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1 Determination of the Efficiency of Filtration of Cultures from Microalgae and Bacteria
2 using Hollow Fiber Filters

3 Robla, J.¹, García –Hierro, J.¹, Alguacil, F. J.¹, Dittami, S.M.², Marie, D.³, Villa, E.⁴,
4 Deragon, E.⁴, Guillebault, D.⁴, Mengs, G.⁵, Medlin, L.K.⁶

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6 +

7 ¹ CENIM- Spanish National Research Council (CSIC), Madrid Spain

8 ²Sorbonne Université, CNRS, Integrative Biology of Marine Models (LBI2M), Station
9 Biologique de Roscoff, 29680 Roscoff, France

10 ³Sorbonne Université, CNRS, Adaptation and Diversity in the Marine Environment (ADME),
11 Station Biologique de Roscoff, 29680 Roscoff, France

12 ⁴ Microbia Environnement, Banyuls sur Mer 66650 France

13 ⁵EcotoxiLab, Madrid, Spain

14 ⁶Marine Biological Association of the UK, Plymouth, PL1 2PBUK

15
16 **Abstract**

17 The most important question in sampling is “*Is the sample representative of the target*
18 *population?*” This question is necessary to understand how valid the sample taken is to the
19 original population and if generalizations can be made from the sample. Samples taken for
20 water quality measurement range from 1 mL for bacterial contamination to 100 mL or up to
21 1000 L for protozoan parasites. With larger samples taken, the confidence in detecting rare
22 events increases dramatically. Here we illustrate that hollow fiber filters as routinely used for
23 kidney dialysis can be adapted for environmental use. The filters retain all organisms down to
24 viral particles and organic matter above 70 kDA, the molecular cutoff for urea, one of the waste
25 products removed in kidney dialysis. With these filters, 50 liters of water can be filtered in
26 about 90 minutes. Backflush of the filters recovers viable cells with minimal cell lysis that can
27 be processed downstream for molecular analysis. Highest recovery rates were as high as 89%
28 and 75% for phytoplankton and bacteria, respectively.

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Introduction

33 Sampling is the process by which scientists choose a sub-group from a population to be the
34 object of their study. It is expected that the selected individuals represent the population from
35 which they were sampled [1]. The population size and diversity of a community can only be
36 accurately estimated if a large sample can be collected. Such a large sample minimizes the
37 variance in measures of abundance (see references in [2]). Researchers want to gather
38 information about an entire group of objects, which can be termed the population. However,
39 researchers observe a sample, which is only a part of the population. After studying the sample,
40 the researchers use their findings to make generalizations about the sample, which is extended
41 back to the population.

42 The most important question in sampling is “*Is the sample representative of the target*
43 *population?*” (<http://korbedpsych.com/R06Sample.html>). This question is paramount to
44 understanding population validity, which is the degree to which the results can be generalized
45 from the sample taken to the original population. If the sample is too narrow, then it is likely
46 that the sample and population are not identical and no generalizations can be made. If the
47 population is too broad, then the sample cannot be representative. The sample will be more
48 representative of the target population and more statistically valid if it can be as large as
49 possible. This question can be answered most easily if the sample taken is random and large,
50 which will make it the more representative of the entire population. Choices have to be made
51 because it is not possible to study all groups of organisms in any area. Larger samples may
52 compromise any experimental and quasi-experimental designs because large sized samples
53 cannot be easily manipulated for experimental purposes, e.g., C¹⁴ incubations.

54 Aquatic habitats are difficult to sample because they include a variety of communities. In
55 this study, we focused on the plankton. Plankton is, by definition, composed of drifting or
56 weakly swimming organisms, and consists of a multitude of small microscopic animals, algae
57 and bacteria. Planktonic assemblages are known to be strongly affected by physical and
58 chemical characteristics of water masses from small bays to entire ocean basins [3]. The depth
59 of the mixed layers, which influences nutrient and light levels to control phytoplankton growth
60 and diversity is also important in deciding how a plankton sample should be taken [3]. Thus, the
61 routine method of taking a planktonic sample is either using a net if larger organisms are the
62 target (zooplankton) or a whole water sample if smaller ones are targeted (viz., phytoplankton,
63 bacteria and viruses), the former can sample the entire water column but the latter cannot.
64 Counts cannot be made of the smallest of organisms in a whole water sample with any accuracy
65 in most waters (< 30 mm) unless the sample is concentrated.

66 Concentration of the cells can be made by sedimentation after fixation or centrifugation of
67 the sample. Usually only a one-liter whole water sample is taken but such a small volume
68 cannot address the scale of plankton variation, which is seldom evenly distributed in the water
69 column but can be concentrated in thin layers or be patchy. Although excellent and authoritative
70 manuals on aquatic biological analyses are available [4], their methods and procedures are often
71 diverse because there is usually a different protocol for each organism to be monitored for
72 regulatory purposes. In most detailed protocols targeted to a specific group of organisms, e. g.,
73 bacteria, a specific minimum volume, often 100 mL, is usually required but there are no
74 instructions as to how to take this minimum volume.

75 The US EPA has standardized methods for enumeration of viruses, bacteria and protozoans,
76 which are the common pathogens that have to be monitored for public health
77 (<https://nepis.epa.gov/Exe/ZyPDF.cgi?Dockey=P100WD73.txt> and [https://www.regulations.gov](https://www.regulations.gov/document?D=EPA-HQ-OW-2017-0284-0021)
78 /document?D=EPA-HQ-OW-2017-0284-0021). Sample volumes range from a minimum of 100
79 mL taken for bacteria, followed by nutrient enrichment to determine CFU, 1L upto 1800L for
80 viruses, and 10L for protozoans. For non- cultural bacteria, PCR methods are added. Viral
81 concentration are usually so low in natural waters that sampling of thousands of liters may be
82 necessary, whereas in waste water much smaller volumes can be taken. Whole water samples up
83 to 1000L can be filtered by membrane or hollow fiber filters to concentrate protozoans for
84 detection by microscopy or PCR or viruses. Each method requires the total number of
85 microorganisms be estimated from the sample taken, except for coliform bacteria where only
86 their presence is required. As sample size increases, the estimated number microorganisms per
87 unit volume approaches a normal distribution. A smaller sample is adequate if the number of
88 microorganisms per unit volume is relatively high. A larger sample must be taken if there are
89 relatively few microorganisms per unit volume. Thus how large the sample must be to achieve a
90 normal distribution should be addressed. This concern is eliminated if hollow fiber filters are
91 used because larger volumes can easily be taken.

92 It is the detection of rare events that makes sampling with hollow fiber filters so attractive.
93 These rare events can be tracked over time to determine if the rare organisms move out of the
94 rare biosphere and into the bloom one to cause a health issue or some other ecological concern,
95 such as invasive species.

96 In three EU projects (μ Aqua, Microkit, and SMS) hollow fiber filters were used to sample
97 large volumes of water (30 to 1000L) and all organisms in the sample volume were
98 concentrated by these filters. In each of these projects, samples were taken over one year. It was
99 our intention to sample as large as volume as possible to detect rare events and to follow the
100 population development of our target organisms from their earliest stage to their demise. The
101 filters were backflushed using a solution with surfactants to remove the organisms trapped by

102 the filter to yield a sample with all organisms down to viruses and all organics greater than 70
103 kDa from the original sample concentrated into a one-liter volume. This concentrate was then
104 processed further in downstream analysis in all projects. The downstream analyses included
105 RNA/DNA extractions, FISH and microarray hybridizations from freshwater pathogens and
106 their toxins to marine toxic algae. In each EU project, the various partners validated the field
107 sample using whatever means were available to each institute. Details of all investigations from
108 these projects can be found in [5-13].

109 Prior to these studies, Veolia Environment, who was a partner in μ Aqua, had used hollow
110 fiber filters in their research [14] and it was upon their recommendation that the FX80 model
111 filters from Fresenius were used in the subsequent EU projects. Fresenius sells these filters as
112 kidney dialysis filters (www.freseniusmedicalcare.com) and in this study we demonstrate that
113 these filters are suitable for environmental use with a high recovery of bacterial and
114 phytoplankton target cells. We have performed several experiments with these filters to
115 determine empirically the % recovery of different fractions of the plankton by the hollow fiber
116 filters. The experiments reported here involve 1) determining the optimal filter model for cell
117 recovery, 2) the % recovery of cultures from the concentrate after filtration and 3) automation of
118 the filtration to ensure constant performance of the filtration system.

119

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Material and Methods

121 *How the filter works:* The filtration system employs a disposable polysulfone hollow-fiber
122 ultrafilter coupled to a peristaltic pump. Water is pushed through the hollow fibers and the filter
123 cartridge is capped at the opposing end, forcing water through the pores of the fibers in a dead-
124 end filtration path (Figure 1). Water and particles smaller than the pore size are discarded,
125 whereas microorganisms and organics greater than 70 kDa are retained between the fibers. The
126 microorganisms trapped between the fiber cores are removed by backflushing in the reverse
127 direction (from the outside to the inside of the filter) with an elution buffer pushed by the
128 peristaltic pump and the concentrate is collected in a beaker (Figure 2).

129 The assembly of the peristaltic pump to the filters as well as the opening and the closing of
130 the filter openings to change from filtration to backflush mode were performed manually in
131 each of the three EU projects mentioned above and by Roscoff and Microbia in the experiments
132 presented here. The manual flow rate for the filtration step is 300 mL/min and the backflush rate
133 is 100 mL/min. This was done automatically in the experiments by CENIM and EcoToxiLab.
134 First, a brushless pump (1500 L/ h), pump 1, is turned on. A set of conductions with solenoid
135 valves (electrovalve 1) opens to allow passage of the sample into the filter; organisms/organics
136 are retained in the filter and cell-free or filtered water is discarded through opening 4 of the

137 filter in Fig. 1. The filtered liquid, that consists of water and particles smaller than the 70kDa,
138 drain through the electrovalve 2. Once the “sample” has been filtered, the filtration pump is
139 stopped and the filling and output solenoid valve of the discarded liquid are closed.
140 Backflushing is performed by starting brushless pump 2 (240 L/h) and by opening electrovalves
141 3 and 4. Electrovalve 4 is located at the exit of the backflush tank and electrovalve 3 at the exit
142 of the filter. At this second step, pump 1 is stopped and valves 1 and 2 are closed. All
143 electrovalves and pumps are controlled automatically, with a PC running the Windows
144 operating system and a custom application developed in the TestPoint programming
145 environment.

146 *Efficiency of the Filter Type:* Six different types of hollow fiber cartridges were provided by
147 Fresenius Medical Care (FX100, FX80, FX10, Diasafe, EMIC2 and Fxpaed dialysis filters
148 (Table 1). Five liters of natural seawater spiked with *Alexandrium minutum* (29 cells per
149 mL) were filtered at a flow rate of 300 mL/min. Cell elution was performed in a final volume of
150 0.5 liter of 0.05% TWEEN- 0,1% NaPP elution buffer. Experiments were performed in two to
151 four replicates for each cartridge for a total of 18 independent filtrations A recycling procedure
152 was included between each replicate to avoid any bias in cell enumeration and was performed
153 as follows: (i) a cleaning step using 0.2 N NaOH, a neutralizing step using 0.1% Sodium
154 Bicarbonate solution and (ii) a rinsing step with double distilled water, each step for 30 minutes.
155 Filtration time varied from 1 hour to 5 hours depending on the cartridge type.

156 Cartridge efficiency was individually evaluated by observing cell integrity and estimating
157 cell concentration either by microscopy observation and counts for *A. minutum* cells or by flow
158 cytometry counts for bacterial cells. Recovery percentages with standard deviation were
159 calculated for all tests (Samples = 18; replicates = 3-5). For flow cytometric analyses, 1 mL of
160 formaldehyde fixed (2% final) natural spiked sample was stained with SYBR Green I
161 (Invitrogen - Molecular Probes) at 0.025% (vol/vol) final concentration for 15 min at room
162 temperature in the dark. Counts were performed with the FacsCanto flow cytometer (Becton
163 Dickinson). Stained bacterial cells, excited at 488 nm, were enumerated according to their right
164 angle light scatter (SSC, related to cell size) and green fluorescence measured at 530/30 nm.
165 The bacterial concentration was determined using a flow rate of 16 $\mu\text{L}/\text{min}$. Fluorescent beads
166 (1 μm ; Polysciences Europe) were systematically added to each sample as internal standard. On
167 the dual distribution green versus red fluorescence, photosynthetic cells were distinguished from
168 non-photosynthetic microorganisms. Analyses were performed before and after filtration to
169 calculate cell recovery percentage. Microscopic counts and observation of cell integrity of *A.*
170 *minutum* were performed with a 1 mL aliquot of the pre and post filtration samples. All cultures
171 that were established with backflush inoculum survived (data not shown).

172 *Experiments performed in Roscoff:* Two cultures, *Alexandrium minutum* (RCC1490) and
173 *Rhodomonas* sp. (RCC1507), from the Roscoff Culture Collection, were grown and two liters
174 were transferred into 50 L of sterile seawater. The final concentrations were 41 algal cells and
175 154,000 bacterial cells per mL for the *A. minutum* culture, and 943 algal cells and 596,000
176 bacterial cells per mL for the *Rhodomonas* culture. The 50 L were filtered at the same rates as
177 above with an FX80 filter by manually adjusting the openings of the filters for each filter
178 direction and with a one-liter backflush in a single volume. These two microalgae were chosen
179 because *A. minutum* has a strong cell wall and *Rhodomonas* is a naked flagellate, thus the
180 effects of filtration on the cell integrity could be expected to be more severe on the naked
181 flagellate than the walled species. Counts for both the algal and bacterial replicates were
182 performed with the FacsCantoII flow cytometer (Becton Dickinson).

183 *Experiments performed by CENIM and EcoToxilab:* Two algal cultures, *Scendesmus armatus*
184 (BEA 1402) and *Alexandrium minutum* (Vigo AL1V) were grown and transferred into 5 L of
185 water or sterile seawater for filtration. The final concentrations of these cultures were 123 and
186 43 cells per mL, respectively. One bacterial culture, *E. coli* (OP₅₀), was grown in 50 L broth
187 with SDS (sodium dodecyl sulfate) 20% (w/v) (final concentration 14,000 cells per mL). To test
188 when the majority of cells were eluted from the filter, the *Alexandrium minutum* culture was
189 backflushed initially with 100 mL elution buffer and then with subsequently with 100 mL
190 backflush volumes until a total of one liter backflush was obtained. Cells were counted in each
191 elution fraction. Filtration rates were tested with *E. coli* using four different filtration rates: 1) a
192 high filtration rate = 2500 mL/min and a high backflush rates =120 mL/min and 2) a low
193 filtration rate = 250 mL/min and a low backflush rate = 100 mL/min and combinations of the
194 two. Counts for the algae were made with a haemocytometer after fixation with Lugol's iodine
195 and formaldehyde 2% for the bacteria to be counted, and labelled with Syto 13, 5%, by flow
196 cytometry with a FACSCan flow cytometer (Becton Dickinson).

197

198

Results

199 *Filter Type:* In this experiment we tested if the type of filter used preferentially recovered either
200 of our two target groups that represent two different size fractions in environmental samples
201 (bacteria vs. phytoplankton). Bacterial cell recovery varied from 27% for the EMIC2 and
202 Fxpaed cartridges to 75% for the Diasafe cartridge, whereas phytoplankton cell recovery varied
203 from 6% for the Fxpaed to 37% for the FX100 using the protocol and buffers described in
204 materials and methods (Tables 2, 3). The Diasafe cartridge is not a dialysis filter but a filter
205 designed to remove small target molecules from water to provide ultra pure water for dialysis,
206 hence its efficacy in removing the bacteria to a high degree.

207 *Experiments by Roscoff*: In this experiment, we tested the fragility of the target organism with
208 respect to its recovery rate. More intact cells of *Alexandrium* (with a cell wall) were recovered
209 than *Rhodomonas* (no cell wall) but both of these were higher than those obtained by Microbia
210 (72-89%) (Tables 3, 4). The initial algal cell concentrations in the Roscoff cultures were about
211 double to 10X that of the experiments by Microbia and by ECT/CENIM. The bacteria
212 associated with these cultures were also very well recovered (70-76%).

213 *Experiments performed by CENIM and EcoToxilab*: In these experiments, we tested various
214 combinations of filtration and backflush flow rates and how the recovery of the target cells
215 changed over the duration of the backflush period to determine an optimal combination for the
216 faster filtration with the maximum recovery. An automated prototype has been developed that
217 allows software to control the elution and backwash times, thus controlling the filtered volumes,
218 which allows us to test which elution fraction contained the highest concentration of cells
219 present, or when all cells have been eluted. With the automated filtration system developed by
220 CENIM, we used at first a higher filtration and backflushing rate so that the entire process took
221 less than 10 min to filter 5 L and backflush into 1000 mL. We recovered 71 % of *Scenedesmus*
222 and 65 % of *Alexandrium* cells (Table 4). When we analyzed the concentration of *Alexandrium*
223 in the different filtered fractions we can verify that, in the first 600 mL, 98% of the total number
224 of recovered cells was recovered (Fig. 4).

225 The % recovery with the faster filtration rate by CENIM was lower than that recovered by
226 Roscoff who used a slower filtration rate. Thus, we performed four experiments, filtering *E. coli*
227 with different combinations of the filtration rate and the backflush rate to determine where we
228 were losing cells. These combinations were tested: high filtration and high backflush, low
229 filtration and low backflush rate, high filtration and low backflush, low filtration and high
230 backflush (Table 4). The low filtration and low backflush rate obtained the best recovery rates
231 recovering 72 % (Table 4) as compared to the 34 % using the fast filtration and backflush rate
232 (Table 4). It would appear from our test that the biggest impact on cell recovery occurs with a
233 fast filtration rate, fast backflush rate. The use of a hydraulic pump in the automatic version
234 versus peristaltic impulse pumps in the manual version may have contributed further to cellular
235 deterioration from mechanical forces.

236

Discussion

237 Hollow fiber filters are, most commonly, used to produce ultra pure water or air
238 [www.kuraray.com/products/filter, 15] whereas it is not usually common to target the organisms
239 retained by the filter. The application of hollow fiber filters to concentrate/target organisms
240 retained by the filter has been used prior to our use in our different EU projects [see references
241 in 16] but they have been mainly used in conjunction with tangential filtration where the filtrate

242 is passed several times through the hollow fiber filter, which can be difficult to implement and
243 is not conducive for field sampling. Because we wanted to use the filters to obtain a more
244 representative sample of the environment from higher volume samples, we needed to test the
245 efficiency of the retention of different size fractions of organisms from higher volumes and test
246 the ability to automate the filtration so that the filtration could be done easily and consistently
247 for field implementation.

248 Leskinen et al. [17] used hollow fiber filters from Fresenius to test for the presence of target
249 pathogens by PCR in 300 mL to 10 L of spiked and non-spiked sewage water samples from
250 various sources at various dilutions and eluted into 250 mL. They only reported the
251 presence/absence of their target pathogen in the spiked/non-spiked sample and not the quantity
252 in all tested scenarios. They suggested that future work should focus on detecting these target
253 organisms relevant for public health from higher volume samples. Hill et al. [18] spiked 100 L
254 tap water with high and low seeds of viruses and parasitic protozoans and estimated their %
255 recovery in a 500 mL concentrate. They found that the hollow fiber filter filtration method with
256 Fresenius filters was as effective, if not more effective, than established microbe-specific US
257 EPA methods for concentration of viruses (VIRADEL-OF) and parasites (Method 1623) from
258 tap water. Low seed tests more indicative of natural concentrations were less effective. Morales
259 et al. [19] used hollow fiber ultra filtration for simultaneous recovery of bacterial, viral, and
260 protozoan pathogens from various surface waters with different turbidities and achieved average
261 recoveries of bacteria up to 92%, viruses up to 63%, and protozoans (32%) and it took only 45
262 minutes to filter the 10L from the samples with the highest turbidity. Smith and Hill [16] tested
263 four brands of hollow fiber filters (including older models of Fresenius' filters) with a filtration
264 rate of 2.1L/min for viruses, bacteria and parasitic protozoans spiked into 100 liters of tap water
265 at three different turbidity concentrations (low, medium and high). The former two types of
266 organisms had been resuspended in a PBS/Tween80/antifoan solution prior to spiking.
267 Backflush rates were 650 mL/min into .533 L. The four brands of filters were tested over nine
268 flow rates and required different amounts of pressure to achieve the same flow rate, with
269 Fresenius filters requiring the most pressure. Because REXEED filters required the least amount
270 of pressure they were chosen for their turbidity tests. Recovery rates were highest (94%) when
271 the test organisms were at their lowest concentration, except for the bacteriophage, which was
272 more efficiently recovered at higher densities. Pressure was also at its highest towards the end
273 of the filtration experiment as the filter began to clog. At the flow rates tested, filtration took 60
274 minutes. Frany et al. [20] tested five different filters (REXEED hollow fiber filters not from
275 Fresenius) to test the recovery of viruses, bacteria and parasitic protozoans spiked into 10L lake
276 water and the recovery of natural bacterial populations from 200L of lake water. The maximum
277 recovery of each target organism varied with the type of filter tested. All elution solutions were

278 further centrifuged to determine % recovery. Hollow fiber filters coupled with tangential
279 filtration performed the best. Highest recovery dead end filtration of hollow fiber filters for
280 bacteria/viruses was higher than that of the parasitic protozoans.

281 Our results show that in standard conditions, most of the cartridges were able to concentrate
282 the different types of cells tested from natural seawater more or less efficiently and that
283 recovery rates were sometimes lower for phytoplankton cells than for bacterial cells, but that
284 was likely dependent on the elution volume in our studies. Our recovery rates were directly
285 affected by cartridge specifications and flow rates. The studies mentioned above [16-20] also
286 had a lower % recovery for the larger cells spiked into the sample than the bacterial and viral
287 fractions. High viral % recovery in high concentrations suggests that perhaps the cells are
288 adhering to one another as they are being concentrated or that the surfactant used in the
289 backflush is especially effective in dislodging them from the filter fibers.

290 We assume that the difference in the % recovery in the test between the two algal species
291 with two different cell coverings is because of their relative fragility with *Rhodomonas* being
292 naked is the most fragile and easily ruptured cell, whereas *Alexandrium* has a cell wall or theca.
293 Presumably any rupture of the microalgae would release their DNA/RNA into the filter cavity
294 and these molecules would be of a higher molecular weight than 70 KDa and would be retained
295 by the filters and would be backwashed. We have not tested a total nucleic acid preparation
296 before and after filtration to determine if DNA/RNA has been retained. However, 70 kDa is
297 equivalent to a 1.8kb length of DNA or approximately the length of the SSU rRNA gene so
298 theoretically free DNA and most RNAs would be trapped by the filter if they were not
299 degraded. This needs to be tested empirically to determine if free DNA /RNA can be removed
300 from the filter if cells are ruptured and whether or not it is degraded after release into the
301 medium being filtered.

302 One of the important reasons for using dialysis filters is that they provide a gentle filtration
303 so that cells (blood cells in dialysis) are not ruptured. In a normal membrane filtration, cells
304 can be easily ruptured especially as the filter begins to clog. The DNA/RNA would likely be
305 lost as it passed through the filters. Visual inspection of the filtrate showed cells freely
306 swimming and little evidence of rupture theca. However, if cells did rupture, then all genetic
307 material from the cells that were destroyed by mechanical forces has likely been recovered been
308 recovered in the filtrate because of the 70 kDa cut off size for organic matter, if not degraded.
309 This will allow a more reliable quantification and identification of the content of the sample
310 using molecular tools but this needs to be tested empirically. It will be necessary to analyze
311 further to determine what caused cell loss, although it is presumed to be the rupture of the cells
312 from the mechanical forces of the system or they are retained in some element of the system as
313 yet unidentified.

314 The optimal flow rate for the best % recovery was about 300mL/min, which is a much
315 reduced flow rate as compared to earlier studies [16-20]. Flow rate most affected the percent
316 recovery of the cells. The earlier studies showed that a faster flow rate can recover high
317 percentages of bacteria but may have an adverse effect on recovery of larger cells. However in
318 our study, where phytoplankton was re-inoculated back into culture after filtration, cultures
319 were viable. Our procedure also did not pre-treat the filters with Tween80 as did those studies
320 but we did add this surfactant and the antifoam to the elution buffer, which may help to loosen
321 the cells from the fibers.

322 The cartridge choice could be essential in the environmental applications to specifically
323 target a particular cell type, i.e., bacteria/viruses or phytoplankton. In the tests performed by
324 Microbia, FX100 was the best filter for the phytoplankton and Diasafe was best for the bacteria.
325 However, in contrast to these results, higher % recovery rates were obtained by the three other
326 labs using the FX80 filter, which was the filter recommended by Veolia prior to the start of the
327 first EU project μ Aqua, using initial concentrations higher (Roscoff) and lower (ECT/CENIM).
328 From the results obtained by CENIM and Ecotoxilab, Microbia used half of the backflush
329 volume as did Roscoff and ECT/CENIM and this likely did not expel all of the cells. Their
330 initial inoculum was intermediate between the other two labs, with all other things being equal
331 except the elution volume.

332 The automatic filtration device allows us to standardize the recovery percentages of
333 microorganisms. The filtration and elution speed are decisive in the percentage of bacteria
334 recovered: low filtration and elution rates recovered the highest number of microorganisms. The
335 slower filtration rates require a lower amount of pressure, thus likely reducing the mechanical
336 damage to the cells but increases the time of the filtration. By means of our automated device,
337 recovery can be optimized by varying the filtration parameters of the system. Furthermore, the
338 handling of the sample is reduced to the maximum, also allowing the obtaining of a high
339 number of samples, which is vital if the filtration is to be done in the field. We were able to use
340 the hollow fiber filtration in the field in the EU MicroCokit project when it was necessary to
341 sample four sites along the entire length of the Tiber River, Italy in one day [9]. Automation of
342 the filtering device requires no more elaborate equipment than to use it in a manual format and
343 should be amenable to field application. We plan to construct the automated version of the
344 hollow fiber filter into a portable box for easy field application. Both our manual and automated
345 filtrations methods used a peristaltic pump and not a tangential flow centrifuge, making it easier
346 to use under laboratory conditions and certainly in field conditions.

347 We are continually trying other times and rates of filtration to maximize the yield but even
348 with a yield of up to 90%, we believe that we are obtaining a sample that is highly
349 representative of the population that was the object of our study. Our best recovery rates

350 exceeded or equaled the recovery rates in the previous studies of environmental samples using
351 Fresenius filters especially for cells in the larger cell fraction [16-20], except for viruses, which
352 we did not test here. Also, we did not recirculate the concentrate and further concentrate the
353 sample by centrifugation, as did the previous studies. There was no need to concentrate by
354 centrifugation for cell counting in our experiments because we measured all cells by flow
355 cytometry before and after each experiment, which is one of the most accurate means of
356 counting cells. Thus, our recovery rates are likely to be more accurate than these in the previous
357 studies as judged by the SD in their percent recovery, which were twice that of our studies.

358 We note that in Roscoff the samples were inoculated into 50L seawater, whereas in both the
359 Microbia and ECT/CENIM samples were inoculated into 5 L. It is possible that the more dilute
360 the original sample is, the more efficient the filters are at concentrating the cells. Roscoff also
361 had the highest initial inoculum. Microbia had a lower initial inoculum but not as low as
362 ECT/CENIM so the elution volume is likely the most critical part of their filtration protocol.
363 These experiments have been done with pure cultures and although the starting cultures were in
364 exponential growth when harvested, the condition of the culture could be an artifact. The next
365 step will be performed controlled experiments on environmental samples whose cell numbers
366 have been verified by a counting, such as flow cytometry.

367 Filters were cleaned and reused up to five times in the EU projects because of their relatively
368 high cost but this was not empirically tested in these projects. Depending on the EU project and
369 the country in which the filters were bought in the EU projects, their cost ranged from 15 to 65€
370 because each country provides a different subsidy for dialysis. The filters were ordered directly
371 from Fresenius and the cost for the filters to each project reflected the subsidy rate for the
372 country in which the filter was ordered. There was a high SD among the bacterial tests
373 performed by Microbia when testing the various filters (Table 2). In their studies, the filters
374 were reused between replicates and this may have had an effect on the% recovery of the
375 bacteria. At present, dialysis filters are not routinely for sale for non-medical use; however,
376 Fresenius is diversifying into non-medical use and is using their knowledge and experience in
377 hollow fiber fabrication to redesign filters for non-renal use with a nominal pore size of 5.5 nm
378 (www.inuvai.com).

379 Organic matter above 70 kDA is retained by the filter and several applications may shed
380 some light on the results obtained by the retention of free organic matter. In the two toxin
381 analyses done using the elution from the filters [7, 10], better resolution of the toxins was
382 obtained with the elution than from samples taken from the unfiltered initial sample, which
383 would suggest that any toxins already liberated in the water were concentrated and flushed from
384 the filter. Scientists on the Tara Expedition have used the FX80 filters. We are awaiting results
385 from the genetic diversity obtained by Millipore filters of smaller volumes vs the genetic

386 diversity obtained from the concentrate eluted from a 50-liter sample (Chris Bowler pers.
387 comm.). In the samples obtained by Millipore filters any eDNA will likely have been lost
388 through the filters but any eDNA trapped by the hollow fiber filters, not degraded, will have
389 been retained. In the three EU projects that used these filters, waters were taken from very
390 different sources, ranging from rivers to reservoirs and from freshwater to marine samples and
391 total cell numbers were extrapolated from the microarray signals and results were verified
392 where possible [5-14]. These are the only studies that we are aware of in which the hollow fiber
393 filters were used on environmental samples that were not experimental samples to test matrix
394 effects. In those few cases in these studies, where discrepancies between the inferred microarray
395 cell counts and the cell counts obtained by microscopy were found, volume differences (50L vs.
396 10-15 mL) were attributed to be the cause.

397 We have tested several attributes of the filters, such as flow rate, size of cells filtered, %
398 recovery over the time the sample is eluted. Although we tried to standardize the filtration rates
399 and the organisms filtered across the labs that tested the filters, the power of the peristaltic
400 pump may affect the length of time taken to filter the sample. Certainly the volume of the
401 backflush is critical to a higher % recovery. It does not seem possible to scale down from the
402 volume filtered to a comparable backflush volume without leaving cells in the filters. Our
403 maximum recovery of the algal cells was 89% +/- 14% SD, so it is possible to have a very high
404 recovery rate from the organisms in a 50-liter sample with a one-liter backflush, which for
405 larger cells is the highest reported to date. As little as fifteen mLs from the one L backflush was
406 sufficient to provide high and good quality RNA for microarray hybridization [9].

407

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Conclusions

409 We have shown here that dead end hollow fiber filters used as normal kidney dialysis filters can
410 effectively filter large quantities of water in a relatively short period of time. The filters do not
411 clog and can be backflushed for further downstream analysis, be it cell isolation or nucleic acid
412 extractions. The filters have been successfully used in three EU projects. The routine use of
413 these filters in environmental sampling will enable researchers to take a larger sample volume
414 that is more representative of the population they are studying, especially if a low flow rate is
415 used that ensures all cell sizes are recovered equally well. Other workers have shown that these
416 filters perform as well or better than USEPA methods.

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427 Author contribution: Robla, J. conceived the automated scheme for the filtration unit, performed
428 the experiments for CEMIN; García –Hierro, J. built the automated prototype; Alguacil, F. J.
429 wrote the software to control the automation; Mengs, G. performed and analyzed the
430 experiments for EcotoxiLab and CENIM; Dittami, S.M. performed and analyzed the
431 experiments for Roscoff, Marie, D. performed the flow cytometry for Roscoff. Villa, E.
432 performed the experiments for Microbia, Guillebault, D. analyzed the experiments for Microbia;
433 Deragon, E. performed the experiments for Microbia; Medlin, L.K. obtained the funding for the
434 project, conceived the idea to automate the filtration, wrote the manuscript.

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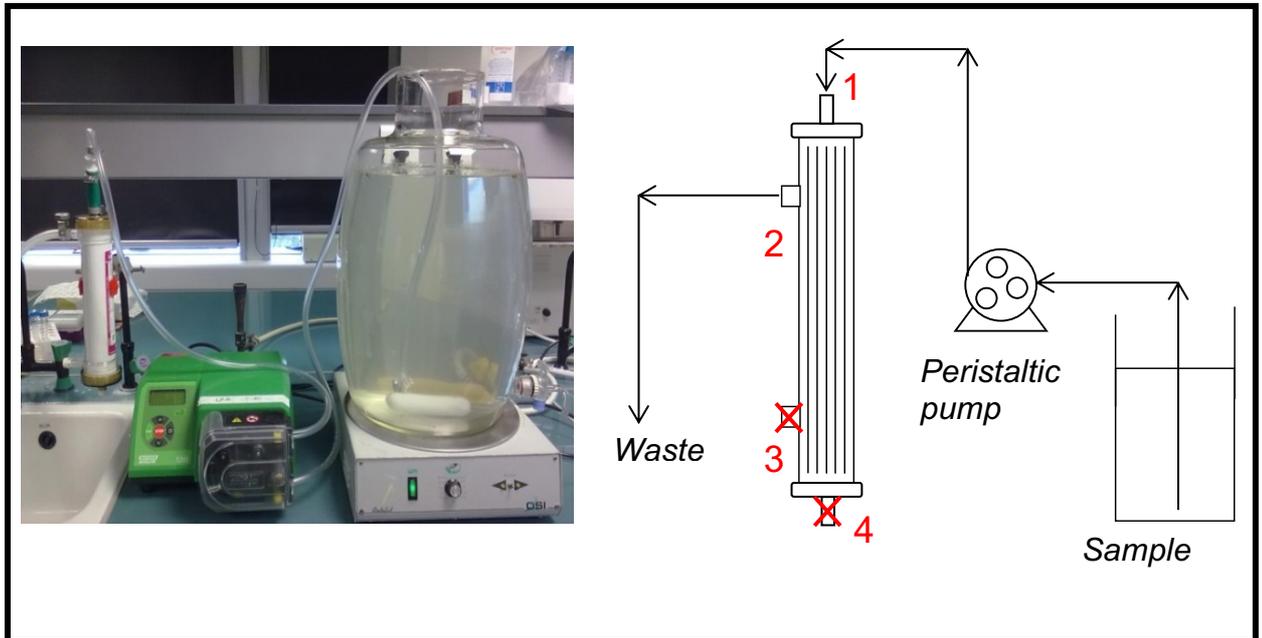
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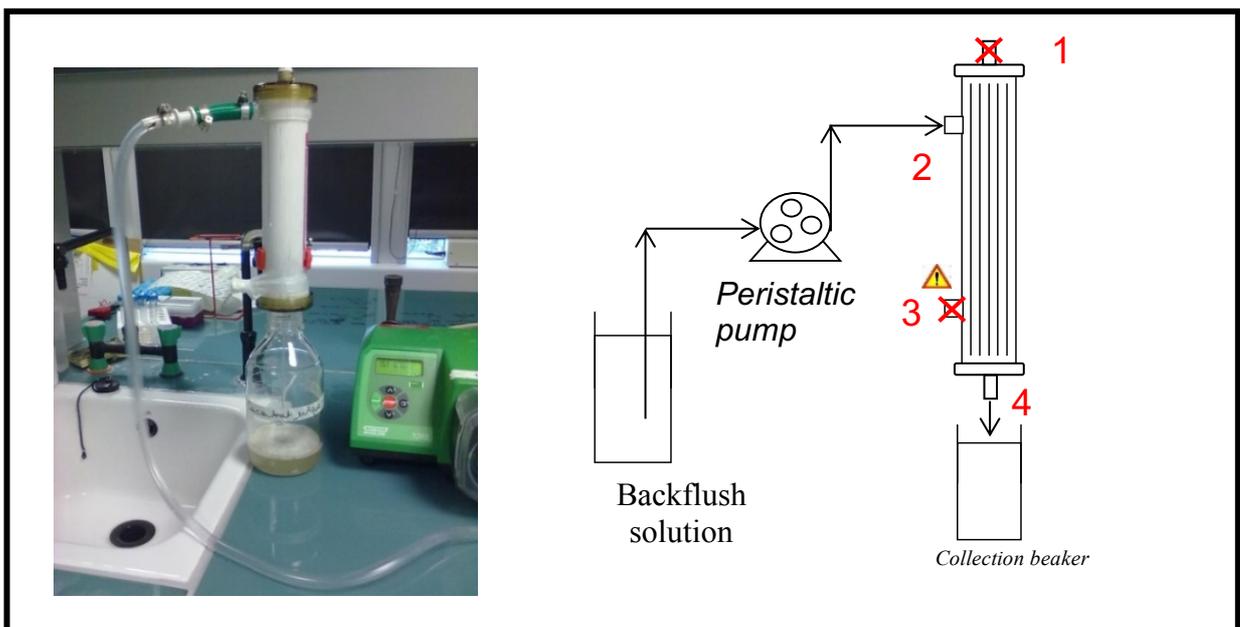
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500 Figure 1. Setup of the filter and its tubing for manual sample filtration. Ports 3 and 4 are sealed
501 for filtration.

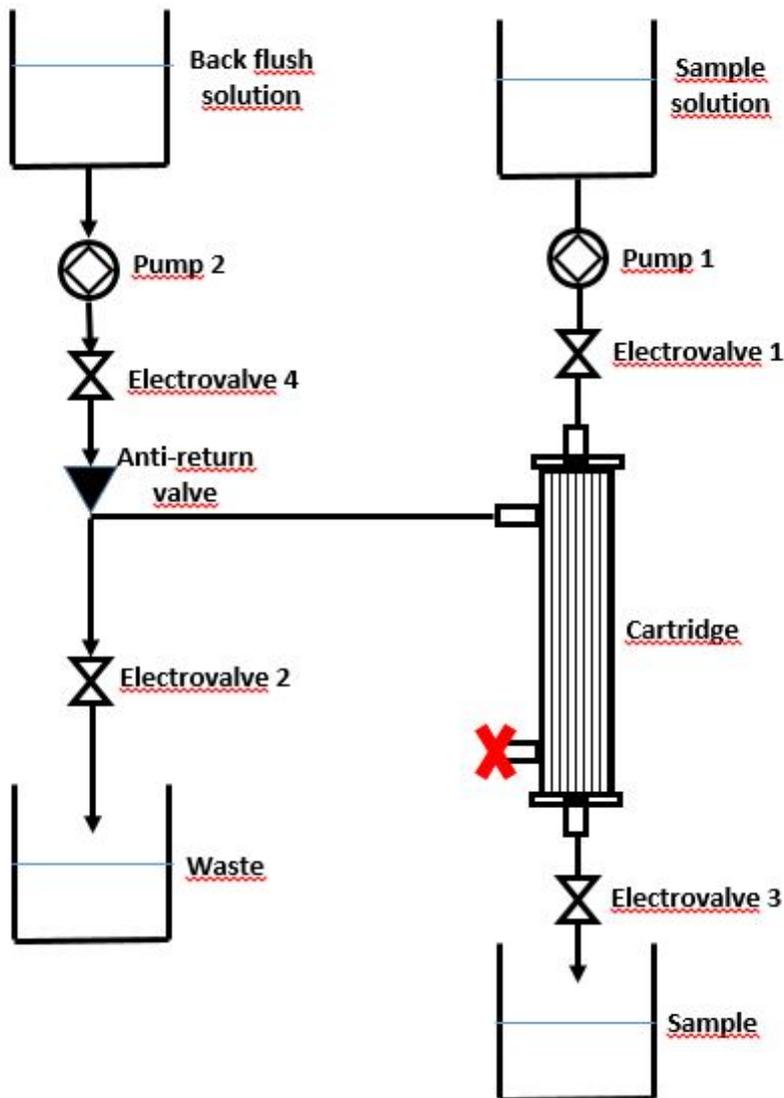


514 Figure 2. Setup of the filter and its tubing for manual backwash and collection of the
515 concentrated sample. Ports 1 and 3 are sealed for sample collection.



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528 Fig. 3. Diagram of the automation of the hollow fiber filters.



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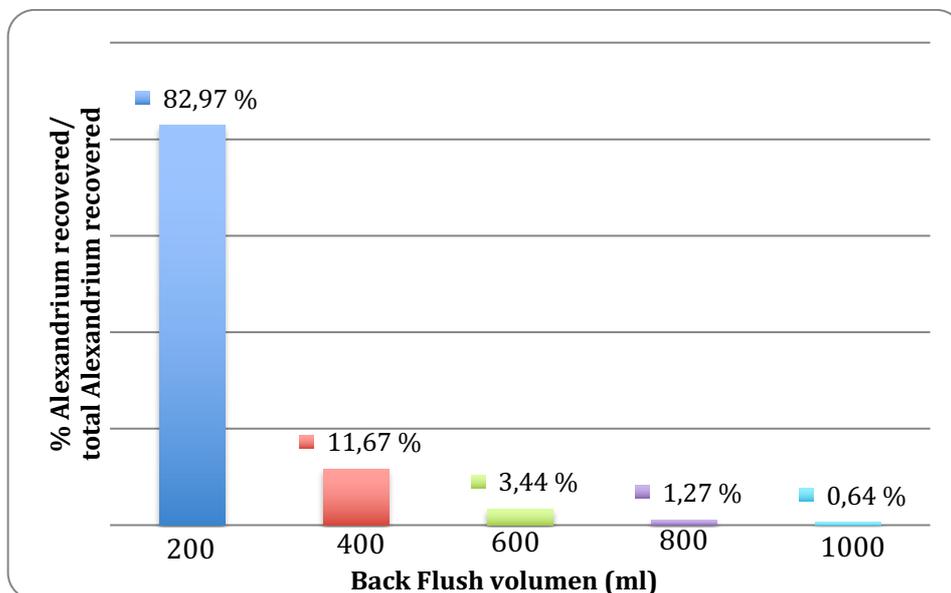
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539 Fig. 4. Comparison of the % recovery of *Alexandrium minutum* with subsequent volumes of
540 backflush until a total of 1 L is recovered by CENIM and Ecotoxilab.

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