physicochemical parameters of the water can affect the microorganism recovery. TOC,
409 turbidity and conductivity are parameters that quantify the presence of organic matter, particles
410 and electrolytes, respectively, which can saturate the filter and reduce the filtered volumes. In
411 spite of the physicochemical parameters interfere with the DEUF method, different studies have
412 promoted its use because it allows for the concentration of different types of microorganisms and
413 pathogens with high microbial recoveries (Francy et al., 2013; Smith and Hill, 2009).

414 In both DWTPs the highest percentages of FIO and pathogen removal were achieved in the
415 clarification stage but in general, each stage contributed to the removal of FIO and pathogen
416 concentrations in both DWTPs, working as an effective multibarrier system. Nevertheless,
417 However, the increased detection of TC after the active carbon filtration could suggest that the
418 organic matter retained by the filters provides the nutrients required for bacterial growth.
419 Furthermore, the carbon particles could protect the bacteria against the treatments by allowing
420 the formation of biofilms (Gibert et al., 2013), which is a critical issue in drinking water treatment.

421 FIO concentrations were higher in the direct samples of both catchments compared to the
422 values obtained by the DEUF method. However, the lower detection limit in the treated water
423 samples concentrated by DEUF resulted in a logarithmic increase in FIO removal detected at all
424 stages of the system since for some samples the FIO values by the direct method were below the
425 limit of detection. In addition, the concentration allowed to improve the percentages of detection,
426 especially in that stages where the FIO concentrations were very low. This issue is crucial for the
427 microbial risk assessment in water for the reason that the DEUF method could increase the safety
428 of the drinking water.

429 The suitability of the DEUF method to perform the concentration of reference pathogens was
430 not compared with direct sample analysis since it is well known that a concentration method is
431 usually needed to quantify pathogens due to the low numbers present in the environment.
432 However, if we compare the obtained results with values obtained in previous studies performed

by our research group in the same catchment area, the EV mean concentration detected in this study (0.01 pfu/100 ml) was very similar to that reported by Costán-Longares and co-workers in a previous study, which reported a mean concentration of 0.04 pfu/100 ml in different rivers in Catalonia using electropositive filter cartridges to concentrate virus by adsorption (Costán-Longares et al., 2008). However, *Campylobacter* spp. concentrations were slightly lower than those of a previous study at the same waterbody using the centrifugation of 3 l as the concentration method (Rodríguez and Araujo, 2010). This difference could have two possible explanations: the increased ecological flow in catchment A over the last decade producing a dilution effect, and fewer sources of *Campylobacter* spp. pollution. Nevertheless, we detected *Campylobacter* spp. in 100% of catchment A samples, which was higher than the 81% of positive samples in the previous study. Our results for *Campylobacter* spp. confirm the high pollution pressure in catchment A and are similar to those of other studies reporting a high percentage of *Campylobacter* spp. in polluted surface waters (Eyles et al., 1998; Stelzer and Jacob, 1991).

The *Cryptosporidium* spp. concentrations detected in this study were higher than the 0.43 - 1.36 oocysts/l previously reported at the same waterbody using Envirocheck® filters (Montemayor et al., 2005). While both studies report 100% of positive samples in catchment A, the DEUF method could explain why we obtained higher concentrations despite an increased ecological flow in catchment A in the last years. The positive result obtained in treated water from DWTP A was obtained after a heavy rainfall event, during which DWTP A stopped operating for 2 days. The sampling campaign was performed 2 days after the DWTP A resumed functioning, when the treated flow, 0.6 m^3/s, was still lower than the normal flow of 1.2 m^3/s, which was subsequently achieved on the same day as the sampling. The turbidity in catchment A was still high (130 NTU) and the concentration of *Cryptosporidium* spp. in the catchment sample was 52 oocysts/l, the highest concentration obtained during the studied period. It should be taken into account that the detection and quantification method performed in this study do not provide information about the viability and infective capacity of the parasite. Previously reported viability levels range from 16% to 28% (Montemayor et al., 2005). Thus, this incident further suggests the important role of heavy rainfall in the mobilisation of waterborne pathogens (Curriero et al., 2001;

García-Aljaro et al., 2017; Kistemann et al., 2002; Tryland et al., 2011), which affects the quality of the surface water utilized by the DWTPs and entails a risk if the pathogens can overcome the different treatment stages.

The treated water of both DWTPs achieved the drinking water quality standards (CEU, 1998; WHO, 2012), as the current law assessing drinking water treatment processes only requires the absence of *E. coli* and *Enterococci* in 100 ml and the analysis of *Clostridium perfringens*. The monitoring not only of FIO but also pathogens, especially protozoa and resistance forms, in the last stages of drinking water treatment is crucial because they can become attached to particles and infrastructures and form biofilms (Wingender and Flemming, 2011). Several waterborne pathogens can be released from biofilms to water and constitute a hazard for consumers (Helmi et al., 2008; Searcy et al., 2006). Moreover, the ability of *Cryptosporidium* to excyst and grow in an environment without host cells such as biofilms has been recently described (Clode et al., 2015; Thompson et al., 2016). This capacity opens a new scenario in water quality monitoring, focusing attention on the treatment stages where multiplication is possible, and the possible addition of new stages to the multibarrier system.

The concentrations of protozoa in this research were in agreement with the results of previous studies, where *Cryptosporidium* spp. were detected more frequently and in higher concentrations than *Giardia* spp. in surface waters (Prystajecky et al., 2014). However, the inverse trend, where the concentrations of *Giardia* spp are higher than the concentrations of *Cryptosporidium* spp. has also been reported by some authors (Burnet et al., 2014; Mons et al., 2009). Several factors such as human and animal parasitization (Fletcher et al., 2012), the physicochemical characteristics of the water, water discharges, and the persistence of oocysts and cysts can contribute to the different densities and predominance of one or another protozoan in surface waters (Wilkes et al., 2009; Xiao et al., 2013).

Although we only found some statistically correlations between SSRC and the analysed protozoa and IE and *Campylobacter* spp. in the DWTP A, this method could be useful to select the most suitable surrogate microbial indicators and pathogens for testing.

Finally, the main advantage of this method is that allows the easy concentration of different microorganism with high recoveries for all of them. The use of the DEUF method to concentrate environmental samples before performing microbial analysis can provide valuable information for water management and for the quantitative microbial risk assessment included in water safety plans. As well as drinking water, DEUF can be applied to concentrate water for usage requiring less quality, such as bathing or irrigation.

CONCLUSIONS

The DEUF method was effective for FIO and pathogen concentration in high volumes of water with different physicochemical characteristics. The physicochemical factors determining the volume concentrated by DEUF were turbidity, conductivity and TOC. The concentration method reduced the FIO detection limit, increasing the logarithms of FIO removal in both DWTPs.

FIO and pathogen removal occurred at all the stages of the DWTP multibarrier systems but in both DWTPs the highest removal was achieved in the clarification stage. The increased detection of indicators after active carbon filtration showed this stage to be a critical point for water quality monitoring.

Concentration by DEUF represents an effective method for monitoring the quality of water for different uses and performing the quantitative microbial risk assessment required by water safety plans. Moreover, it allows for the identification of critical points during the water treatment processes and conditions that can compromise the microbial water quality.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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716 **TABLES AND FIGURES**

717 **Table 1:** Number of samples, mean and standard deviation of concentrated sample volumes;
718 turbidity, conductivity and TOC at the different stages of DWTP A and DWTP B.

	Stage	n	Filtered volume (l)	Turbidity (NTU)	Conductivity (µS/cm)	TOC (mg/l)
DWTP A	Catchment	12	175.6 ± 27.2	36.6 ± 37.6	1246 ± 368	3.8 ± 0.8
	Clarification	12	244.0 ± 98.1	1.3 ± 0.5	1256 ± 365	3.0 ± 0.6
	Sand Filtration	12	504.0 ± 8.3	0.4 ± 0.2	1251 ± 367	2.9 ± 0.6
	Active carbon filtration	12	504.4 ± 8.0	0.3 ± 0.1	1250 ± 368	1.8 ± 0.4
	Chlorination	12	505.1 ± 6.8	0.4 ± 0.3	862 ± 394	1.4 ± 0.3
DWTP B	Catchment	9	503.3 ± 5.7	1.5 ± 0.6	416 ± 31	2.9 ± 0.5
	Clarification	9	506.1 ± 14.5	0.6 ± 0.2	448 ± 34	2.5 ± 0.5
	Active carbon filtration	9	464.6 ± 43.0	0.3 ± 0.2	450 ± 36	2.0 ± 0.4
	Chlorination	9	501.2 ± 14.3	0.3 ± 0.2	451 ± 25	1.9 ± 0.4

719

720 **Table 2:** Percentage of positive FIO results in direct and concentrated samples at the different
721 stages of the DWTPs (n=12 in DWTP A; n=9 in DWTP B).

			TC	EC	IE	SSRC	SOMCPH	CB390PH	FRNAPH
DWTP A	Catchment	Direct	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Concentrated	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	Clarification	Direct	83.3	83.3	83.3	91.7	66.7	66.7	33.3
		Concentrated	83.3	83.3	66.7	83.3	75.0	58.3	33.3
	Sand filtration	Direct	8.3	0.0	0.0	0.0	0.0	0.0	0.0
		Concentrated	25.0	25.0	0.0	0.0	0.0	0.0	0.0
	Active carbon filtration	Direct	0.0	0.0	8.3	0.0	0.0	0.0	0.0
		Concentrated	41.7	0.0	0.0	8.3	0.0	0.0	0.0
	Chlorination	Direct	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Concentrated	0.0	0.0	0.0	0.0	0.0	0.0	0.0
DWTP B	Catchment	Direct	100.0	100.0	88.9	100.0	22.2	22.2	0.0
		Concentrated	100.0	100.0	100.0	100.0	88.9	100.0	44.4
	Clarification	Direct	11.1	0.0	0.0	0.0	0.0	0.0	0.0
		Concentrated	11.1	0.0	0.0	0.0	0.0	0.0	0.0
	Active carbon filtration	Direct	11.1	0.0	0.0	0.0	0.0	0.0	0.0
		Concentrated	22.2	0.0	0.0	0.0	0.0	0.0	0.0
	Chlorination	Direct	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Concentrated	0.0	0.0	0.0	0.0	0.0	0.0	0.0

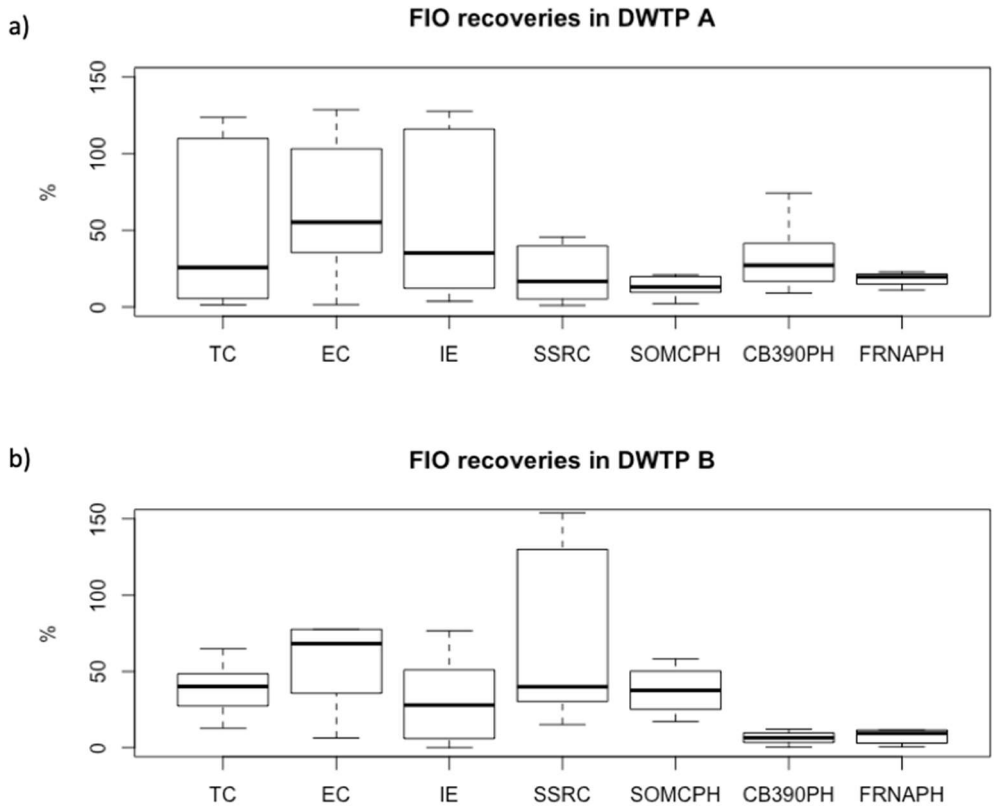
722

723 **Table 3:** Percentage of positive pathogen results after the different stages of both DWTPs (n=12
724 in DWTP A; in DWTP B n=9 for EV, n=8 for *Cryptosporidium* and *Giardia*, n=7 for
725 *Campylobacter*).

		Campylobacter	EV	*Cryptosporidium*	*Giardia*
DWTP A	Catchment	100.0	83.3	100.0	91.7
	Clarification	41.7	16.7	33.3	8.3
	Sand filtration	0.0	0.0	0.0	0.0
	Active carbon filtration	0.0	0.0	0.0	0.0
	Chlorination	0.0	0.0	8.3	0.0
DWTP B	Catchment	85.7	0.0	100.0	62.5
	Clarification	0.0	0.0	12.5	0.0
	Active carbon filtration	0.0	0.0	12.5	0.0
	Chlorination	0.0	0.0	0.0	0.0

726

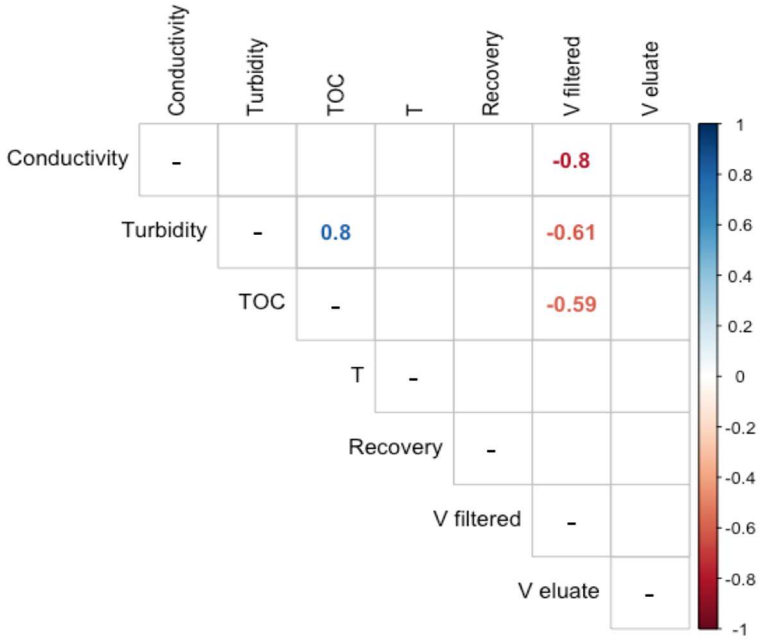
727 **Fig. 1:** Percentage of FIO recoveries in a) DWTP A catchment and b) DWTP B catchment. (n=12
728 in DWTP A and n=9 in DWTP B).



729

730 **Fig. 2:** Statistically significant Spearman's correlations (P <0.01) between FIO recoveries and

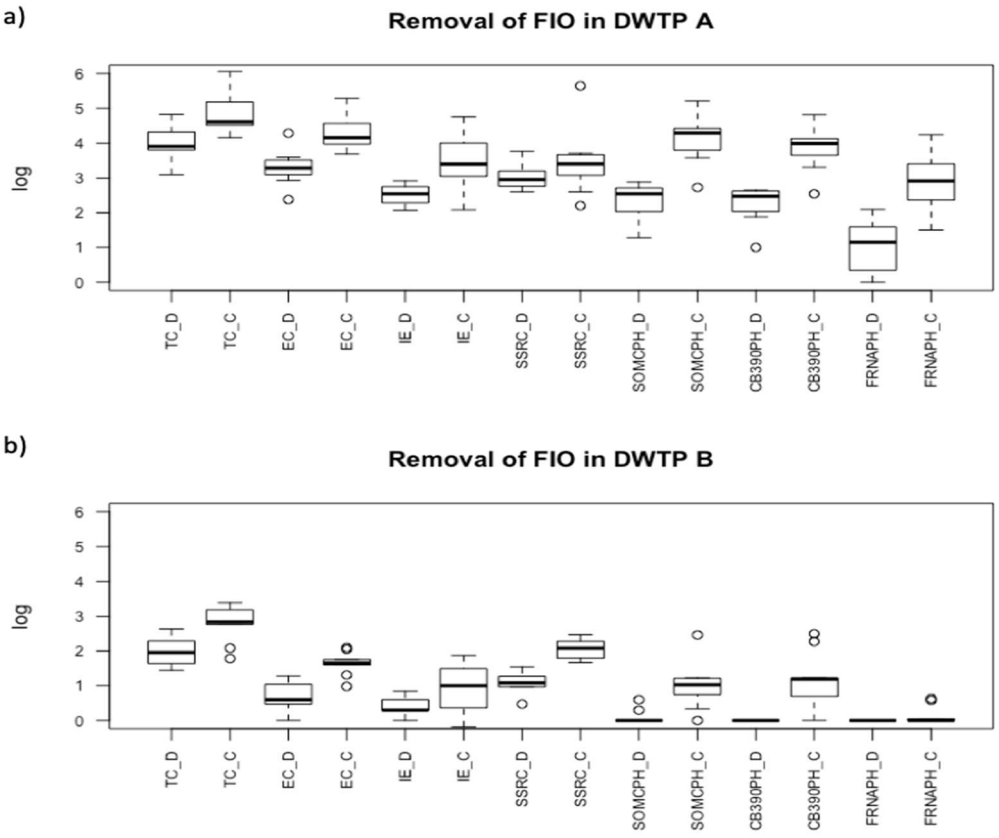
731 the main physicochemical parameters.



732

733 **Fig. 3:** Removal of faecal indicator organisms in direct samples (D) and concentrated samples

734 (C) from the catchment to treated water in a) DWTP A and b) DWTP B.



735

APPENDIX A: Supplementary Data

Fig. A.1: Spearman's correlation of streamflow ((log (Q), in log(m^3/s)) and Turbidity (log (Turbidity), in log (NTU)) in the catchment of DWTP A.

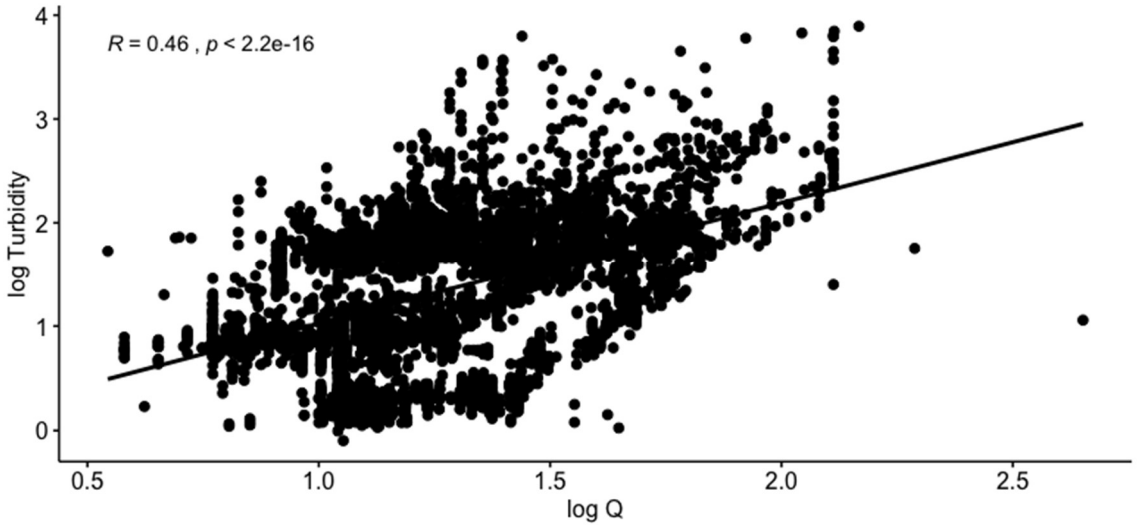


Fig. A.2: Concentration of faecal indicator organisms in a) direct samples from catchment A, b) concentrated samples from catchment A, c) direct samples from catchment B and d) concentrated samples from catchment B.