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Evaluation of quantitative recovery of bacterial cells and DNA from different lake sediments by Nycodenz density gradient centrifugation

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ABSTRACT

While purified bacterial cells and DNA – the signature of life – from soil and sediment matrices have been extensively studied in a wide range of environments and in different microbial ecosystems, the paucity of data on DNA extraction from contaminated sediments emphasizes the need for further research on the isolation and quantification of bacterial cells and DNA in sediments. Consequently, the Nycondez gradient centrifugation method was applied to extract bacterial cells from contaminated and uncontaminated sediments. Quantitative estimates of recovered bacterial cells were obtained from direct counts performed using DAPI (4′,6′-diamino-2-phenylindole hypochloride) staining couples with fluorescence microscopy and indirect counts (colony-forming units). The estimation was improved by using an efficient method of comparing sediment types composed of quantifying bacterial densities in three steps: S_1 the initial freshwater sediments; S_2 the first supernatant recovered after mixing the sediments with sodium hexametaphosphate solution followed by centrifugation; and S_3 the extracted cells. Total and extracellular DNA were extracted and quantified in each of the three steps. Additional analysis of faecal indicator bacteria (FIB) including *E. coli* and *Enterococcus* (ENT) was also performed in each step. The results display considerable variability in the quantity of bacteria cells depending on sediment type, ranging from 1.2×10^5 to 6.2×10^9 cell g^{-1} dry sediments. The treatment with sodium hexametaphosphate solution (2%) leads to the desorption of bacterial populations which were firmly adsorbed on contaminated sediment surfaces resulting in more than 90% of the FIB being recovered. The Nycondez density gradient centrifugation method makes it possible to extract bacterial cells from freshwater sediments without extracellular DNA so it is ideal for metagenomic analysis of bacteria.

1. Introduction

Bacteria are the most ubiquitous organisms and are found in all environments including soils and sediments. The latter are important reservoirs of microbial diversity. Since microbes are associated with different types of particles, the separation of the microbes is very important as it has multiple applications in microbial ecology. Bacterial cells are not uniformly distributed in soil and sediments. They can be adsorbed onto particles including clay, organic matter, and humic acids. Therefore, techniques that guarantee desorption of bacterial cells from sediment components are required for efficient bacterial enumeration (Fry, 1990; Furtado and Casper, 2000). Soil particles and bacteria are almost complete separated by Nycondenz density gradient centrifugation with both preservation of the bacterial structure (Lindahl, 1996) and bacterial recovery rates in a wide variety of soil types being excellent (Priemé et al., 1996; Lindahl and Bakken, 1995). Furtado and Casper (2000) report several techniques for extracting bacteria from sediments but most of these are very time consuming and also require very large amounts of materials in order for them to be efficient. Craig et al. (2002) evaluate a technique for the separation of faecal indicator bacteria (FIB) from sediments that includes hand shaking and sonication bath. Their results suggest that treatment using sonication bath is relatively efficient in removing bacteria from sandy sediments.

DNA and purified bacteria cells from soil and sediment matrix have been widely studied for natural gene exchange, soil and sediment bioremediation, genetically modified organisms, biotechnological metagenomic library, physiological studies, exobiology, and paleontology (Furtado and Casper, 2000; Courtois et al., 2001; Poté et al., 2005; Bertrand et al., 2005; Patra et al., 2005; Desai and Madamwar, 2006). However, polluted aquatic

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ecosystems have largely been ignored and only a few studies, which deal with DNA extraction, have been carried out on polluted sediments. Consequently, little information is available concerning the extraction and quantification of bacteria cells from different sediment types (Fortin et al., 2004; Desai and Madamwar, 2006).

Sediments are both the most complex of habitats and the most intensively colonized by microorganisms—of many different types (e.g., bacteria, cyanobacteria, viruses, fungi, algae, and protozoans) which are extremely important in aquatic food webs. Quantification of their abundance and the extent of their diversity are critical in modern biological oceanography (Li et al., 1995; Urakawa et al., 1999). Therefore, bearing in mind the importance of microbial communities and their role in aquatic ecosystems, there is a great need to develop suitable methods of bacteria cell extraction and purification from different sediment types. Previous studies have been performed to evaluate the construction and screening of metagenomic libraries derived from enriched soil culture bacteria (Ogram et al., 1987; Courtois et al., 2001; Entcheva et al., 2001; Knietsch et al., 2003; Bertrand et al., 2005). It has been demonstrated that the cell extraction technique using the Nycondez purification method provides a major advantage in overcoming the bias of cultivation avoiding the co-extraction of bacteria cells and extracellular DNA.

The extraction and quantification of microbial populations from soil and sediments depends on the procedures employed (Lindahl, 1996: Richaume et al., 1993). Standard methods of bacteria enumeration are in many cases inadequate for investigating microbial abundance and distribution in subsurface environment. For instance, the indirect bacteria cell count (IBC, cfu: colony-forming units) is based on culture-dependent methods that require the growth of bacteria in the media, e.g. Tryptic Soy Agar medium (TSA). Thus, IBC only takes culturable bacteria into account which represent less that 5% of the total (Porter and Feig, 1980; McDaniel and Capone, 1985; Richaume et al., 1993; Stevens and Holbert, 1995). The development of fluorescence microscopy for direct bacteria cell counts using dyes such as DAPI (4′,6′-diamino-2-phenylindole hypochloride) and acridine orange could estimate the number of bacteria by staining solvently fixed cells, including culturable and unculturable forms (Porter and Feig, 1980; Crozat et al., 1982; Haglund et al., 2003).

The aim of this study is to provide a standard procedure for the effective quantification and purification of bacteria cells from sediments. Contaminated and non-contaminated sediments from Lake Geneva (Switzerland) were used as end members in order to test this methodology. According to previous studies (Loizeau et al., 2004; Wildi et al., 2004; Pardos et al., 2004; Poté et al., 2008; Haller et al., 2009), the sediments from different areas of Lake Geneva vary in particle grain size, water and organic matter content. The shallow Bay of Vidy for instance is the most contaminated area of Lake Geneva as shown by its heavy metal concentration, total organic matter content, faecal indicator bacteria and hydrophobic organic compounds.

The influence of sediment type was further analyzed and both the amount of bacteria cells recovered and the DNA extracted during different steps of the method were quantitatively evaluated. Faecal indicator bacteria (FIB) analyses, including *E. coli* and *Enterococcus* (ENT), were also performed.

2. Materials and methods

2.1. Sediment sampling

Bottom sediments (0–3 cm thickness) were collected from four sites in Lake Geneva: (i) the Bay of Vidy (BV) at the outlet of a discharge pipe from a wastewater treatment plant representing contaminated sediment in heavy metals, organic matter content and faecal indicator bacteria; (ii) the mouth of the Chamberonne River (CR) that presently drains surface water from its natural drainage basin and some untreated wastewater as shown by the presence of contaminated sediment in faecal indicator bacteria; (iii) the Creux-de-Genthod region (CDG); and (iv) the mouth of the Versoix River (VE). Thus, BV and CR represent the contaminated sediments whereas the Versoix and Creux-de-Genthod regions of Lake Geneva, known to be free of pollution, represent the non-contaminated end members.

The "Büchi grab sampler" on the research vessel "La Licorne" from the Institute F.A. Forel was used to collect the sediments from CDG and VB. The sediments from VE and CR were collected manually. After sampling, the sediments were kept at 4 °C and immediately transported to the laboratory where they were analyzed within 24 h.

2.2. Particle grain size and analysis of sediment contaminants

Grain size measurements were performed with a laser Coulter® LS-100 diffractometer (Beckman Coulter, Fullerton, CA, USA) following ultrasonic dispersal in de-ionized water. The sediment total organic matter content was measured by calcination at 550 °C for 1 h in a Salvis oven (AG Emmenbrucke, Lucerne, Switzerland). The sediment total organic carbon content (TOC) was determined by titrimetry following acid oxidation. Total nitrogen (TN) was obtained using the Kjeldhal's method (Pardos et al., 2004). Total phosphorus (TP) was measured using a spectrophotometer (Thermo Electroporation, AG Digitana, Yverdon-les Bains, Switzerland) at 850 nm by following the method described in Murphy and Riley (1962) and Harwood et al. (1969).

2.3. Extraction of bacteria cells from sediments

The separation of bacteria and sediment matrix was performed using Nycondez density gradient centrifugation (with three steps; S_1 initial sediment, S_2 supernatant after low centrifugation, S_3 extracted cells, Fig. 1) as previously described (Lindahl, 1996; Bertrand et al., 2005) but with minor modifications. Briefly, 100 g (wet weight) of freshwater sediments and 500 ml of 0.2% sodium hexametaphosphate ($Na_2(PO_3)_6$, E. Lotti S.A, Switzerland) were dispersed in 1 L sterile plastic bottles and mixed for 1 h using the agitator rotary printing-press Watson-Marlow 601 controller (SKAN, Switzerland) at room temperature. The sample was then centrifuged at 750 × *g* for 15 min at 15 °C in a 3K-1 (Sigma, centrifuge). The supernatant (S_2) was collected and filtered with sterile gauze. For this step, the extraction procedure (pellet re-extracted with $Na_2(PO_3)_6$) was repeated three times with a series of fractions designated f_1, f_2, and f_3. The supernatant was then centrifuged at 7500 × *g* for 30 min at 10 °C. The microbial cell fraction-containing pellet was resuspended in sterile 0.8% sodium chloride solution by vortexing. 25 ml of the homogenous solution was transferred to an ultracentrifuge tube containing 11 ml of Nycodenz solution (Axis-Shield, Oslo, Norway) at 1.3 g/ml density (8 g of Nycondez to 10 ml of sterile ultrapure water). Bacterial cells and sediment particles were separated by high-speed centrifugation (15,000 × *g* for 1 h at 10 °C) in centrikon T-1080 (Kontron, Instrument, ultracentrifuge). A white layer of bacterial cells was obtained at the interface between the Nycodenz-soil mix particles and the overlying aqueous layer. This white layer was carefully recovered, and mixed with an equal volume of sterile ultrapure water, then centrifuged at 7500 × *g* for 20 min at 10 °C. To remove traces of Nycodenz, pelleted bacteria were resuspended in 20 ml sterile ultrapure water and centrifuged for 20 min at 7500 × *g* (S_3).

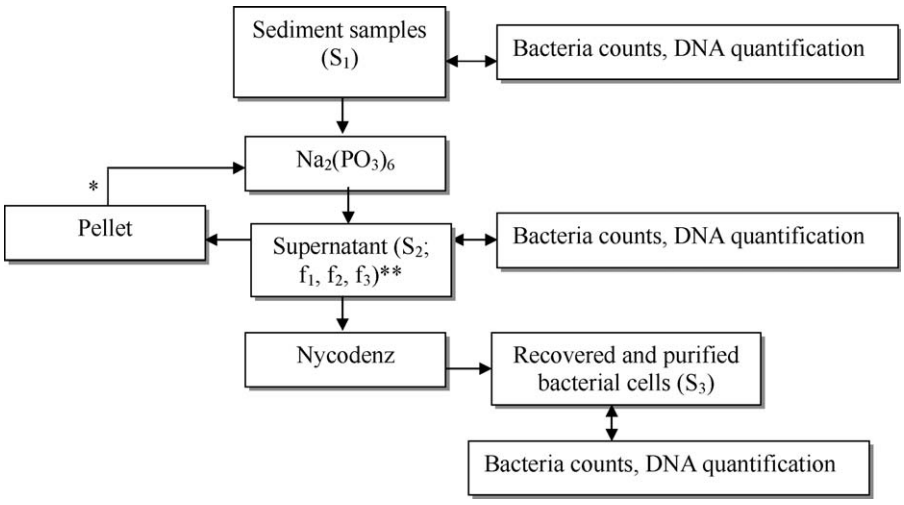

Fig. 1. Different steps (S_1, S_2, and S_3) of bacteria densities and DNA quantification. *Repeated extraction to recover maximum of DNA and bacteria (three times; f_1, f_2, f_3). **f_1, f_2, f_3 first, second and third fractions of S_2 (low centrifugation at 750 × *g* after mixing initial freshwater sediments with $Na_2(PO_3)_6$ 2%).

2.4. DNA extraction and quantification

Total DNA in the initial freshwater sediments and in step S_3 was extracted using a PowerMax™ Soil DNA Isolation Kit (Mo Bio laboratories, Inc.) according to the manufacturer recommendations. Extracellular DNA was extracted using buffer TENP (50 mM Tris, 20 mM EDTA, 100 mM NaCl, 1% [wt/vol] polyvinylpolypyrrolidone) according to the methodology described by Frostegärd et al. (1999). Then the DNA suspension was passed through purification Microspin S-400 (Pharmacia Biotech) to remove all traces of humic acids (Poté et al., 2003, 2007). The concentration of recovered DNA was quantified spectrophotometrically (OD_{260}).

2.5. Bacterial counts and analysis of faecal indicator bacteria

Indigenous bacteria were quantified using both direct and indirect counting methods for the various steps summarized in Fig. 1. Fluorescence microscopy enumeration of the total bacterial cells was performed by DDC (DAPI direct count). Consequently, 45 ml aliquots of three each fractions (with dilution; for step S_3, e.g., the dilution was performed 10^2 to 10^5 times using 200–500 μL of bacteria cells suspension) were fixed with 5 ml particle-free formaldehyde, then stained with DAPI, and filtered onto 0.2-μm pore size black polycarbonate filters (Millipore). Cell binding was evaluated as a proportion of 20 randomly selected fields per filter. The results are expressed in number of cells g^{-1} dry sediment.

The indirect bacteria cell counts (IBC) was performed to determine the colony-forming units (cfu) using 10% tryptic soil agar (TSA) (OXOID, LTD, Basingstoke, England) medium supplemented with the antifugal (Nystatin solution; 50 mg ml^{-1}, Sigma–Aldrich, Gmbh, Germany). The cfu were counted after 3 days of plate incubations at 28 °C. The results were expressed as colony-forming units per 100 g of sediment (cfu 100 g^{-1} dry sediment).

The faecal indicator bacteria (FIB) analyses, including *E. coli* and *Enterococcus* (ENT), were carried out according to the Swiss standard methods for water quality determination using the membrane filtration method (OHyg, 2005). Consequently, 100 ml of suspension (with dilution) from different steps was passed through a 0.45 μm filter (47 mm diameter, Millipore, Bedford, USA), which was then placed on a different culture media (Biolife, Italiana) using the following incubation conditions: *E. coli* on Tryptic Soy Agar medium plates incubated at 20 °C for 24 h and transferred to ECD Agar Mug medium at 44 °C for 24 h; ENT on Slanetz Bartley Agar plates incubated at 37 °C for 48 h and transferred onto Bile Aesculin Agar at 37 °C for 4 h. The results were expressed as colony-forming units per 100 g of dry sediment (cfu 100 g^{-1} dry sediment).

All analyses were conducted in triplicate for each set of conditions. In addition, three plates per dilution were inoculated for cfu and FIB. SigmaStat 3.0 software (Systat Software, Inc., USA) was used for the statistical analysis of the data.

3. Results and discussion

3.1. Sediment characterization

Grain size analysis shows that the sediments are composed of a clastic sandy fraction of various sizes and organic matter. The sediments were classified as loamy sand with wide variations in organic matter content and particle grain sizes (Table 1). Thus,

Table 1
Sediment characteristics and GPS location (in Swiss coordinates) of sampling sites in Lake Geneva.

	CDG	VE	CR	BV
Depth (m)	50	<1	<1	35
Distance to coast (m)	1000	5	4	300
Water content (%)	43	29	36	68
Organic matter content (%)	8	2	2.3	28
pH	7.4	7.2	7.2	7.8
Sand (%)	14.87	51.74	68.97	27.23
Silt (%)	85.	48.16	30.85	72.67
Clay (%)	0.11	0.10	0.18	0.10
GPS location in Swiss coordinates	*X*: 502613, *Y*: 122938	*X*: 502287, *Y*: 125630	*X*: 534626, *Y*: 152154	*X*: 534676, *Y*: 151543

CDG (Creux-de-Genthod region), VE (mouth of Versoix River), CR (mouth of Chamberonne River), BV (Bay of Vidy, at wastewater treatment plant outlet pipe discharge).

Table 2
Concentration values of total organic carbon (TOC), total nitrogen (TN) and total phosphorous (TP) in sediments.

Sampling site	Concentration (mg kg^{-1} dry weight sediment)		
	TOC	TN	TP
CDG	58675.4	2206	1252
VE	70745.5	3090	1118
CR	88599.5	4432	1216
BV[a]	143886	14383	18886

CDG (Creux-de-Genthod region), VE (mouth of Versoix River), CR (mouth of Chamberonne River), BV (Bay of Vidy, at wastewater treatment plant outlet pipe discharge).

[a] Values at closest to the outlet pipe of sewage treatment plant in the lake, at 35 m depth (Poté et al., 2008).

these sediments present different characteristics due to the sand, silt, water and organic matter content. The sediments sampled from the sites close to the outlet pipe of the WWTP present high organic matter content of approximately 28% (measured by loss on ignition at 550 °C). These values were much higher than the values measured on the other sample sites: 2.3%, 8%, and 2% for CR, CDG and VE, respectively. The average values of sedimentary organic matter content from many sampling sites in Lake Geneva as well as from other Swiss lakes range from 0.2 to 8%. Sediments with more than 10% are considered as rich and polluted in organic matter content (Pardos et al., 2004). The sediments from the Bay of Vidy can reach values of 30% and thus are considered as polluted in organic matter (Wildi et al., 2004; Poté et al., 2008; Haller et al., 2009).

The sediments from BV are generally soft, muddy, and black in color with a mean grain size of 63.66 μm, and have a strong odor resulting from the reduced layer. The coarser sediments close to the outlet pipe of the WWTP are mainly composed of organic debris. The Chamberonne River delivers coarse sediments close to its mouth with a mean grain size of 86.6 μm whereas this value drops to 25.9 and 23.8 μm, respectively, at CDG and VE.

The TOC, TN, and TP concentration values for all four sites are reported in Table 2. The highest concentration for each of these parameters was measured in the sediments from BV confirming that this is the most contaminated site.

3.2. Quantitative estimation of sediment bacterial densities in each fraction

The DDC and IBC counts of bacteria cells extracted (using Nycondez gradient centrifugation) from all the sediments ranged, respectively, from 5.5×10^6 to 2.6×10^8 and from 1.2×10^5 to 1.4×10^6 cell g^{-1} sediment (Table 3). Indeed, in all cases culturable bacterial densities (IBC, cfu) represent less than 3% of total DDC (total cell counts) ($P < 0.05$), indicating that the bacterial densities enumerated by DDC count were considerably higher than the number obtained by IBC plate counting, as expected. For both the DDC and IBC enumeration methods, fraction f_1 of step 2 gave high recovery followed by f_2 and f_3. The cumulative enumeration of bacterial populations in the three fractions (f_1–f_3) in step S_2 was higher than the quantity counted in the initial freshwater sediments (step S_1). The highest bacterial cell counts in each step were found in BV sediment (Table 3).

The percentages of cell recovery (calculated as the number of extracted cells per number of cell quantified in freshwater sediments) by DDC were 4.6, 0.45, 0.74, and 9% for the sediments from CDG, VE, CR, and BV, respectively. The percentages of cell recovery for IBC were 0.4, 0.4, 0.2, and 0.5 for the sediments from CDG, VE, CR, and BV, respectively.

The results presented in Table 3 demonstrate that a significant amount of bacteria remains unquantified by the DDC and IBC count methods in step S_1 mainly in the BV sediments; e.g. DDC count for the sediments from CDG and VE are both in S_1 and S_2 in the order of 10^8 and from BV in order of 10^9 in S_1 and 10^{10} in S_2 (f_1). It was observed a positive effect of $Na_2(PO_3)_6$ on the desorption of bacteria from the sediments. These results indicate that a 2% solution of $Na_2(PO_3)_6$ is the best when using the DDC and IBC methods to quantify indigenous bacteria in sediments. In order to

Table 3
Quantification of bacteria by DDC (DAPI (4′,6′-diamino-2-phenylindole hypochloride) direct count, cell g^{-1} dry sediments) and by IBC (indirect bacteria count: colony-forming units (cfu g^{-1} dry sediment)), quantity of total and extracellular extracted DNA (μg g^{-1} dry sediment) in selected steps of the method.

	S_1[b]	S_2[b]			S_3[b]
		f_1[c]	f_2[c]	f_3[c]	
DDC (cell g^{-1} sed.)[a]					
CDG	4.8×10^8 (1.4×10^8)	7.5×10^8 (3.8×10^8)	4.9×10^6 (1.7×10^6)	8.6×10^5 (4.1×10^5)	2.2×10^7 (3.3×10^7)
VE	3.3×10^8 (5.1×10^7)	6.8×10^8 (3.5×10^7)	8.6×10^6 (4.2×10^6)	3.2×10^5 (8.2×10^4)	5.5×10^6 (2.4×10^5)
CR	4.6×10^9 (3.6×10^8)	5.7×10^9 (6.1×10^8)	5.2×10^6 (2.6×10^6)	1.2×10^5 (9.3×10^4)	3.4×10^7 (4.8×10^6)
BV	2.9×10^9 (8.3×10^8)	6.2×10^{10} (4.1×10^{10})	4.5×10^8 (2.3×10^8)	3.8×10^7 (7.4×10^6)	2.6×10^8 (1.3×10^8)
IBC (cfu g^{-1} sed.)[a]					
CDG	1.9×10^6 (3.1×10^6)	3.1×10^6 (4.3×10^6)	1.3×10^6 (4.4×10^5)	5.8×10^5 (3.3×10^5)	1.7×10^6 (2.1×10^5)
VE	2.6×10^6 (2.4×10^6)	5.9×10^6 (2.8×10^6)	2.8×10^6 (1.7×10^6)	1.8×10^5 (1.3×10^5)	1.2×10^6 (1.7×10^5)
CR	5.6×10^7 (7.8×10^6)	9.1×10^7 (5.3×10^7)	9.2×10^6 (4.9×10^7)	1.9×10^6 (1.6×10^6)	1.9×10^6 (4.5×10^5)
BV	1.7×10^7 (1.9×10^7)	8.2×10^7 (1.6×10^7)	9.3×10^6 (1.8×10^6)	5.4×10^6 (1.5×10^6)	1.4×10^7 (3.2×10^6)
Total DNA extracted (μg g^{-1} sed.)[a]					
CDG	57.5 (10.3)	–	–	–	1.3 (0.6)
VE	36.6 (3.5)	–	–	–	0.9 (0.4)
CR	62.8 (9.1)	–	–	–	1.5 (0.8)
BV	84.2 (15.4)	–	–	–	2.1 (1.3)
Extracellular DNA extracted (μg g^{-1} sed.)[a]					
CDG	12.5 (2.8)	8.6 (1.2)	–	–	b.d.l.
VE	13.8 (4.3)	9.3 (1.7)	–	–	b.d.l.
CR	26.5 (7.2)	17.5 (3.6)	–	–	b.d.l.
BV	35.8 (11.4)	29.8 (3.7)	–	–	b.d.l.

[a] Standard deviation is indicated in parentheses (statistically significant coefficients are in bold $P < 0.05$).

[b] S_1 initial sediment, S_2 supernatant after centrifugation, S_3 extracted cells, summered in Fig. 1.

[c] f_1, f_2, f_3 first, second and third fractions of S_2 (low centrifugation at 750 × g after mixing initial freshwater sediments with ($Na_2(PO_3)_6$ 2%); '–' analysis not performed; b.d.l.: below detection limit.

optimize the efficiency of step S_2, fractions f_1, f_2, and f_3 were mixed and bacteria cells were recovered. No great difference was observed in the amount of bacterial cells recovered by mixing all the fractions compared to using only fraction f_1. Therefore, the fraction f_1 can be used to quantify and extract bacterial cells from both contaminated and uncontaminated sediments. Consequently, there is no need for fractioning the step S_2.

The sediment characteristics including particle grain size and organic matter content have a considerable influence on the separation of bacteria from the sediments (DeFlaun and Mayer, 1983; Furtado and Casper, 2000; Craig et al., 2002). The results of our study show that the highest percentage of purified cells was recovered in the sediments rich in organic matter; 9% from Bay of Vidy (28% of OM), 4.6% from Creux-de-Genthod (8% of OM), 0.74% from mouth of Chamberonne River (2.3% of OM) and 0.45% from mouth of Versoix River (DDC count). Therefore, the application of Nycodenz gradient centrifugation can optimize the separation of bacteria and loads particles of soil, aquifers and sediments (Caracciolo et al., 2005).

3.3. Quantitative estimation of recovery DNA

Total and extracellular DNA concentrations are reported in Table 3. With regard to bacterial cells, the higher amounts of DNA were obtained in sediments from BV. The concentration of total DNA in the freshwater sediments ranged from 36.6 ± 3.5 to $84.2 \pm 15.4\ \mu g^{-1}$ dry sediments whereas for extracellular DNA it ranged from 12.5 ± 2.8 to $35.8 \pm 11.4\ \mu g^{-1}$ dry sediments. Interestingly, fraction f_1 of step S_2 contains a huge amount of extracellular DNA ranging from 8.6 ± 1.2 to 29.8 ± 3.7, as expected. The amounts of total DNA recovered from the bacteria cells extracted ranged from 0.9 ± 0.4 to 2.1 ± 1.3.

An important quantity of extracellular DNA from different environment samples is recovered from plants, animals, bacteria, and viruses after death of these organisms (Paget and Simonet, 1994). The amount of extracellular DNA depends on the type of aquatic environments and the extraction methods used (Paul et al., 1987; Karl and Bailiff, 1989; England et al., 2005). It has recently been found that the concentration of extracellular DNA in coastal sediments ranged from 6.7 to 22.6 $\mu g\ L^{-1}$ (Corinaldesi et al., 2007). In this study, the concentration of extracellular DNA recovered ranged from 12.5 to 35.8 and from 8.6 to 29.8 $\mu g\ g^{-1}$ dry sediments in the primary sediment samples (S_1) and in fraction S_2, respectively. These values include the prokaryotic and eukaryotic amounts of extracellular DNA.

The DNA yield recovered from bacteria cells previously extracted from sediment matrix using Nycodenz gradient centrifugation ranged from 0.9 to 2.1 $\mu g\ g^{-1}$ dry sediments. These values correspond respectively to the range of 5.5×10^6 to 2.6×10^8 extracted cells g^{-1} dry sediments (DDC direct count). These results suggest that the total DNA recovered from fraction S_1 contained more DNA than could originate from the bacteria cells present. Since a direct lysis procedure has been used (Ranjard et al., 1998), contamination by eukaryotic cells or the presence of extracellular DNA could contribute to the excess of DNA. Assuming a mean DNA content of 1.5–9 fg/cell, and compared to previous studies (Bakken and Olsen, 1989; Ranjard et al., 1998; Courtois et al., 2001; Maron et al., 2006), the DNA yields recovered from bacterial cells in the present study could be a significant factor in evaluating the microbial diversity found in sediments samples.

3.4. Faecal indicator bacteria

Table 4 shows the *E. coli* and *Enterococcus* densities measured in steps S_1, S_2, and S_3. In the sediments from the Chamberonne River (CR), the *E. coli* concentration ranged from 5×10^4 ($\pm 1.5 \times 10^4$) to 8.3×10^5 ($\pm 2.6 \times 10^5$) CFU $100\ g^{-1}$ dry sediments and *Enterococcus* ranged from 4.7×10^3 ($\pm 3.2 \times 10^3$) to 5.9×10^4 ($\pm 2.7 \times 10^4$) $\mu g\ 100\ g^{-1}$ dry sediments. The *E. coli* concentration in the Bay of Vidy (BV) sediments ranged from 5×10^4 ($\pm 1.5 \times 10^4$) to 8.3×10^5 ($\pm 2.6 \times 10^5$) $\mu g\ 100\ g^{-1}$ dry sediments and *Enterococcus* from 4.7×10^3 ($\pm 3.2 \times 10^3$) to 5.9×10^4 ($\pm 2.7 \times 10^4$) $\mu g\ 100\ g^{-1}$ dry sediments. Highest FIB concentrations in all four cases were detected in the supernatant of S_2. Compared to the FIB densities recovered in step S_1, the increasing factor (IF) showed that 90–96.4% of FIB was recovered in step S_2 and 13.3–47.8% in the recovery cell (step S_3). No FIB was detected in the sediments from CDG and VE. These two regions are known to have no faecal pollution.

Table 4
Faecal indicator bacteria *E. coli* and *Enterococcus* concentration ($\mu g\ g^{-1}$ dry sediment) in selected steps of the method.

	S_1[b]	S_2[b] (f_1[c])	S_3[b]
E. coli ($\mu g\ g^{-1}$ dry sediment)[a]			
CDG	b.d.l.	b.d.l.	b.d.l.
VE	b.d.l.	b.d.l.	b.d.l.
CR	4.5×10^4 (1.5×10^4)	8.3×10^5 (2.6×10^5)	5.8×10^4 (2.2×10^4)
BV	2.6×10^5 (1.3×10^4)	6.4×10^6 (5.2×10^6)	0.3×10^6 (7.4×10^5)
E. coli (%) IF[d]			
CR		94.6	22.4
BV		95.9	13.3
Enterococus ($\mu g\ g^{-1}$ dry sediment)[a]			
CDG	b.d.l.	b.d.l.	b.d.l.
VE	b.d.l.	b.d.l.	b.d.l.
CR	4.7×10^3 (3.2×10^3)	5.9×10^4 (2.7×10^4)	0.9×10^4 (8.4×10^3)
BV	3.2×10^4 (2.4×10^4)	8.8×10^5 (1.6×10^5)	4.9×10^4 (1.5×10^4)
E. coli (%) IF[d]			
CR		92.0	47.8
BV		96.4	34.7

[a] Standard deviation is indicated in parentheses (statistically significant coefficients are in bold $P < 0.05$).
[b] S_1 initial sediment, S_2 supernatant after centrifugation, S_3 extracted cells, summered in Fig. 1.
[c] f_1 first fraction of S_2 (low centrifugation at $750 \times g$ after mixing initial freshwater sediments with $(Na_2(PO_3)_6$ 2%).
[d] IF: increasing factor of FIB recovered, calculated as (number of bacteria in S_2 or S_3) – number of bacteria in S_1)/number of bacteria in S_2 or S_3); b.d.l.: below detection limit.

Sediment in the aquatic environment may constitute a reservoir of different pollutants including heavy metals and microorganisms. Some studies assess the presence of FIB in both sediments and in water column (LaLiberte and Grimes, 1982; Anderson et al., 2005; Evanson and Ambrose, 2006) demonstrating the influence of environmental conditions on the persistence and accumulation of sediment hosted bacteria at levels 100–1000 times higher than found in the water column, with a potential risk of pollution for recreational coastal water. As sediments can be a reservoir for FIB, only sampling overlying water may greatly underestimate the risk of exposure to potentially pathogenic micro-organisms in recreational water (An et al., 2002; Craig et al., 2002).

One of the aims of this study was to evaluate how efficiently the Nycondez density gradient centrifugation method extracts FIB from contaminated sediments. Compared to FIB concentration obtained in the initial sediments and in the recovered bacteria cells, more than 90% of *E. coli* and *Enterococcus* were recovered in step S_2. Several previous studies have evaluated the techniques of separating FIB from different sediment characteristics (McDaniel and Capone, 1985; Craig et al., 2002). It has been demonstrated that sediment characteristics including particles size and organic carbon content have a considerable influence on the separation of FIB from sediments, and it has also been demonstrated that the

optimal treatment time needs to be established. The results of this study suggest that using sodium hexametaphosphate solution 2% following the method described is effective in isolating FIB from contaminated sediments.

4. Conclusions

The results of this study showed considerable variability on the quantification of indigenous bacteria and faecal indicator bacteria including *E. coli* and *Enterococcus* in the different steps of the method. The amount of bacteria quantified for all investigated sediment samples increased when using a 2% sodium hexametaphosphate solution. Direct and indirect bacteria counts showed a decreasing amount of recovered purified cells using Nycondez density gradient centrifugation (S_3). The results of this study show that Nycondez gradient centrifugation may be a useful alternative procedure to extract purified bacteria cells from complex matrix as freshwater sediments, even rich in organic matter content. As in soils, this method (step S_3) allows to obtain high purity of bacteria cells from sediments avoiding problems of co-extraction of large amounts of extracellular DNA as well as contaminating with organic and inorganic impurities.

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