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Running title: FCM for counting benthic prokaryotes

Abstract

Flow cytometry offers an easy and powerful way to assess multi-parametric data in different domains, notably in the environmental sciences. Because evaluating heterotrophic prokaryotic abundance is crucial to understand an ecosystem's functioning, we propose a quick and efficient protocol for 1) cell's detachment in muddy coastal sediments followed by 2) enumeration of prokaryotes by flow cytometry compared to epifluorescence microscopy and 3) a type of storage adapted for benthic samples. First, samples preparation by incubation in a detergent mix containing sodium pyrophosphate (0.01 M final concentration) and Tween 80 (0.1% final concentration) drastically increased cell detachment from sediment particles (+130.40 %) compared to extraction with sodium pyrophosphate only. Cell sorting allowed to control the efficiency of the extraction as few cells were found attached to sediment particles in epifluorescence microscopy after sorting. Flow cytometry gave consistent results with strong reliability by counting 1.81 times more cells compared to epifluorescence microscopy. Thirdly, results revealed that sediment samples fixed with formaldehyde and then liquid-N_2 frozen and directly stored at -80°C can be analysed within 3 months. In routine, our method of extraction and counting allowed to evaluate 83.67% of the real abundance in a sediment sample. Finally, this optimized technique was applied on sandy and muddy coastal and freshwater sediments and allowed us to prove the high efficiency of this new method. Flow cytometry is a fast, replicable and low-cost method for counting heterotrophic prokaryotes, even for sediment samples. The two-step method that we developed enables high frequency analyses (30 samples in less than 4 hours).

Keywords : prokaryotes; cell enumeration; flow cytometry; coastal sediments

1 Introduction

Microorganisms dominate marine ecosystems (DeLong et al., 2006) and were often considered as a "Black Box" by scientists (Fuhrman et al., 2002). To investigate this "Black Box", many studies have focused on new technologies and advances in molecular biology methods, allowing researchers to acquire a huge quantity of phylogenetic and potential physiologic information from oceans and marine coastal ecosystems. But these revolutionary "omics" data need to be completed by single-cell analysis, metabolic studies, and basic determination of prokaryotic abundances. Prokaryotes (Bacteria and Archaea) are key organisms in biogeochemical cycles in all marine environments (Azam and Malfatti, 2007, DeLong, 2009) and the first step to study these communities is to estimate their abundance within the microbial assemblage. Estimations of abundance showed that prokaryotes are as abundant as 10^6 cells.mL^{-1} in ocean water (Whitman et al., 1998) and more than 10^8 cells.mL^{-1} in marine surface sediments (Jorgensen and Boetius, 2007). In sediments, prokaryotes play a crucial role in the food web as they remineralize organic matter and lead major cycles such as nitrogen and carbon cycles. Most specifically, heterotrophic prokaryotes are a majority throughout the whole sediment depth and can be present until almost 2 km below the surface (Ciobanu et al., 2014).

The first advances in heterotrophic prokaryotic enumeration were made using epifluorescence microscopy (EFM) (Porter and Feig, 1980). Fluorochromes combined with EFM have been used to develop standardized methods to successfully count bacteria in freshwater and marine water columns (Daley, 1979). The most widespread way of staining cells is to target DNA with a fluorescent dye such as DAPI (4', 6-diamidino-2-phenylindole), currently used in microscopy (Porter and Feig, 1980). Montagna (1982) showed under Acridine Orange (AO)-EFM observation that bacteria in muddy sediments occur at levels two orders of magnitude greater than in sandy sediments. Nowadays, EFM is still the most

widespread technique for estimating the abundance of prokaryotes (see Supplementary Information Table 1 for references). Nevertheless, Robertson and Button (1989) were the first to use flow cytometry (FCM) to enumerate heterotrophic prokaryotic cells by DAPI-staining in marine and freshwater samples. Even if FCM seemed to be an accurate and rapid method for determining heterotrophic prokaryotic cells, advances were needed in storage conditions or fixative effects on benthic samples. These fixatives were known to permeate cells (Troussellier et al., 1995); consequently, the interactions between dyes and fixatives needed to be taken into account when choosing dyes. During the exponential phase of FCM utilization for environmental marine samples, many dyes have been reported in the literature such as DAPI, Hoechst 33342, TO-PRO-1, SYBRGreen (I or II), SYTO13 etc… (details are reviewed in Gasol and Del Giorgio (2000)). With technological advances, FCM became more and more useful in marine microbiology and offered new challenges to scientists, such as the prokaryotic enumeration in soils and sediments and the use of specific probes (Fluorescence in situ hybridization - FISH) (Llobet-Brossa et al., 1998). For sediment and soil analysis, the dye mostly used to stain DNA is SYBRGreen I (Kallmeyer et al., 2008), and many authors fixed cells with formaldehyde (Epstein and Rossel, 1995) preferentially, but the best temperature for long time storage is still unclear.

FCM is now widely used for water column samples, but sediment samples carry the difficulties of a solid matrix rich in detritus, minerals and exopolymeric substances (EPS). Indeed, sediments are particularly hard to study because dyes (e.g. AO or DAPI) can produce a high fluorescence background with clay and silt-rich sediments containing a high quantity of detritus and EPS (Kuwae and Hosokawa, 1999). Additionally, in such environments, prokaryotic cells are often attached to sediment particles by EPS (Decho, 2000), creating a complex with organic and mineral particles (Epstein and Rossel, 1995, Kallmeyer, et al., 2008). The point of divergence between microbiologists remains the separation method to

92 detach cells from the solid matrix. In order to improve counting yield in sediment, chemical
93 dispersion and physical detachment should be applied. Physical detachment can be achieved
94 by isoelectric method (Jaspers and Overmann, 1997), capillary electrophoresis
95 (Schneiderheinze et al., 2000), sonication bath (Duhamel and Jacquet, 2006, Ellery and
96 Schleyer, 1984, Gasol, 1993), or probes (Albright et al., 1986, Epstein and Rossel, 1995,
97 Kallmeyer, et al., 2008), vortexing (Frischer et al., 2000, Whiteley et al., 2003), or by blender
98 homogenization (Lindahl and Bakken, 1995, Maron et al., 2006, Yamamoto and Lopez,
99 1985). The sonication probes appear to be the best way to mechanically detach prokaryotic
100 cells from sediment particles, and applying it with an intensity of 60W for 30 seconds has
101 been shown to be a good compromise between high counting yield and avoiding lysing cells
102 (Garet, 1996, Lei et al., 2010). Concerning the chemical dispersion solution, the most cited is
103 the sodium pyrophosphate (NaPp), which is often found in detergent solutions in combination
104 with Tween 20 (Amalfitano and Puddu, 2009) or Tween 80 (Duhamel and Jacquet, 2006,
105 Epstein and Rossel, 1995), the phosphate buffer saline (PBS) solution (Barra Caracciolo et al.,
106 2005), the sodium chloride (Fazi et al., 2005), or methanol (Kallmeyer, et al., 2008, Lunau et
107 al., 2005). Moreover, it is possible to apply a density gradient (Kallmeyer, et al., 2008,
108 Morono et al., 2013) after the chemical separation in order to improve the time and reliability
109 of the counting (Fazi, et al., 2005). However, in the literature, it remains unclear which best
110 dilution and detergent mix need to be applied to sediment samples in order to detach the
111 maximum of aggregates and cells adsorbed on particles.

112 Nowadays, no simple and standardize method existed to study microorganisms in
113 different type of sediments. On that basis, this study aims at optimizing sediments fixation
114 and storage, cells separation and comparing two analysis methods (EFM and FCM) to count
115 benthic heterotrophic prokaryotes.

2 Materials and Procedures

2.1 Sample collection, fixation and storage

Muddy sediments from the French Atlantic coast were sampled in the Moëze Bay and the Aiguillon Bay from the surface to 10 cm-deep. The sediment samples were collected in 2012 and 2013 at low tide using cores (15 cm diameter). Back in the laboratory (less than one hour), the sediments were homogenized, and sub-samples were put in containers using sterile 50 mL syringes with cutoff tips. Finally, subsamples were fixed with 0.2 µm-filtered formaldehyde solution (vol/vol, 2% final concentration) and kept according two conditions: at +4°C in the dark or frozen in liquid-N_2 directly followed by storage at -80°C. Different storage times were tested on five different muddy samples in duplicates: 1 week, 1 month, 3 months, and 6 months after sampling.

2.2 Protocol development

Here, we described the proceedings of the protocol development. Different steps of the procedure were investigated in order to improve the enumeration of prokaryotes in sediment: 1) sample preparation before mechanical extraction; 2) utility of centrifugation to remove sediment particles; 3) repetitive steps of extraction to improve cell counting yield.

2.2.1 Sample preparation before mechanical extraction

First tests were achieved by preparing sediment slurries with a solution of NaPp 0.01M (Pascal et al., 2009, Tso and Taghon, 1997). Then, in order to disaggregate the attached cells, Tween 80 (0.1% final concentration) was added to the NaPp solution (0.01 M). Indeed, Tween 80 is a non-ionic surfactant known to decrease particle aggregates and to enhance detachment of cells from particles in sediment samples (Velji and Albright, 1986, Yoon and Rosson, 1990). The influence of Tween 80 addition on the cell recovery efficiency was

evaluated on 55 samples. According to preliminary tests, using NaPp solution or NaPp+Tween 80 mixture, slurries were prepared by processing to successive dilutions until 1:2,000 (1:10; 1:100; 1:500; 1:1,000 and 1:2,000) to reduce sediment background (dos Santos Furtado and Casper, 2000, Duhamel and Jacquet, 2006). A vortexing step of 5 sec was applied before and after each successive dilution.

According to Epstein and Rossel (1995) and Velji and Albright (1986) recommendations, 30 minutes of incubation at +4°C was used to detach cells from sediment particles. Then, mechanical extraction consisted of sonicating samples 30 s at 60W in ice with a sonicator probe (Branson, SLPE-150, 1/8" or 3 mm microtip, 40KHz) (Lei, et al., 2010).

2.2.2 Utility of centrifugation to remove sediment particles

The impact of a centrifugation step was tested by applying or not a low speed centrifugation. A first part of extracted samples was centrifuged at 1 000 g at +4°C during 1 min, after which supernatant was transferred and then stained for EFM and FCM analysis (see below for staining settings). On the second part of extracted samples, a proportion was mixed, transferred and then stained for EFM and FCM analysis and centrifugation was applied on the remaining proportion to collect sediment and attached cells for a second extraction step.

In any case, after centrifugation step, the remaining supernatant was discarded and the pellet was re-suspended in the detergent mix [0.01 M NaPp and Tween 80 (0.1% final conc.)] with the same volume of supernatant. By this way, the cells remaining attached to the sediment particles in the pellet can be detached and counted in a second step.

2.2.3 Repetitive steps of extraction to improve cell counting

Because cells can be still attached to sediment particles even after the first extraction, we evaluated the number of repetitive extraction steps needed to improve cell counting yield. The second step was processed as the first one, by incubating the samples in the detergent mix

163 [0.01 M NaPp and Tween 80 (0.1% final conc.)] for 30 min at +4°C. Then, sonication was
164 repeated (same settings as above) before the analysis.

165 Aiming at evaluating how many steps were needed, extraction process was repeated
166 until cells recovery reaches a plateau on 15 different samples. Thus, the total prokaryotic cell
167 abundance corresponded to the sum of all the counting values obtained in each extraction
168 step.

169 *2.3 Microscopic count*

170 Extracted samples were stained with 4',6-diamidino-2-phenylindole (DAPI, 250
171 µg.ml^{-1}, 15 min, +4°C) and filtered through black polycarbonate membrane (0.2 µm pore size,
172 25 mm, Nucleopore) (Porter and Feig, 1980). Next, filters were mounted on slides using anti-
173 fading oil type F (Olympus, Japan), and conserved at -20°C until counting. Finally, counts
174 were made with an epifluorescence microscope (Axioskop2, Carl Zeiss Microscopy, LLC,
175 United-States) at 1,000 x magnification under UV excitation (Filter set 01, 397 nm – Zeiss).
176 For each sample, a minimum of 20 fields (> 600 cells) were counted and averaged (Lebaron
177 et al., 1994).

178 *2.4 Flow cytometric and cell sorting analysis*

179 For each extraction step, the flow cytometric analysis consisted of SYBRGreen I-
180 stained (1:10,000 final concentration) extracted sample during 15 min in the dark at room
181 temperature. Fluorescent beads (Fluoresbrite Multi fluorescent microsphere 1.0 µm,
182 Polysciences, Germany) were added simultaneously to each sample in order to analyze cell
183 fluorescence and scatter properties of samples. Each sample was analyzed for 30 s at low flow
184 speed with FacsCanto II cytometer (3-laser, 8-color (4-2-2), BD Biosciences) equipped with a
185 20-mW 488-nm coherent sapphire solid state blue laser. Data were acquired using DIVA
186 software provided by BD-Biosciences.

187 A cell sorter (FACSAria BF-Biosciences) was used to control extraction yield on
188 prokaryotic population. Then, sorted fraction were observed using EFM (BX300, Olympus)
189 to take pictures.

190 Stained cells were discriminated according to green fluorescence (FL1) from
191 SYBRGreen staining and side scatter properties (SSC). Picophytoplanktonic cells are also
192 discriminated based on their red fluorescence (FL3) and SSC properties and excluded from
193 final prokaryotic counts measured on a gate SSC-FL1 (Marie et al., 2001).

194 Accurate cell concentrations were performed using TruCount beads from BD-
195 Biosciences (excitation: red laser 633 nm; emission: FL5 660/20 nm).

196 *2.5 Statistical analysis*

197 All statistical analysis was performed using R software (R core Team, 2013). The
198 effect of the addition of Tween 80 was tested with a Paired t-test. The influence of the
199 parameters tested or percentage of sand in samples was evaluated by applying a Kruskal-
200 Wallis rank sum test for one sample and Wilcoxon test for two paired samples on cell
201 abundance values. The relationship between FCM and EFM was shown by fitting a
202 significant linear regression. Effects of storage conditions were tested with a 2-way analysis
203 of variance (ANOVA), residuals were tested for application validation and a TukeyHSD post-
204 hoc test was used.

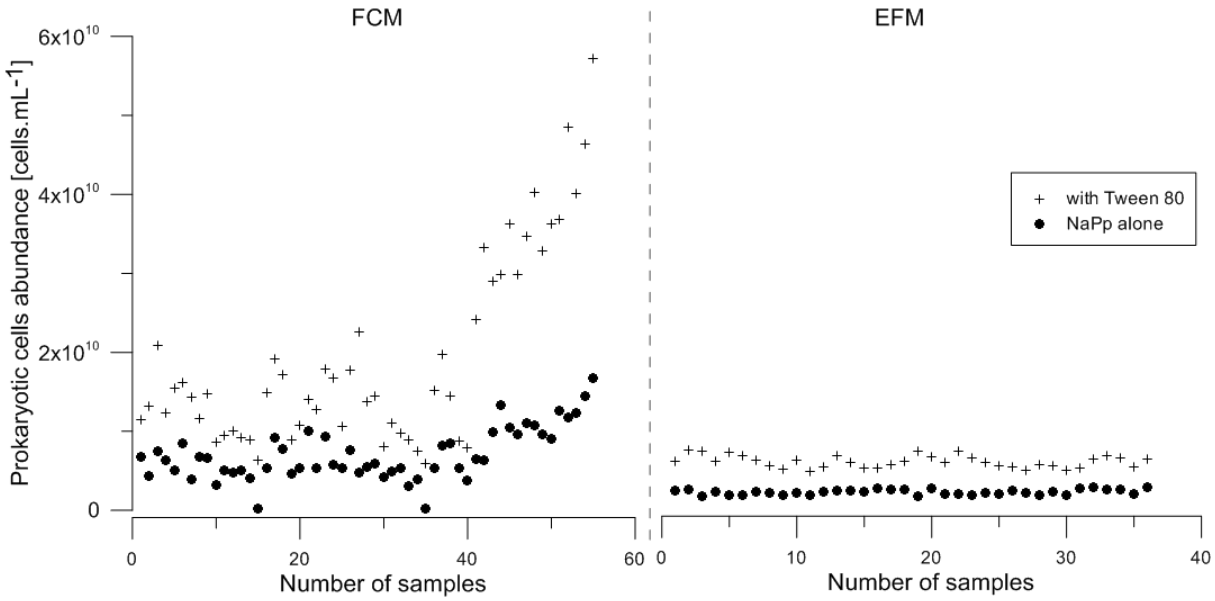
3 Results

Hereby, we described the results of the protocol development proceedings and then the final protocol that we succeed to establish.

3.1 Sample preparation

First, slurries were prepared with NaPp 0.01M alone and cell sorting flow cytometry followed by EFM observations were used to visualize the extracted populations. Two populations were observed: free prokaryotic cells population and attached-prokaryotic cells population. The NaPp alone at 0.01 M was apparently not efficient enough to separate cells from sediment particles, because attached prokaryotic cells were still present in the samples and represented 27.7% of the total abundance. On this basis, the effect of adding Tween 80 was evaluated to minimize cell aggregation (Yoon and Rosson, 1990). Both FCM and EFM counting revealed a higher cell counting yield (Fig. 1), with an increase of 130.40 % ± 12.49 SE and 176.79 % ± 14.25 SE respectively, compared to the treatment without Tween 80. Adding Tween 80 to NaPp in the mixture significantly improve the number of cells counted in FCM (Paired t-test: t = -9.6127, df = 54, *p*-value <0.001) and EFM (Paired t-test: t = -27.1056, df = 35, *p*-value <0.001). After two repetitive steps, cell recovery efficiency rose from 43.3 % ± 2.0 SE without Tween to 92.5% ± 2.0 SE with addition of Tween 80. Moreover, microscopic analysis on sorted populations confirmed that the counted cells were free cells, clearly separated from sediment particles when Tween 80 was added to the mixture. Consequently, it appears that Tween 80 disaggregated efficiently benthic cells and therefore improved the cell counting results (Fig. 1). The recommended protocol is thus to use sodium pyrophosphate (NaPp) and Tween 80 treatment to prepare sediment samples for heterotrophic prokaryotes enumeration by FCM (Fig. 5).

228



229

230 **Fig. 1.** Effects of the addition of Tween 80 to sodium pyrophosphate (NaPp) on prokaryotic
231 cell abundance in sediment samples (FCM counts from SYBRGreen: n = 55; EFM counts
232 from DAPI: n= 36). Values for all the samples tested are presented. Crosses represented
233 samples extracted with Tween 80 and NaPp, and circles represented samples extracted with
234 NaPp alone.

235

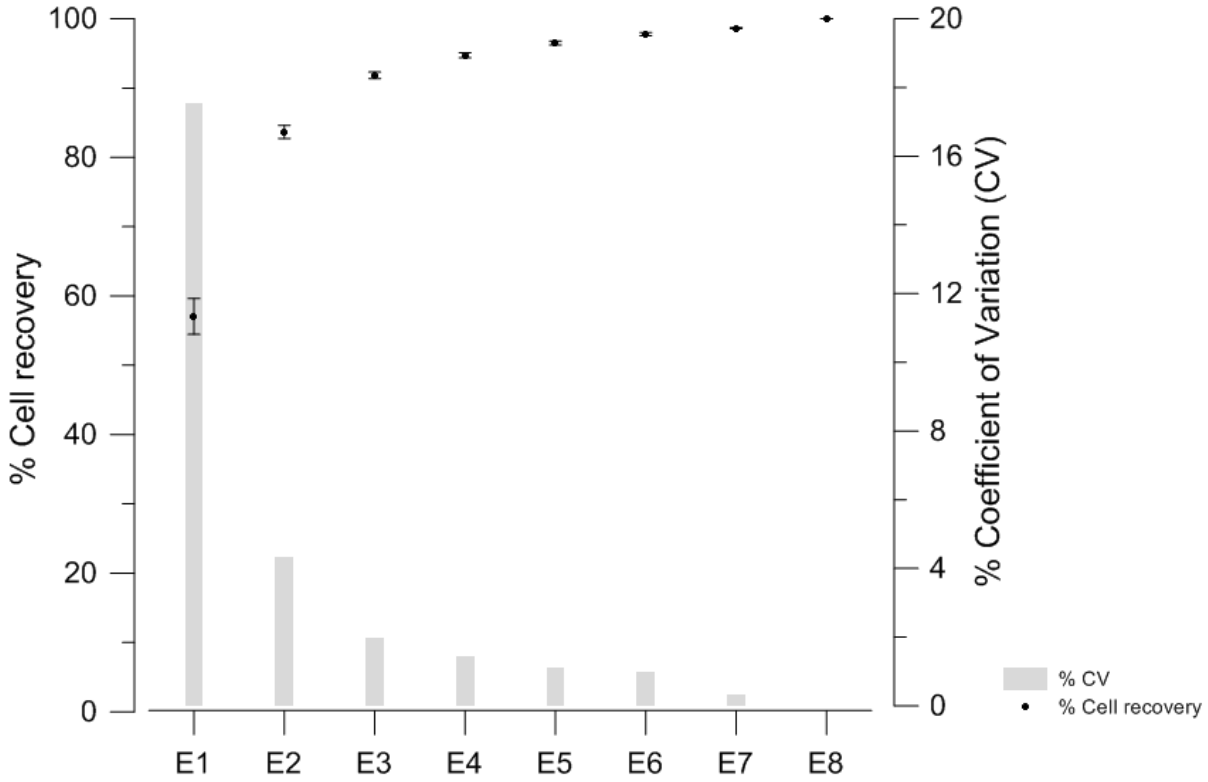
2-column	fitting

3.2 Utility of centrifugation

The utility of a centrifugation step before counting analysis was tested by performing an experiment with and without low speed centrifugation (1,000 g, 1 min, +4°C). Experiments showed that centrifugation moderately decreased cell counts (-1.71%) in FCM and EFM because cells that remained attached to the particles were removed (data not shown). Settling velocities of sands have been calculated according to Soulsby (1997), and we evaluated that the settling time of particles is inferior to the standing step applying on cytometer to acquire data before recording. Indeed, particles of 62 µm will take 4.8 seconds to settle in the tube and the cytometer takes 5 seconds to acquire and 10 seconds to record. The particles cannot collapse the flow cell chamber. Thus, we proposed to analyse samples without the centrifugation step and then to centrifuge samples afterward to proceed to a second cells extraction on the pellet.

3.3 Repetitive steps of extraction

After the first extraction, 57.04 % ± 2.58 SE of cells were extracted and counted. The cumulative cell recovery increased strongly and reached a plateau after the fourth extraction (Fig. 3), showing that in routine analysis it will not be necessary to do more than four extractions. The coefficient of variation of the first extraction was the highest, reaching 17.52 %. The strongest decrease in CV was observed between the first and the second extraction (Fig. 2) showing that the second extraction allowed counting a higher number of cells (83.67% ± 0.94 SE) with a lower imprecision (CV < 5%). After that, the CV continued to decrease with lower range values. Doing eight extractions can be time consuming and expensive. In our case, with sediment samples, eight extractions were not necessary. Thus, for routine analysis of benthic samples by FCM, we propose a 2-step extraction as a good compromise among 1) cell recovery efficiency (83.67% ± 0.94 SE) and accuracy (CV = 4.34); and 2) analysis time (4 hours for 30 samples) and cost.



261

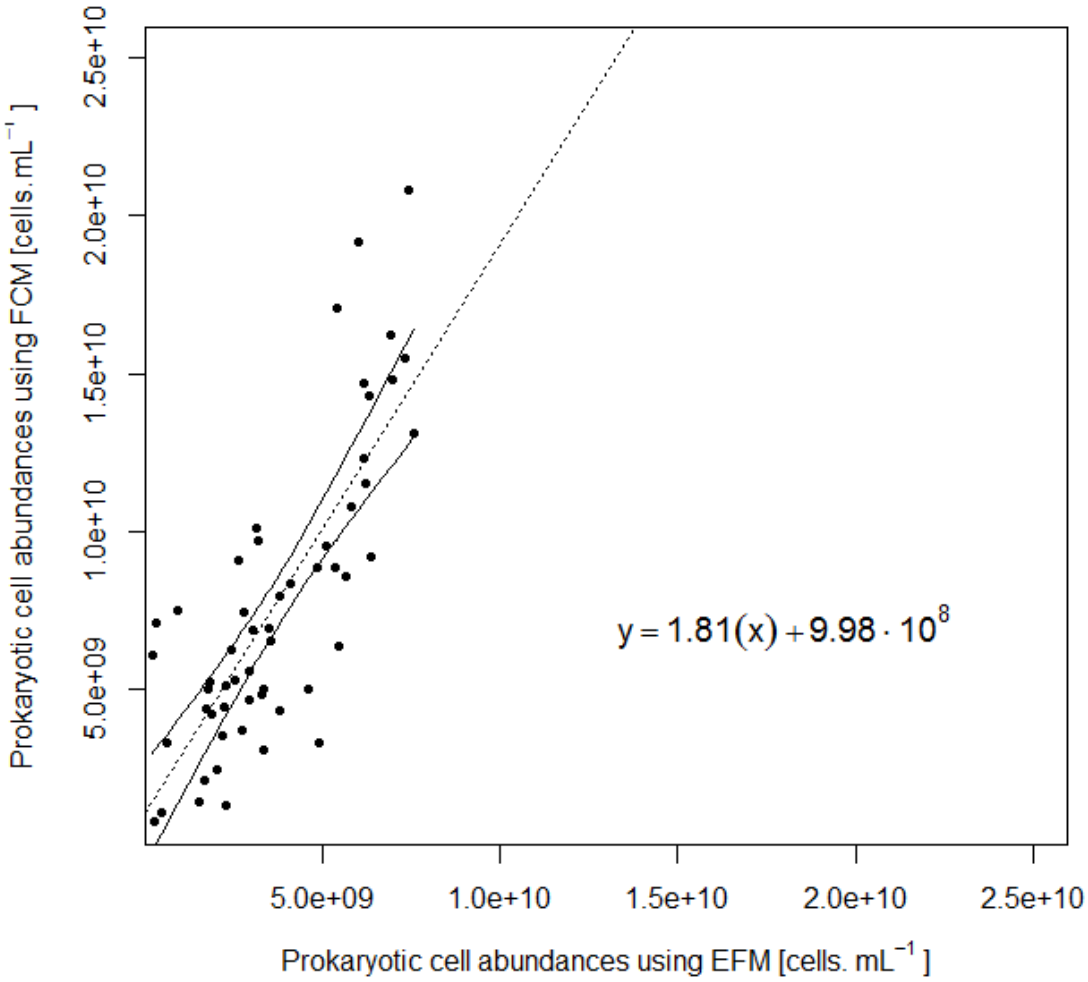
262 **Fig. 2.** Cumulative percentage of cell recovery using the final extraction protocol (extended to
263 eight extractions), counting by flow cytometry (FCM) and percentage coefficient of variation
264 (% CV) (n=15). Black dots represent cell recovery efficiency with standard errors and grey
265 bars represent % CV, E = extraction number.

266

single column fitting image

3.4 Microscopy versus flow cytometry

Fifty five muddy samples were tested and highly significant correlations were found between EFM and FCM counts (Fig. 3; t-test: R^2= 0.615, df =53, p-value <0.001). Moreover, cell abundance estimated by FCM was always higher than cell abundance counted by EFM, by a factor of 1.81. Thus, EFM and FCM results followed the same trends but FCM always allowed detecting more cells than EFM. We prove by this way that the traditional method by EFM need to be re-evaluated and that FCM can be a better method to assess the heterotrophic prokaryotic abundance.



275

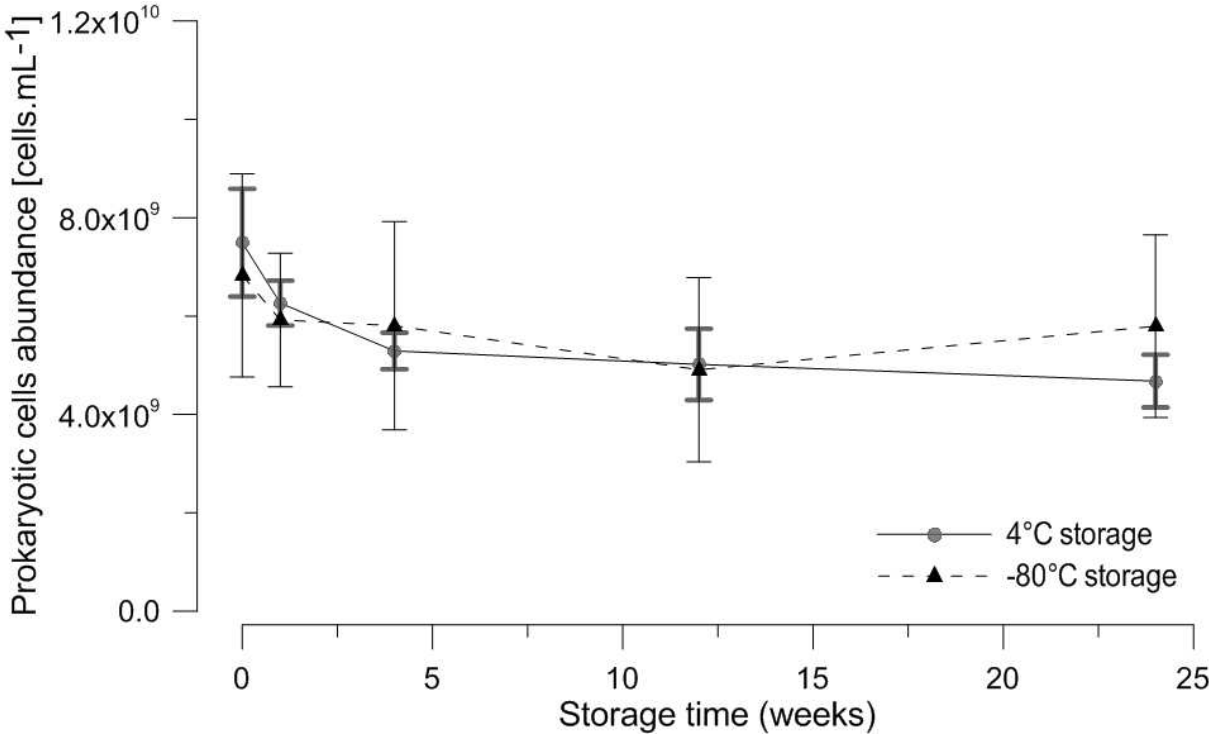
276 **Fig. 3.** Linear regression between prokaryotic cell abundance determined by flow cytometry
277 (FCM) and observed by epifluorescence microscopy (EFM). Samples come from the Moëze
278 mudflat at different depths and sampling seasons (n=55), dot line corresponding to the
279 regression line and envelopes represent 95% confidence intervals. Significant adjusted R-
280 squared: 0.615 (t-test: F-stat : 87.3, df =53, p-value <0.001).

281

single column fitting image

3.5 Which type of storage?

The influence of storage conditions on prokaryotic cells was studied. After the first month of storage, a loss of 24.46 % ± 4.5 SE of cells was observed under the two storage conditions (-80°C and +4°C). After that, prokaryotic abundances remained stable until 6 months (-4%; Fig. 4). High standard error bars on Fig. 4 were due to the differences in prokaryotic abundances between sample depths, but the results brought out that prokaryotic abundances in samples stored at -80°C tend to be higher than in those conserved at +4°C. A significant difference was detected between T0 and 12 weeks after sampling (Tukey HSD; p-value<0.05), nevertheless, neither the temperature of storage nor the interaction with time influenced the abundance of prokaryotes counted (2-way ANOVA, p-value > 0.05). After 3 months, the abundances measured with the final protocol were more variable. The recommended protocol is to store fixed sediment samples at -80°C (as for water samples, (Marie et al., 1997)) and to analyse samples within 3 months after sampling.



295

296 **Fig. 4.** Effects of storage temperature and time on heterotrophic prokaryotic cell abundance
297 obtained by flow cytometry (FCM) with the two-step protocol (mean values +/- standard
298 errors from five samples in duplicate are shown).

1.5-column fitting image

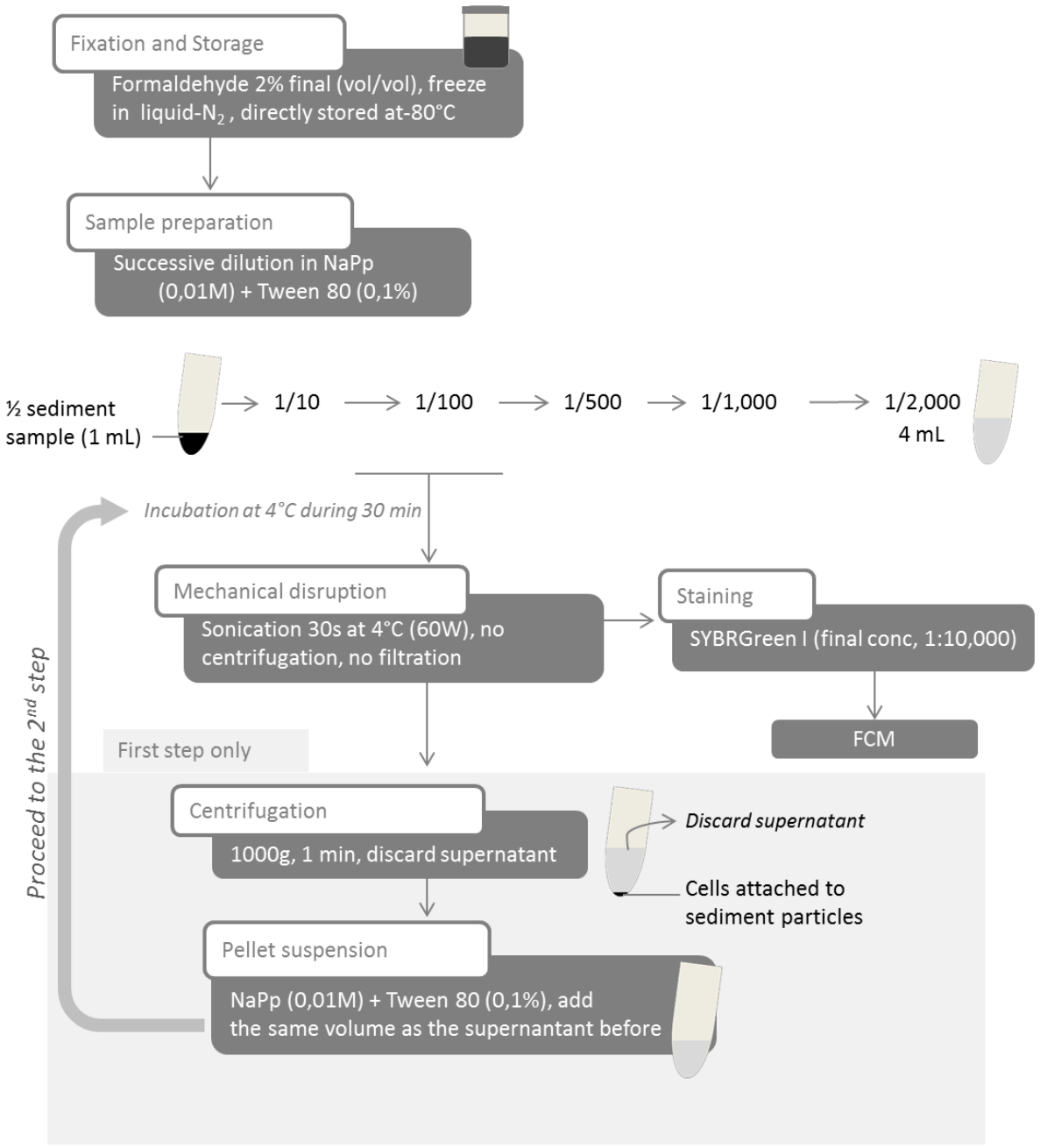
3.6 Final protocol

All the assessments above resulted in a final protocol. This final protocol (Fig. 6) detached and homogenized cells in sediment samples thanks to 2 successive extractions (§ 3.3) and allowed to count the prokaryotic cells using FCM.

1) Samples were prepared and extracted using: a dilution (1:1,000 to 1:2,000) in a detergent mix (§ 3.1) [sodium pyrophosphate (0.01 M) + Tween 80 (0.1%)] and a vortexing step and 30 min of incubation at +4°C. After the vortexing step, a sonication separation for 30 sec (60W) in ice with a sonication probe (3 mm) was applied. Without any centrifugation step (§ 3.2), an aliquot of the sample was stained with SYBRGreen I (1:10,000) 15 min in the dark and analyzed by flow cytometry (FCM).

2) the remaining part of the sample was centrifuged at low speed (1 min at 1,000 g at +4°C); the pellet was then resuspended in the detergent mix and step 1 was repeated once more.

Using this two-step protocol, 83.67% ± 3.63 SD (§ 3.3) of total cells can be extracted from a solid matrix and counted by SYBRGreenI-stained FCM.



314

315 **Fig. 5.** Final protocol of the improved two-step separation method. FCM = Flow Cytometry ;

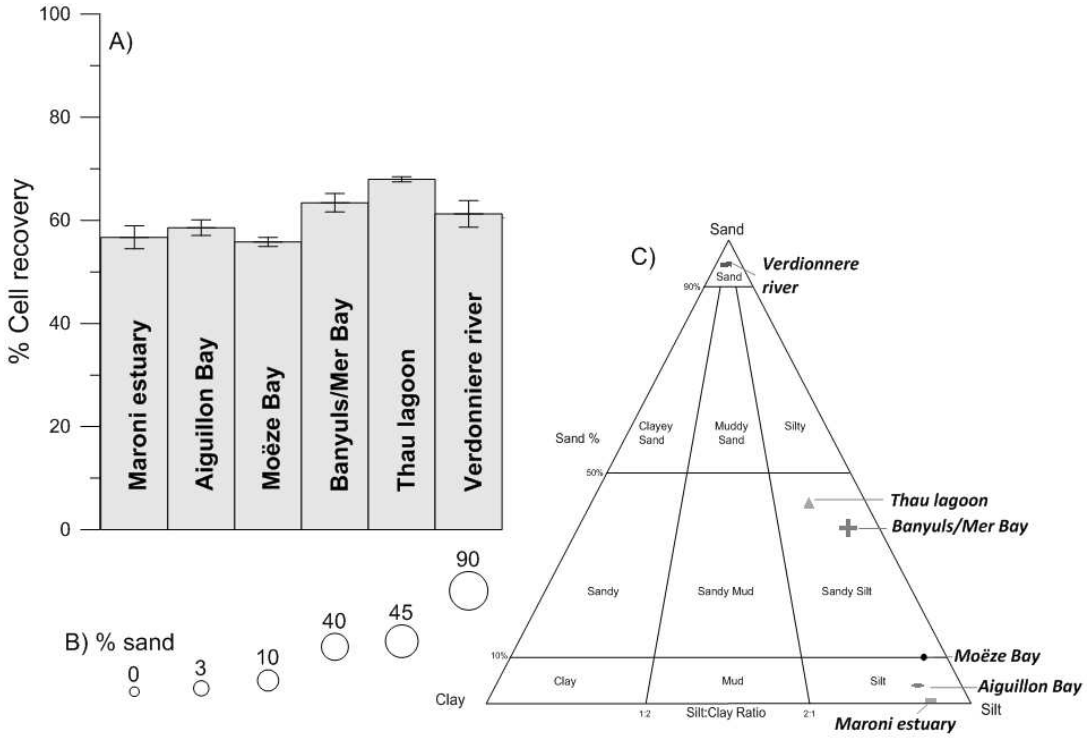
316 NaPp = sodium pyrophosphate.

317

2-column fitting image

3.7 Application on different types of sample

To validate the protocol, the method for FCM analysis was applied to sandy, sandy-mud and muddy sediments from different locations with different values of sand contents (from 0 to 90%) following a range of silt/sand content. For each sample, the cell recovery percentages of the first step extraction were high, by mean 61% ranging from 55% to 68% (Fig. 6; Supplementary Information Table 2.). The cell recovery efficiencies of these samples were in the same range and there were no significant effects of the sand content on the cell recovery of the first extraction (Kruskal-Wallis test; $\chi^2 = 5$; df = 5; *p*-value = 0.4159). These results showed that our developed method is efficient for sandy, sandy-mud and muddy sediments tested whatever the location and sand content or composition.



329

330 **Fig. 6.** A) Percentage of cells extracted after the first step by applying our two-step extraction
331 method followed by FCM heterotrophic prokaryotic enumeration on sandy and muddy
332 sediment samples from diverse locations with a range of sand percentages. B) Sand
333 percentages for each sample. C) Sand/Silt/Clay diagram for each sample.

334

2-column fitting image

4 Discussion and Conclusions

Flow cytometry (FCM) is now widely used for water column analysis in order to estimate autotrophic diversity and abundance, and to enumerate heterotrophic prokaryotes (Legendre et al., 2001). For sediment analysis, technical advances have been made but not well optimized to get an efficient and rapid method for FCM. The main difficulty remains the necessary step of cell preparation and separation from sediment which need to be adapted for each sediment type (Duhamel and Jacquet, 2006). Many authors have tried to find the best protocol for separating prokaryotic cells from a solid matrix. One particularly powerful way seems to be the density gradient application on various sediments (Aakra et al., 2000, Amalfitano and Fazi, 2008, Kallmeyer, et al., 2008, Lunau, et al., 2005, Morono, et al., 2013, Whiteley, et al., 2003), but these techniques are relatively time-consuming and expensive. The present study demonstrates a quick and efficient protocol for the enumeration of heterotrophic prokaryotes in coastal sediments.

Since EFM is the classic method of counting heterotrophic prokaryotic cells in sediment, our protocol of extraction followed by FCM analysis needs to be validated by EFM. We compared the cell abundance obtained by EFM (DAPI-stained cells) and FCM (SYBRGreenI-stained cells). The staining protocol used was different with the two methods (i.e. DAPI preferentially bind on A and T bases). The choice was to compare the traditional protocol (DAPI-stained cells) and the most used protocol in FCM (SYBRGreenI-stained cells). It is known that the two dyes may differ in terms of binding and affinity on DNA and RNA (simple and double-stranded) (Marie, et al., 1997, Troussellier et al., 1999) and thus can produce different results. Nevertheless, DAPI-stained samples (sorted and non-sorted sediment samples) were counted using FCM equipped with a violet laser (407 nm). Cytograms were weakly exploitable because DAPI fluorescence yield was not optimal at 407 nm (optimal wave length = 375 nm) resulting in a low signal resolution and thus high

360 fluorescence background of DAPI (data not shown). Moreover, observations of SYBRGreenI-
361 stained cells under EFM were not satisfactory.

362 Finally, FCM appeared to be a consistent method to count benthic prokaryotes as it
363 allows the detection of 1.81 times more cells than EFM with a significant correlation. The
364 explanation could be that EFM is human-dependent and biased by the cell location of the
365 filter (can be hidden by sediment particles). FCM allows assessment of particle count as well
366 as multi-parameters analysis for each cell (Bouvier et al., 2001, Porter et al., 1997). The use of
367 FCM increases counting efficiency as compared to the classic EFM method, and the
368 estimation of prokaryotic abundance is consistent.

369 Liquid N_2 storage is the most widely used method of conserving prokaryotic cells in
370 water samples for FCM analysis (Vaulot et al., 1989), but in the case of benthic prokaryotic
371 cells, samples are usually directly counted or stored at +4°C before EFM counting (Ellery and
372 Schleyer, 1984, Epstein and Rossel, 1995). We then propose to fix the sediment samples with
373 2% formaldehyde solution and after liquid-N_2 fixation, store them immediately at -80°C and
374 then count within 3 months after sampling.

375 Many authors had proposed protocols for FCM analysis including a centrifugation step
376 and/or a filtration through 5 µm because it can limit detritus clogging in the cytometer nozzle
377 (Duhamel and Jacquet, 2006). The present study brings out that without centrifugation, the
378 sediment particles can settle in the tube and did not accumulate in the flow cytometer nozzle,
379 and FCM analysis was possible. To develop a rapid and easy protocol, we proposed to analyse
380 samples without the centrifugation step and then to centrifuge samples afterward to proceed to
381 the second extraction (on the pellet).

382 Taking into considerations all these features, the development of the protocol focused
383 on a method of cell separation to define a simple, inexpensive, and rapid method to enumerate
384 prokaryotes in sediment.

385 Kallmeyer, et al. (2008) extracted between 65 and 100% of prokaryotic cells in deep
386 subsurface sediments by applying a bilayer density gradient. In soil, Barra Caracciolo, et al.
387 (2005) also used a bilayer gradient density as well, and were able to extract up to 77% of total
388 prokaryotic cells. More recently, Morono, et al. (2013) applied a multilayer density gradient
389 on samples from marine subsurface and obtained from 50 to 80% cell recovery. Lunau, et al.
390 (2005) worked on muddy and sandy sediments and opted for a low-speed centrifugation
391 method combined with a methanol purification step; by an EFM analysis, they achieved 54 to
392 114% cell recovery. Even if the cell recovery had been found to be variable among the
393 physico-chemical parameters of a solid matrix (Maron, et al., 2006), we confirmed that our
394 method is consistent and quicker, and can be applied to different kind of benthic samples. It
395 allows the extraction of a large quantity of prokaryotic cells (between 10^8 and 10^{10} cells.mL^{-1})
396 and the possibility of determining prokaryotic abundances (83.67% mean cell recovery).

397 Nevertheless, it is clear that some optimization details must be done for each type of
398 samples. We suggest staining cells with more concentrated SYBRGreen I (1:5,000 final
399 concentration) if the prokaryotic population is not easily distinguishable from background
400 noise due to organic and mineral matter. Obviously, during the FCM analysis, threshold and
401 fluorescent parameters must be adapted to population characteristics as well as the dilution of
402 the sample.

403 Our two-step extraction method is simple to apply, as it allows the estimation of
404 heterotrophic prokaryotic abundance of 30 sediment samples within 4 hours. This method was
405 applied successfully on different types of sediments (muddy and sandy, coastal marine
406 sediments and freshwater sediment) and among the different types of sediments, our method

was reproducible. Moreover, these applications showed that our method was suitable not only for coastal sediments but also for freshwater sediment (from the Verdonniere river streambed, France). Aiming at finding a method suitable for a large range of sediments samples, this study prove that our optimized method offers a better efficiency for different marine sediment types even for freshwater sediments. In soils, Williamson et al. (2013), showed a strong influence of clay content and recommended testing the influence of the extraction mixture prior to analyse the samples. Further analyses remain possible to establish whether our two-step protocol is efficient on clay-containing sediments.

This fast protocol using FCM is a methodological issue but is also crucial for ecological studies by allowing in the end a better understanding of marine benthic ecosystems. Finally, we can confirm that our protocol worked well for turbid water with high organic matter content in a study on prokaryotic community distribution among a salinity gradient in the Charente River (France). For this study, the dilution was adapted because prokaryotic abundance was between two and three orders of magnitude less than in the sediment samples.

In the future, we are convinced that our study can be useful for assessing activity, productivity or diversity analysis in sediments. Although, the abundance of prokaryotes is a central parameter to measure in all ecosystems, the next step to understand the functioning is evaluating the unknown genetic diversity (DeLong, 2009, Karl, 2007). And activity and productivity are powerful indices to characterize the community and are needed to show the key role of prokaryotic community in biogeochemical cycles. The combinations of our protocol and cell sorting (Wang et al., 2010) can be a great progress for sediment analysis. With cell sorting, it will be possible to sort cells according to their average side-angle-scattered (SSC) light (Bernard et al., 2000) (proxy of size-class) for example and then do diverse analysis on different population of the prokaryotic community.

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5 Figures

Fig. 1. Effects of the addition of Tween 80 to sodium pyrophosphate (NaPp) on prokaryotic cell abundance in sediment samples (FCM counts from SYBRGreen: n = 55; EFM counts from DAPI: n= 36). Values for all the samples tested are presented. Crosses represented samples extracted with Tween 80 and NaPp, and circles represented samples extracted with NaPp alone.

Fig. 2. Cumulative percentage of cell recovery using the final extraction protocol (extended to eight extractions), counting by flow cytometry (FCM) and percentage coefficient of variation (% CV) (n=15). Black dots represent cell recovery efficiency with standard errors and grey bars represent % CV, E = extraction number.

Fig. 3. Linear regression between prokaryotic cell abundance determined by flow cytometry (FCM) and observed by epifluorescence microscopy (EFM). Samples come from the Moëze mudflat at different depths and sampling seasons (n=55), dot line corresponding to the regression line and envelopes represent 95% confidence intervals. Significant adjusted R-squared: 0.615 (t-test: F-stat : 87.3, df =53, p-value <0.001).

Fig. 4. Effects of storage temperature and time on heterotrophic prokaryotic cell abundance obtained by flow cytometry (FCM) with the two-step protocol (mean values +/- standard errors from five samples in duplicate are shown).

Fig. 5. Final protocol of the improved two-step separation method. FCM = Flow Cytometry ; NaPp = sodium pyrophosphate.

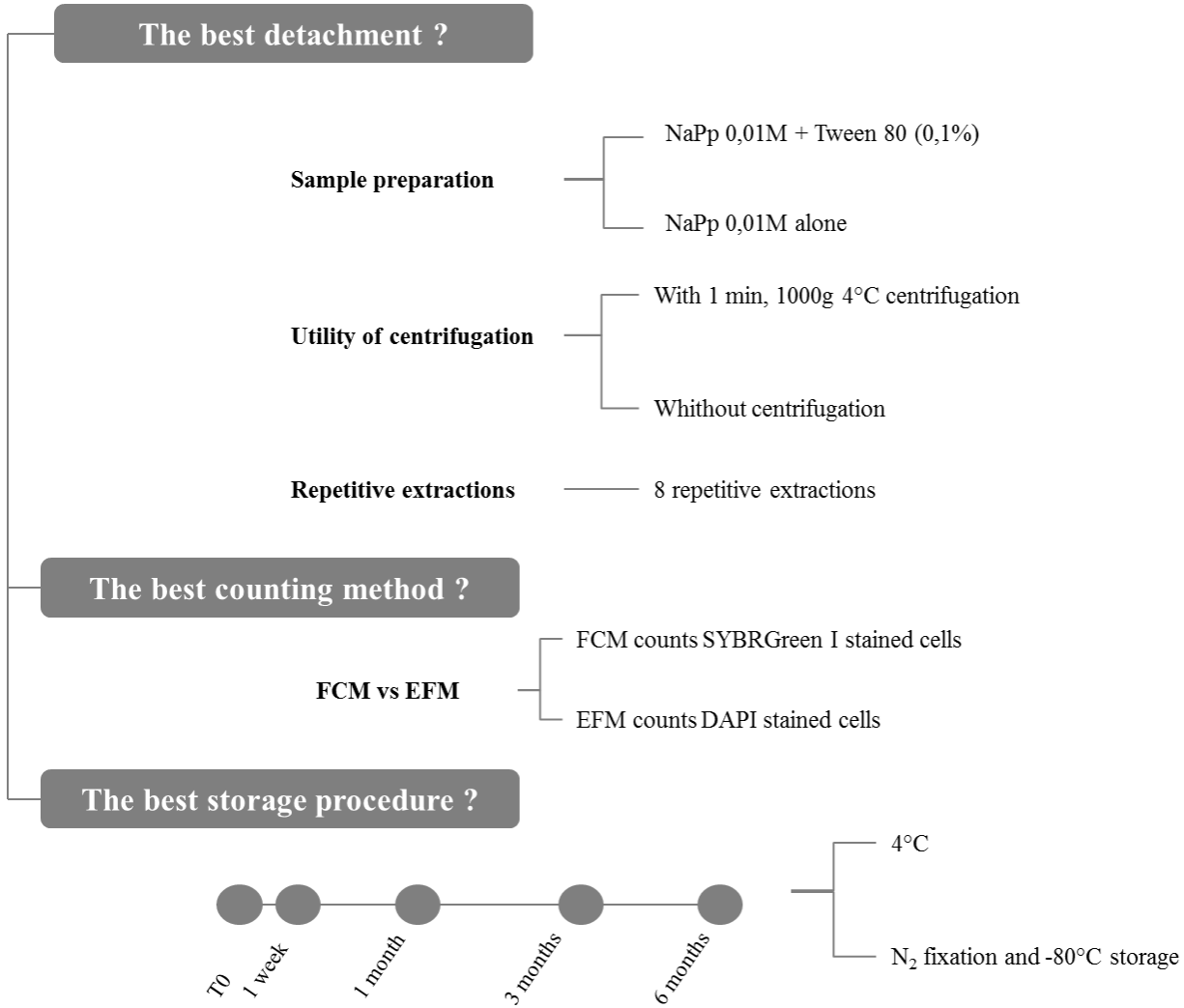
Fig. 6. A) Percentage of cells extracted after the first step by applying our two-step extraction method followed by FCM heterotrophic prokaryotic enumeration on sandy and muddy sediment samples from diverse locations with a range of sand percentages. B) Sand percentages for each sample. C) Sand/Silt/Clay diagram for each sample.

6 Supplementary information

Figure 1. Searching for the best protocol for enumeration of heterotrophic prokaryotes in sediments

Table 1. Extraction, fixation and staining methods in literature

Table 2. Heterotrophic prokaryotes abundance (mean ± SD) in different sediments and cell recovery (% with mean ± SD, min and max) of the first extraction using the two-step extraction protocol analysed by flow cytometry (FCM).



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634 **Figure 1.** Searching for the best protocol for enumeration of heterotrophic prokaryotes in sediments

635 **Table 1**. Extraction, fixation and staining methods in literature

Sample type	Fixation (final concentration)	Chemical separation	physical disruption	Centrifugation	Additional step	% recovery efficiency	Organisms	Staining (method)	References
Turbid seawater	Glutaraldehyde (2%)	Sterile seawater + 0.001% Tween 80	Sonication probe 10W 30s + blending 5 min at 22000 rpm	-			Prokaryotes	DAPI (EFM)	Yoon and Rosson (1990)
Lake sediments	Formaldehyde (2%)	10 mM NaPp + 10% Tween 80 + MilliQ Water	Sonication 3 min stopped for 30s every minute+ shaking	800g 1 min RT	Filtration through 5-µm filter	-	Prokaryotes, Viruses	SYBRGreen II (EFM + FCM)	Duhamel and Jacquet (2006)
	Formalin (3%) 4°C	-	-	750g 10 min 4°C	-	-	Prokaryotes	DAPI (EFM)	dos Santos Furtado and Casper (2000)
	Paraformaldehyde (4%) 4°C	10 mM NaPp + 120 mM NaCl + 10 mM NaPO4	Sonication bath 15 min	-	-	-	Prokaryotes	DAPI (EFM)	Gough and Stahl (2003)
Streambed sand	Formaldehyde (2%)	0.1 % NaPp + 0,5% Tween 20	Shaking 30 min, 720 rpm + sonication 1 min 20W	14000 g 90 min 4°C	NGD[b]	93%	Prokaryotes	DAPI (EFM) SYTO13 (FCM)	Amalfitano and Fazi (2008)
Marine Sediments									
- *Sands and muddy sediments*	Glutaraldehyde (2%)	10 mM P2O7	3 min sonication	800g 1 min RT		60%	Virus	SYBRGreen I	Danovaro et al. (2001)
- *Sandy sediments*	Formaldehyde (4%)	1:1 PBS/Ethanol	Sonication min power 20s	-	3 washes before storage	-	Prokaryotes	DAPI (CARD-FISH)	Ishii et al. (2004)
	Formaldehyde	Sterile seawater + 0.0001 % Tween80	Sonication bath 200W 2.5 min	-	-	-	Prokaryotes	AO (EFM)	Ellery and Schleyer (1984)
	Formaldehyde (4%) 4°C	0.1 mM NaPp + 0.0001% Tween80	Sonication probe 3x60s 109µm	500g 5min RT	8 washes		Prokaryotes	DAPI (EFM)	Epstein and Rossel (1995)
- *Deep subsurface sediments*	Formaldehyde (2%) 6h 4°C + washing steps	DT[a]	Shaking 60 min 500 rpm + sonication probe 20 W 1 min	4500g 15 min and 15000 300 min	MlGD[c]		Prokaryotes	SYBRGreen I (FCM)	Morono, et al. (2013)
	Formaldehyde (2%) 4°C	DT[a]	Vortexing 60 min + sonication probe 5x10 s	3000g 10 min RT	Carbohydrates dissolution + 2 NGD[b] steps	65 to 100%	Prokaryotes	SYBRGreen I + 0.1% *p*-phenylenediamine (EFM)	Kallmeyer, et al. (2008)
- *Muddy sediments*	-	10% methanol	Sonication bath 320W 15 min 35°C	190g 1min	-	54-114%	Prokaryotes	SYBRGreen I (EFM)	Lunau, et al. (2005)
Soil	no fixation	0.2% NaP2O7	0.5h intensive shaking	600g 5 min RT		45%	Bacteria, fungi	DAPI (EFM)	Riis et al. (1998)
	Formaldehyde (2%)	PBS+Tween20 + NaPp	Shaking 15 min at 400 rpm (orbital shaker)	14000g 90 min 4°C	NGD[b] + filtration through 0,2-µm filter	77%	Prokaryotes	DAPI (FISH)	Barra Caracciolo, et al. (2005)
Aquifere	Formaldehyde (0,5 %)	0.1% NaP2O7	Shaking 60 min at 155 rpm at 25°C	-	-	-	-	Propidium iodide (EFM)	DeLeo and Baveye (1996)
	Formaldehyde (2%)	PBS+ 0,5% Tween20	Shaking 15 min at 400 rpm (orbital shaker)	14000g 90 min 4°C	NGD[b] + filtration through 0,2-µm filter	78%	Prokaryotes	DAPI +(FISH)	Barra Caracciolo, et al. (2005)

[a]DI-H2O + 100mM EDTA + 100mM NaPp+ 1% Tween80 + NaCl + MeOH

[b]Nicodenz gradient density

[c]Multilayer Gradient density

636

637 **Table 2**. Heterotrophic prokaryotic abundance (mean ± SE) in different sediments tested and
638 cell recovery (% with mean ± SE, min and max) of the first extraction using the two-step
639 extraction protocol analysed by flow cytometry (FCM).

	Prokaryotes abundance [cells.mL^{-1}] mean +/- SE			% sand	Cell recovery [%]				
					Mean (+/- SE)			Min	Max
Salt Lagoon sediment									
Thau lagoon (n=48), France	$1.53.10^{9}$	+/-	*$4.46.10^{7}$*	45 [a]	67.98	+/-	*0.49*	60.72	75.06
Intertidal Mudflat sediment									
Moëze Bay (n=45), France	$7.31.10^{9}$	+/-	*$2.92.10^{8}$*	10	55.81	+/-	*0.87*	40.77	65.96
Aiguillon Bay (n=42), France	$1.40.10^{10}$	+/-	*$4.34.10^{8}$*	3 [b]	58.57	+/-	*1.51*	32.31	69.35
Maroni estuary (n=9), Surinam	$3.99.10^{9}$	+/-	*$3.37.10^{8}$*	0	56.70	+/-	*2.23*	38.16	68.72
Sandy muddy coastal sediment									
Banyuls s/ mer Bay, France Proteic enrichment (n=32)	$2.81.10^{10}$	+/-	*$5.52.10^{8}$*	40	62.81	+/-	*1.29*	38.14	72.90
Banyuls s/ mer Bay, France Glucidic enrichment (n=60)	$1.46.10^{10}$	+/-	*$3.47.10^{8}$*	40	64.03	+/-	*0.88*	41.56	72.75
Sandy streambed sediment									
Verdonniere River (n=11)	$6.75.10^{9}$	+/-	*$1.41.10^{9}$*	90	61.25	+/-	*2.57*	48.86	81.59
				TOT	**61.02**	+/-	***1.40***		

[a] Ifremer (2008)

[b] Bocher et al. (2007), Compton et al. (2008)

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