Bacterial community analysis of activated sludge: an evaluation of four commonly used DNA extraction methods

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ABSTRACT

The effectiveness of three commercially available direct DNA isolation kits (Mobio, Fast, Qiagen) and one published direct DNA extraction protocol (Bead) for extracting bacterial DNA from different types of activated sludge was investigated and mutually compared. The DNA quantity and purity were determined using real-time PCR targeting the bacterial 16S rDNA gene. Microbial community fingerprints were assessed by automated ribosomal intergenic spacer analysis. The resulting community profiles were analyzed with canonical correspondence analysis. Our results clearly demonstrate that direct DNA extraction methods can significantly influence the DNA quantity, purity and observed community patterns of microbiota in activated sludge. Fast and Mobio generated high amounts of good quality DNA compared to Bead and Qiagen. Mobio also resulted in the detection of the highest number of species while Fast scored the best in discriminating between the community patterns of different activated sludge types. With respect to the characterization of community profiles, our analyses demonstrated a strong sludge type dependent variability among methods. Taking into account our results, we recommend Fast as the most suitable DNA extraction method for activated sludge samples used for bacterial community studies.

Keywords: activated sludge, ARISA, community fingerprinting, direct DNA extraction, real-time PCR

Introduction

To date, most wastewater treatment processes rely on the use of activated sludge, which is a common term used to define a very heterogeneous assembly of microorganisms, mainly dominated by a number of bacterial taxa (Gerardi 2006). Especially thanks to the presence of some of these bacterial communities, undesirable organic compounds and micropollutants, such as pharmaceuticals, hormones and pesticides, can effectively be degraded and removed from the wastewater (Radjenovic et al. 2009; Stasinakis 2009).

In order to determine which factors influence the efficiency and stability of biological wastewater treatment plants and to develop strategies for an improved process performance (Nielsen et al. 2009a), we need to gain a better understanding of the community composition and functioning of activated sludge microbiota and how these microbiota are related to environmental variables (e.g., influent quality) and both system and operational parameters. Such knowledge improvement largely depends on molecular techniques that allow the reliable assessment of bacterial community composition. Automated Ribosomal Intergenic Spacer Analysis (ARISA), a genetic fingerprinting technique with a high resolution and sensitivity, is commonly used to assess the composition of complex prokaryotic communities in a rapid and accurate way (Danovaro et al. 2006; Xin et al. 2008; Steele et al. 2005; Lear et al. 2008). ARISA is based on the amplification of the intergenic region between the 16S en 23S rDNA genes in the rDNA operon (known as the Internal Transcribed Spacer (ITS) region), which is characterized by a significant variability in length and nucleotide sequence among different bacterial taxa (Daffonchio et al. 2003). Essential for most molecular biodiversity analyses is the use of samples consisting of pure nucleic acids that provide a realistic representation of the community composition. However, it is commonly acknowledged that fingerprinting patterns can be influenced by the used DNA extraction method (Carrigg et al. 2007). Efficient

extraction of good quality bacterial DNA remains a challenge, especially when working with samples consisting of a complex microbial matrix and a potentially high concentration of PCR inhibitors like soil and activated sludge. It is suggested that direct DNA extraction methods, based on cell lysis within the sample matrix and subsequent separation of DNA from the matrix and cell debris, are best suited for complex samples. Numerous lab-designed and commercial DNA extraction methods are currently available. Despite the common use of the latter ones, they have never been compared for specific applications (Maier et al. 2009). Depending on sample characteristics, some methods provide a high DNA yield while others seem to generate a better DNA purity. For example, Chen and coworkers (2006) compared a solvent-based and a commercially available DNA extraction method in order to enumerate fecal coliforms in biosolids by competitive PCR. They found that the commercial DNA kit provided a low recovery due to an inefficient cell lysis. In contrast, a study performed by Maarit Niemi *et al.* (2001), where different DNA extraction and purification methods were evaluated for soil samples by PCR-DGGE, showed that the commercial method yielded the highest DNA concentration and the most intensive and reproducible banding pattern.

When working on sludge samples, the majority of studies are using protocols intended for habitats like soil without adaptation (McIlroy et al. 2009). We therefore evaluated and compared four direct DNA extractions in their ability to extract good quality DNA of three sludge samples from different origins, and we compared the fingerprinting patterns and determined which DNA extraction method yields the most robust and suitable results for application on activated sludge.

Material and Methods

Experimental design

An experiment with a two-factorial design was performed, by applying four direct DNA extraction methods on three types of activated sludge. Bacterial cell densities (number of cells/g sludge) and DNA purity (presence/absence of PCR inhibitors) were determined by means of a real-time PCR targeting the bacterial 16S rDNA gene. ARISA was used to generate microbial community profiles and to determine the bacterial species richness (total amount of Operational Taxonomic Units (OTUs)). Prior to DNA extraction, each sludge sample was divided into seven independent replicate subsamples [250 ± 10 mg sludge (wet weight)].

Sludge type

Three different sludge samples from Agristo (Harelbeke, Belgium), Danone (Rotselaar, Belgium) and Waterleau (Wespelaar, Belgium), treating wastewater from the potato, diary and sugar industry, respectively, were collected. Upon arrival in the lab, the mixed liquor suspended solids (MLSS) was measured in accordance with standard methods (APHA 1995) and the structure of the three sludge communities was visualized by means of inverted microscopy (CKX41, Olympus). Afterwards, the samples were stored at -80°C until use.

DNA extraction method

Three harsh lysis DNA extraction methods based on bead beating and one soft method that is based on chemical lysis were selected.

Concerning the harsh lysis methods, two commercial DNA extraction methods, namely, Mobio Ultraclean™ soil DNA kit (Cambio) and the Fast DNA spin kit (Bio 101, Qbiogene Inc) and one DNA extraction method based on a previously published technique (Boon et al.

2000), were used. The three mentioned methods are further referred to as 'Mobio', 'Fast' and 'Bead' respectively. The Bead extraction procedure was undertaken as described by Boon *et al.* (2000) and adapted to an initial sludge weight volume of 250 mg. The Mobio and Fast extraction methods were performed as recommended in the manufacturer's instruction.

For the soft lysis method, a combination of the QIAamp® DNA Stool Mini Kit and the QIAamp DNA Blood Mini kit (Qiagen GmbH), as described by Weiss *et al.* (2007), was selected. This method will further be referred to as 'Qiagen'. The reaction volumes were adapted to an initial sludge weight volume of 250 mg.

For the four extraction procedures, a final elution volume of 50 µl was obtained and aliquots of the extracted DNA were stored at -20°C until further use.

Assessment of the DNA extraction efficiency

The bacterial DNA concentrations and amplification efficiency (presence of PCR inhibitors) by using a quantitative Taqman based 16S rDNA real-time PCR were determined. The amplification efficiencies were evaluated using a Taqman® Exogenous Internal Positive Control (IPC) (Applied Biosystems) (Hartman et al. 2005). For each sludge type, three randomly chosen subsamples were assayed together with their 10-fold dilution in duplo in a 25 µL real-time PCR reaction containing 2.5 µL of template DNA, 12.5 µL of 10xTaqMan® Universal PCR Master Mix (Applied Biosystems), 12.5 pmol of forward P891F and reverse primer P1033R, 3.75 pmol of the FAM® labeled probe and 5 µL 2% bovine serum albumin (Sigma-Aldrich) (Nonnenmacher et al. 2004). The IPC was co-amplified in each PCR reaction, according to the manufacturer's instructions. We used double distilled water (ddH_2O) as a negative control and 10-fold serial dilutions of known amounts of *Escherichia coli* DNA (positive control DNA) were included in triplo in each run. The positive control DNA was prepared by culturing *E. coli* for 24h in Luria-Bertani (LB) medium in a shaking

incubator at 120 rpm. The genomic DNA was extracted using a Nucleospin® Tissue Kit (Macherey-Nagel), according to the manufacturers' instructions. Subsequently, the concentration and the purity of the positive control DNA was measured by spectrophotometric measurements (Nanodrop). The initial amount of bacterial cells was enumerated by plating serial dilutions on LB-agar plates. The 16S rDNA copy number was estimated on the basis of the genome size (4.64 Mbp) and seven copies of the 16S rDNA per *E. coli* genome (Okano et al. 2004). DNA amplification and PCR product detection were performed using the ABI Prism® 7000 Sequence Detection System (Applied Biosystems). A standardized real-time PCR reaction contained the following steps: 2 min at 50 °C, 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. The *E. coli* standard curve was automatically generated by the ABI Prism system by plotting the cycle threshold (Ct), at which the threshold fluorescence was reached, versus the logarithmic concentration of positive control DNA. The Ct is defined as the number of cycles required for the fluorescent signal of the target DNA to cross the threshold (i.e. exceeds background signal). Ct levels are inversely proportional to the amount of target nucleic acid in the sample.

We analyzed the real-time PCR data using the SDS 1.2.3. software (Applied Biosystems).

PCR inhibition was determined by comparing the amplification plot of the IPC of the samples with the amplification plot of the IPC of the positive control DNA. An IPC amplifies under the same PCR conditions as the target DNA but by using its own primer and probe set (Hartman et al. 2005).This means that the target DNA amplification efficiency will never be affected by competition of both IPC and target DNA for the same primer and probe set. As PCR inhibition mostly concerns inhibition of the DNA polymerase (Stark et al. 2000), the IPC and the target DNA are exposed to the same degree of amplification inhibition.

We defined "good quality DNA" as DNA that did not show amplification inhibition in real-time PCR analyses.

Assessment of bacterial community profiles

ARISA PCR was performed using approximately 10 ng of sludge DNA and the ITSF/ITSReub primerset, as previously described by Cardinale *et al.* (2004). Five µL of each PCR amplicon were visually checked on a 1.5% agarose gel after GelRed™ staining (Biotium, Hayward). Hereafter, 1 µL of each amplicon was mixed with 0.3 µL internal size standard (GeneScan™ 1200 Liz® Size Standard, Applied Biosystems) and 8.7 µL deionized formamide. ARISA-PCR fragments were separated by capillary electrophoresis on an ABI Prism 3130 Genetic analyser using POP7 polymer (Applied Biosystems). The ARISA profiles were analyzed using Genemapper (version 4.0, Applied Biosystems). To obtain a maximum number of peaks, defined as OTUs, we used a threshold of 50 fluorescence units (baseline). Two peaks were considered identical if the difference between both was less than 0.5 bp. Due to a potential bias in DNA extraction and PCR amplification, peak height cannot be considered proportional to the relative abundance of bacterial species (Suzuki and Giovannoni 1996; Yannarell and Triplett 2004) and hence, OTU presence/absence data were used for analysis. For each sludge subsample, bacterial species richness was expressed as the total number of OTUs within the corresponding electropherogram.

Data analysis

The amount of extracted DNA (experessed as bacterial cell density) and bacterial species richness (total number of OTUs detected) were analyzed with two-way ANOVA, with sludge type and DNA extraction methods as explanatory variables. Being mainly interested in the overall performance of DNA extraction methods across sludge types, we further explored the main effects of this factor by comparing all combinations of its levels using contrast analysis.

A contrast analysis tests the statistical significance for an a priori defined set of comparisons among the levels of experimental factors. Bacterial cell densities were logarithmically transformed prior to analysis. All univariate analyses were performed with Statistica 9.0.

The relationship between the observed microbial community composition (presence/absence data of OTUs) and the experimental factors (sludge type and DNA extraction method) was explored by means of Canonical Correspondence Analysis (CCA). CCA is a direct gradient ordination technique that allows to formally test for the significance of experimental factors on community data, as well as the interaction effects among factors (Lepš and Šmilauer 2003). Significance tests were performed with random Monte Carlo permutation tests (9999 unrestricted permutations per test). The CCA was performed using the software package Canoco for Windows, version 4.5 (Biometris Plant Research International).

Results

Sludge characterization

The Agristo (mainly starch content), the Danone (mainly protein content) and the Waterleau sludge (mainly saccharides content), contained a MLSS of 17.8 ± 2.1 g/L, 13.4 g/L ± 1.4 and 6.5 ± 0.5 g/L, respectively. The Danone sludge contained a very high amount of filamentous bacteria compared to Agristo and Waterleau (Fig 1).

DNA extraction efficiency

Two-way ANOVA revealed a highly significant DNA extraction method and sludge type interaction effect and a significant main effect of the DNA extraction method on the observed bacterial cell density (Table 1). Contrast analysis showed significant differences among all DNA extraction methods, except between ‘Qiagen’ and ‘Bead’ (Table 2). Concerning the Danone sludge, Fast and Mobio showed a high and comparable DNA yield, in contrast to Bead and Qiagen. While for the Agristo and Waterleau sludge, Fast appeared to be the best method (Fig 2).

Fast and Mobio always yielded good quality DNA without any PCR amplification inhibition. In contrast, when microbial DNA was extracted using the Qiagen and Bead methods, inhibition of IPC amplification was detected in both the original sample and the 10-fold dilution (Fig 2).

Bacterial community assessment

Electropherograms from the three different sludge types and the four tested DNA extraction methods are shown in Fig. 2. In general, OTUs ranging from 121 to 1074 bp were obtained and the majority of them had a length between 200 and 700 bp. For all samples a total of 182 distinct OTUs were identified.

As for the bacterial cell density, the observed bacterial species richness was significantly influenced by an interaction between DNA extraction method and sludge type (Table 1). By comparing the different methods mutually, the contrast analysis indicated significant differences among all DNA extraction methods, except between 'Qiagen' and 'Fast' (Table 2). For all three sludge types, Mobio yielded the highest bacterial richness (4).

CCA-analysis showed significant effects of DNA extraction method and sludge type on the bacterial community composition (Fig 5, Table 3). Samples treated with the 'Bead' and 'Fast' techniques yielded very similar community profiles along the first and second axis compared to results obtained from the 'Qiagen' and 'Mobio' methods (Fig 5A, Table 3). Sludge type and DNA extraction method both explained 18% of total bacterial community variation (Fig 5A and B, Table 3), and sludge types tended to yield different community profiles when subjected to different DNA extraction techniques (Fig 5C, Table 3). This sludge x DNA extraction interaction was more pronounced than the main effects of the experimental factors as it explained 49% of the total amount of community variability in the dataset.

Discussion

Assessment of the DNA extraction efficiency

Although the DNA-yield of each of the four DNA-extraction techniques depended on the sludge type (cf. extraction method x sludge type interaction), we observed systematic differences in performance of the extraction methods (cf. main effect of extraction method).

We used three DNA extraction methods in which cells were lysed by means of beads, namely: Fast and Mobio, which are two commercial kits, and the commonly used standard bead beating lab protocol. Fast and Mobio tended to yield the highest amounts of goof quality DNA for all sludge types. The Danone sludge scored surprisingly high for Fast and Mobio compared to Bead and Qiagen. Microscopic analyses demonstrated that this sludge

community was dominated by filamentous bacteria (Fig 1A) compared to the other sludge types (Fig 1B, 1C). Bourrain *et al.* (1999) tested different floc dispersion and cell lysis treatments on different sludge types as a pretreatment step for DNA-extraction and concluded that the lysis efficiency strongly depends on the amount of filamentous bacteria present in the samples. Most filamentous bacteria, originating from industrial WWTPs, are usually Gram positive (Nielsen et al. 2009b; Martins et al. 2004; Nielsen et al. 2009a) and thus characterized by a thick cell wall containing covalently associated peptidoglycans, which are critical for maintaining cell shape and -rigidity (Madigan et al. 2009). Due to the resistant cell wall, Gram-positive bacteria show a high resistance to enzymatic lysis (Niwa et al. 2005). Compared to the other two extraction methods Qiagen and Bead, we thus found Mobio and Fast to be the more appropriate methods for the lysis of foaming or bulking sludge, based on the DNA yields for Danone sludge.

For all three sludge types, both Fast and Mobio were successful in removing PCR amplification inhibitors. Our results confirm other studies where these two commercial kits were used for extracting DNA from other environmental samples like soil and sediments (Jiang et al. 2005; Webster et al. 2003; Maarit Niemi et al. 2001; Stach et al. 2001).

DNA extracted by means of the bead beater protocol was commonly hampered by PCR inhibition. In contrast to Mobio and Fast, which are spin column based methods, the Bead protocol contains a chloroform/isoamyl alcohol purification and isopropanol precipitation. The purification with phenol requires several steps transferring the DNA-containing aqueous supernatant to new tubes without disturbing the interphase. As a consequence, it is not feasible to always transfer all supernatant from one tube to another and loss of DNA is unavoidable (Merk et al. 2001). Despite the fact that the spin column technology doesn’t have a 100% recovery, it is a simple and quick method to extract nucleic acids from biological samples. Furthermore, most column-based procedures do not require hazardous chemicals

like chloroform, which are commonly used in traditional nucleic acid extraction procedures (Tolosa et al. 2007).

The fourth DNA extraction method we used (Qiagen) was a combination of two commercial kits and relied on chemical cell lysis. Real-time PCR results showed a strong amplification inhibition resulting in a quite low DNA amount for all samples. This probably might be due to the absence of a physical lysis of the sludge flocs. In order to extract DNA from activated sludge, it is necessary to disrupt and destruct bacterial cells, regardless of their biochemical composition or their localization in the floc community (Bourrain et al. 1999). Although the Qiagen method has been successfully applied on tissues (Chan et al. 2001), soils (Zoll et al. 2005), fluids (Santos et al. 2010), oil (Ben Ayed et al. 2009) and plants (Elizaquivel and Aznar 2008), it underperformed in our study. Concerning the extraction efficiency, we can conclude that the Qiagen and Bead method are not to be recommended as extraction methods for DNA from activated sludge.

Bacterial community assessment

Analyses of the ARISA data show that the DNA extraction method can strongly affect both the observed bacterial species richness and community profile patterns (Table 2 and 3).

Concerning the bacterial species richness, Mobio yielded the highest bacterial richness for every sludge type. It was found to be highly significantly different compared to the other extraction methods in the contrast analysis. In relation to the sludge type, the differences are not so pronounced as found for the DNA extraction efficiencies (Fig 2). But, regarding the community profile, the CCA plot (Fig 5C) illustrates that the ‘Bead’ and ‘Fast’ techniques tend to group together and yield similar community profiles, whereas the Mobio and Qiagen techniques result in profiles that tend to group along the first but not along the second CCA-axis. A large amount of the variation was explained by the interaction among extraction

method and sludge type, indicating that the relative difference in community profiles among extraction techniques varied strongly with sludge type (Fig 5C). Indeed, differences among extraction techniques tended to be small for all Waterleau analyses, whereas these differences tended to be more pronounced for the Agristo and Danone sludge. The observed interaction highlights a potentially important problem related to the study of sludge community profiles, as the observed community patterns may be partly determined by the specific performance of a given DNA extraction method for a give sludge type, and there is no objective reference available that allows evaluating which method leads to the best approximation of the real community composition for a give sludge type. The three sludge types analyzed in our study differed very strongly in floc structure (e.g. high amount of filamentous bacteria in Danone versus low amount of suspended solids in Waterleau) and origin (potato-, sugar- and diary industry) (Fig 1). Perhaps studies comparing community profiles among samples of a similar sludge type may be less affected by the substrate dependency of method efficiencies and biases. Furthermore, some criteria, such as DNA yield, degree of PCR inhibition and observed number of species may be useful as potential guidelines to evaluate the reliability of community profiles. For example, in our study, we would tend to suggest the patterns delivered by the Mobio and the Fast method, since both methods had a high DNA yield, did not suffer from PCR inhibition in any of the samples, and also resulted in the detection of the highest number of species, suggesting to be the methods with the highest overall performance. However, concerning the ability to distinguish between different sludge types, Fast delivered better results (Fig 5C). When using the Mobio method it's difficult to distinguish the Waterleau and Danone sludge. From this, it can be expected that the Fast method would also result in the most reliable community patterns.

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REFERENCES

APHA (1995) Standard methods for the examination of water and wastewater. American Public Health Association, Washington, DC 20th ed

Ben Ayed R, Grati-Kamoun N, Moreau F, Rebai A (2009) Comparative study of microsatellite profiles of DNA from oil and leaves of two tunisian olive cultivars. Eur Food Res Technol 229 (5):757-762. doi:10.1007/s00217-009-1111-3

Boon N, Goris J, De Vos P, Verstraete W, Top EM (2000) Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading comamonas testosteroni strain, i2gfp. Appl Environ Microbiol 66 (7):2906-2913. doi:10.1128/aem.66.7.2906-2913.2000

Bourrain M, Achouak W, Urbain V, Heulin T (1999) DNA extraction from activated sludges. Curr Microbiol 38 (6):315-319

Cardinale M, Brusetti L, Quatrini P, Borin S, Puglia AM, Rizzi A, Zanardini E, Sorlini C, Corselli C, Daffonchio D (2004) Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. Appl Environ Microbiol 70 (10):6147-6156. doi:10.1128/aem.70.10.6147-6156.2004

Carrigg C, Rice O, Kavanagh S, Collins G, O'Flaherty V (2007) DNA extraction method affects microbial community profiles from soils and sediment. Appl Microbiol Biotechnol 77 (4):955-964

Chan PKS, Chan DPC, To KF, Yu MY, Cheung JLK, Cheng AF (2001) Evaluation of extraction methods from paraffin wax embedded tissues for PCR amplification of human and viral DNA. J Clin Pathol 54 (5):401-403

Chen Y-C, Higgins MJ, Maas NA, Murthy SN (2006) DNA extraction and *Escherichia coli* quantification of anaerobically digested biosolids using the competitive touchdown pcr method. Water Res 40 (16):3037-3044

Daffonchio D, Cherif A, Brusetti L, Rizzi A, Mora D, Boudabous A, Borin S (2003) Nature of polymorphisms in 16s-23s rrna gene intergenic transcribed spacer fingerprinting of bacillus and related genera. Appl Environ Microbiol 69 (9):5128-5137. doi:10.1128/aem.69.9.5128-5137.2003

Danovaro R, Luna GM, Dell'Anno A, Pietrangeli B (2006) Comparison of two fingerprinting techniques, terminal restriction fragment length polymorphism and automated ribosomal intergenic spacer analysis, for determination of bacterial diversity in aquatic environments. Appl Environ Microbiol 72 (9):5982-5989

Elizaquivel P, Aznar R (2008) Comparison of four commercial DNA extraction kits for PCR detection of *Listeria monocytogenes, Salmonella, Escherichia coli* O157:H7, and *Staphylococcus aureus* in fresh, minimally processed vegetables. J Food Prot 71 (10):2110-2114

Gerardi MH (2006) Wastewater bacteria. Wastewater microbiology series. Wiley-Interscience, Hoboken, New Jersey

Hartman LJ, Coyne SR, Norwood DA (2005) Development of a novel internal positive control for Taqman® based assays. Mol Cell Probes 19 (1):51-59

Jiang J, Alderisio KA, Singh A, Xiao L (2005) Development of procedures for direct extraction of cryptosporidium DNA from water concentrates and for relief of PCR inhibitors. Appl Environ Microbiol 71 (3):1135-1141. doi:10.1128/aem.71.3.1135-1141.2005

Lear G, Anderson MJ, Smith JP, Boxen K, Lewis GD (2008) Spatial and temporal heterogeneity of the bacterial communities in stream epilithic biofilms. FEMS Microbiol Ecol 65:463-473

Lepš J, Šmilauer P (2003) Multivariate analysis of ecological data using Canoco. Cambridge University Press, Cambridge, New York

Maarit Niemi R, Heiskanen I, Wallenius K, Lindström K (2001) Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. J Microbiol Methods 45 (3):155-165

Madigan MT, Madigan MT, Brock TD (2009) Brock biology of microorganisms. Pearson/Benjamin Cummings, San Francisco, California

Maier RM, Pepper IL, Gerba CP (2009) Environmental microbiology. Academic Press, Burlington, MA

Martins AMP, Pagilla K, Heijnen JJ, van Loosdrecht MCM (2004) Filamentous bulking sludge--a critical review. Water Res 38 (4):793-817

McIlroy S, Porter K, Seviour R, Tillett D (2009) Extracting nucleic acids from activated sludge which reflect community population diversity. Antonie van Leeuwenhoek 96 (4):593-605

Merk S, Neubauer H, Meyer H, Greiser-Wilke I (2001) Comparison of different methods for the isolation of *Burkholderia cepacia* DNA from pure cultures and waste water. Int J Hyg Environ Health 204 (2-3):127-131

Nielsen PH, Daims H, Lemmer H (2009a) Fish handbook for biological wastewater treatment. IWA publishing,

Nielsen PH, Kragelund C, Seviour R, J., Nielsen JL (2009b) Identity and ecophysiology of filamentous bacteria in activated sludge. FEMS Microbiol Rev 33 (6):969-998

Niwa T, Kawamura Y, Katagiri Y, Ezaki T (2005) Lytic enzyme, labiase for a broad range of gram-positive bacteria and its application to analyze functional DNA/rna. J Microbiol Methods 61 (2):251-260

Nonnenmacher C, Dalpke A, Mutters R, Heeg K (2004) Quantitative detection of periodontopathogens by real-time PCR. J Microbiol Methods 59 (1):117-125. doi:10.1016/j.mimet.2004.06.006

Okano Y, Hristova KR, Leutenegger CM, Jackson LE, Denison RF, Gebreyesus B, Lebauer D, Scow KM (2004) Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. Appl Environ Microbiol 70 (2):1008-1016. doi:10.1128/aem.70.2.1008-1016.2004

Radjenovic J, Petrovic M, Barcelo D (2009) Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. Water Res 43 (3):831-841. doi:10.1016/j.watres.2008.11.043

Santos A, Cremades R, Rodríguez JC, García-Pachón E, Ruiz M, Royo G (2010) Comparison of methods of DNA extraction for real-time PCR in a model of pleural tuberculosis. APMIS 118 (1):60-65

Stach J, E. M. , Stephan B, Clapp Justin P, Burns Richard G (2001) PCR-SSCP comparison of 16s rdna sequence diversity in soil DNA obtained using different isolation and purification methods. FEMS Microbiol Ecol 36 (2-3):139-151

Stark PC, Mullen KI, Banton K, Russotti R, Soran D, Kuske CR (2000) Pre-PCR DNA quantitation of soil and sediment samples: Method development and instrument design. Soil Biol Biochem 32 (8-9):1101-1110

Stasinakis AS (2009) Diuron biodegradation in activated sludge batch reactors under aerobic and anoxic conditions. Water Res 43 (5):1471

Steele JA, Ozis F, Fuhrman JA, Devinny JS (2005) Structure of microbial communities in ethanol biofilters. Chem Eng J 113 (2-3):135-143

Suzuki MT, Giovannoni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16s rrna genes by PCR. Appl Environ Microbiol 62 (2):625-630

Tolosa JM, Schjenken JE, Civiti TD, Clifton VL, Smith R (2007) Column-based method to simultaneously extract DNA, RNA, and proteins from the same sample. Biotechniques 43 (6):799-804. doi:10.2144/000112594

Webster G, Newberry CJ, Fry JC, Weightman AJ (2003) Assessment of bacterial community structure in the deep sub-seafloor biosphere by 16s rDNA-based techniques: A cautionary tale. J Microbiol Methods 55 (1):155-164

Weiss A, Jérôme V, Freitag R (2007) Comparison of strategies for the isolation of PCR-compatible, genomic DNA from a municipal biogas plants. J Chromatogr B 853 (1-2):190-197

Xin G, Gough HL, Stensel HD (2008) Effect of anoxic selector configuration on sludge volume index control and bacterial population fingerprinting. Water Environ Res 80:2228-2240

Yannarell AC, Triplett EW (2004) Within- and between-lake variability in the composition of bacterioplankton communities: Investigations using multiple spatial scales. Appl Environ Microbiol 70 (1):214-223. doi:10.1128/aem.70.1.214-223.2004

Zoll G, Grote G, Dierstein R, Köhne S (2005) Rapid isolation of anthrax DNA from large-volume soil samples using QIAamp kits. http://www1.qiagen.com/literature/qiagennews/0102/1019238_QNews12002_22_23.pdf.

	SS	*df*	*MS*	*F*	*p*
Bacterial cell density					
DNA extraction	*15.3*	*3*	*732.5*	*62.2*	*< 0.001*
Activated sludge type	*24.3*	*2*	*1745.4*	*167.1*	*< 0.001*
DNA extraction*activate sludge type	*10.2*	*6*	*245.5*	*61.6*	*< 0.001*
Species Richness					
DNA extraction	*3408.7*	*3*	*1136.2*	*23.7*	*< 0.001*
Activated sludge type	*2609.6*	*2*	*1304.8*	*27.2*	*< 0.001*
DNA extraction*activated sludge type	*8026.6*	*6*	*1337.7*	*27.9*	*< 0.001*

Table 1: Two-factorial ANOVA results for bacterial cell density (log cells/g activated sludge) and species richness as dependent variables and DNA extraction method and sludge type as predictor variables. Bacterial cell density data were log transformed before analysis.

	SS	*df*	*F*	*p*
Bacterial cell density				
Fast – Qiagen	*10.6*	*1*	*1529.8*	*< 0.001*
Fast – Bead	*9.9*	*1*	*1431.2*	*< 0.001*
Fast – Mobio	*1.2*	*1*	*181.5*	*< 0.001*
Bead –Mobio	*4.2*	*1*	*593.4*	*< 0.001*
Bead – Qiagen	*0.2*	*1*	*1.6*	*0.21*
Mobio – Qiagen	*4.6*	*1*	*657.4*	*< 0.001*
Species Richness				
Fast – Qiagen	*14*	*1*	*0.3*	*0.59*
Fast – Bead	*405.4*	*1*	*8.5*	*0.005*
Fast – Mobio	*1249.5*	*1*	*32.1*	*< 0.001*
Bead –Mobio	*3169.1*	*1*	*66.1*	*< 0.001*
Bead – Qiagen	*268.2*	*1*	*5.6*	*0.02*
Mobio – Qiagen	*405.4*	*1*	*26*	*< 0.001*

Table 2: Contrast analysis results for bacterial cell density and species richness, exploring pair wise differences among DNA extraction methods. Bacterial cell density data were log transformed before analysis.

	λ_{tot}	*F*	*P*
DNA extraction	*18%*	*6,43*	*< 0.001*
Sludge type	*18%*	*9,95*	*< 0.001*
DNA extraction*Sludge type	*49%*	*13,54*	*< 0.001*

Table 3: Results of CCA permutation analyses on bacterial community data derived from ARISA, testing for the effect of DNA extraction method, sludge type and the interaction between both factors. λ_{tot} represents the percentage of total community variation explained by the experimental factors.

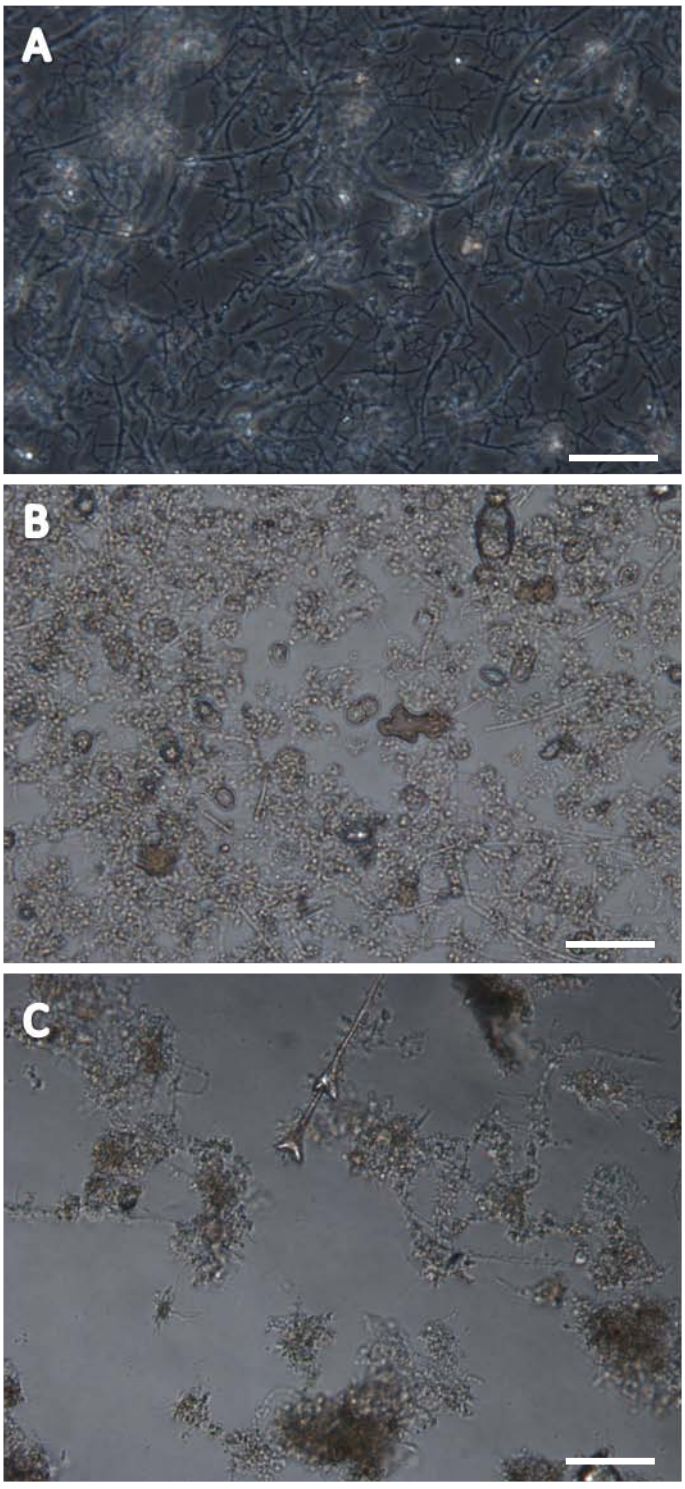

Fig 1: Transmitted light micrograph of the Danone sludge (A), the Agristo sludge (B) and the Waterleau sludge (C). The scale bar represents approximately 100 µm.

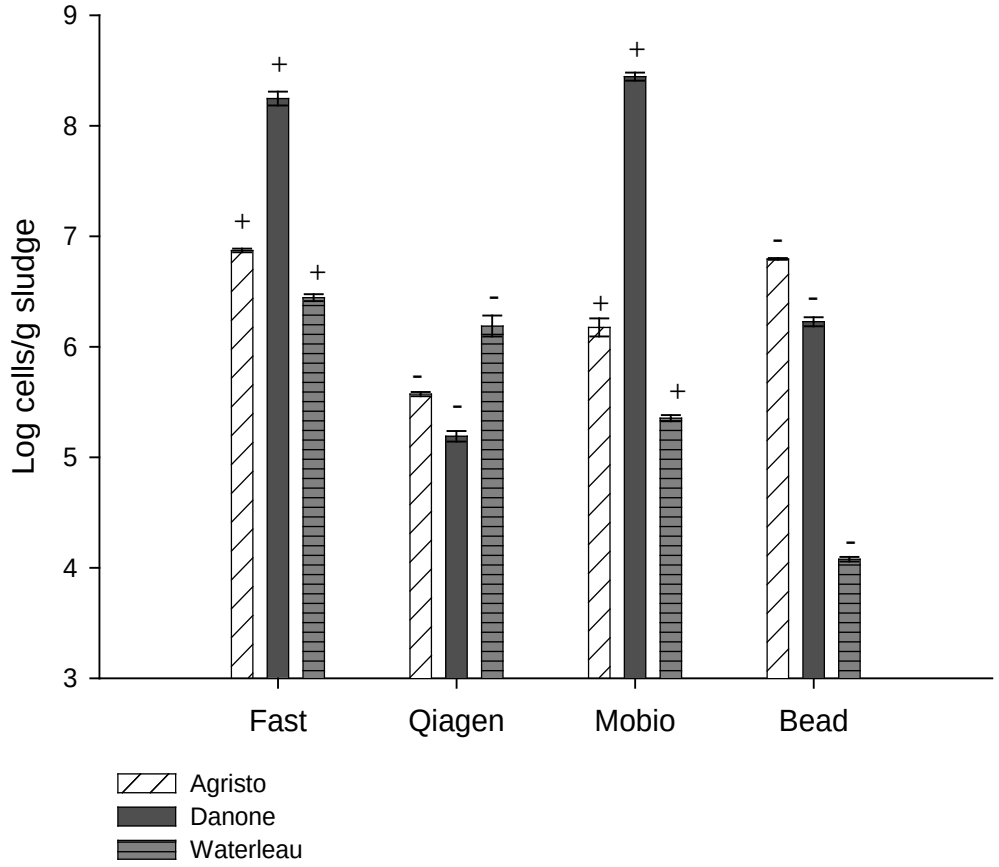

Fig 2: Average yield of DNA (expressed as bacterial cell density) for four different extraction methods using real time PCR targeting the bacterial 16S rNA gene. Error bars represent standard errors (n=3). + means that no inhibition was detected; - means detection of amplification inhibition

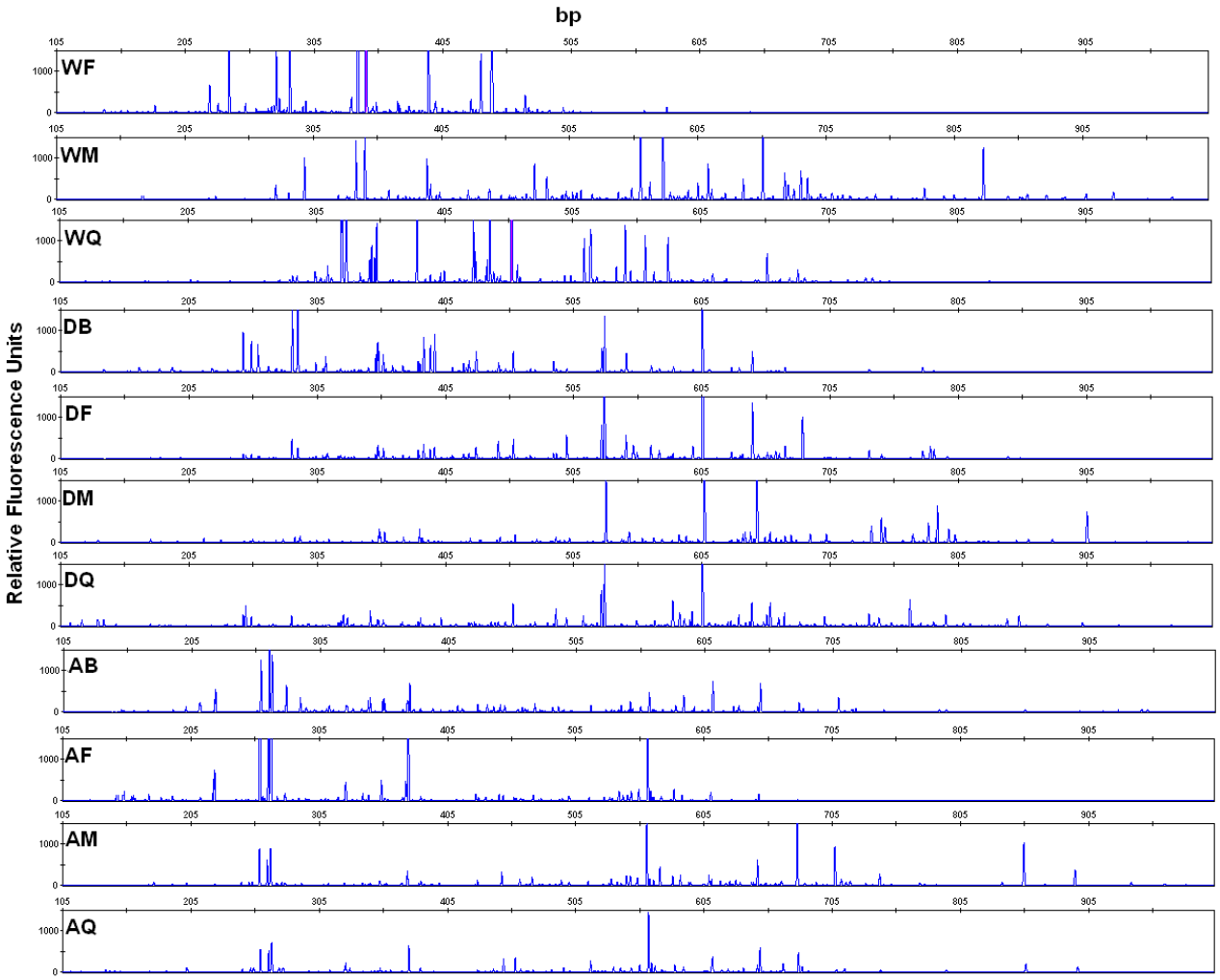

Fig 3: ARISA profiles of Waterleau: (WF) Fast, (WM) Mobio, (WQ) Qiagen; Danone: (DB) Bead, (DF) Fast, (DM) Mobio, (DQ) Qiagen and Agristo: (AB) Bead, (AF) Fast, (AM) Mobio, (AQ) Qiagen. ARISA profiles for the Waterleau sludge, extracted with the Bead method are not available due to a repeated unsuccessful PCR amplification.

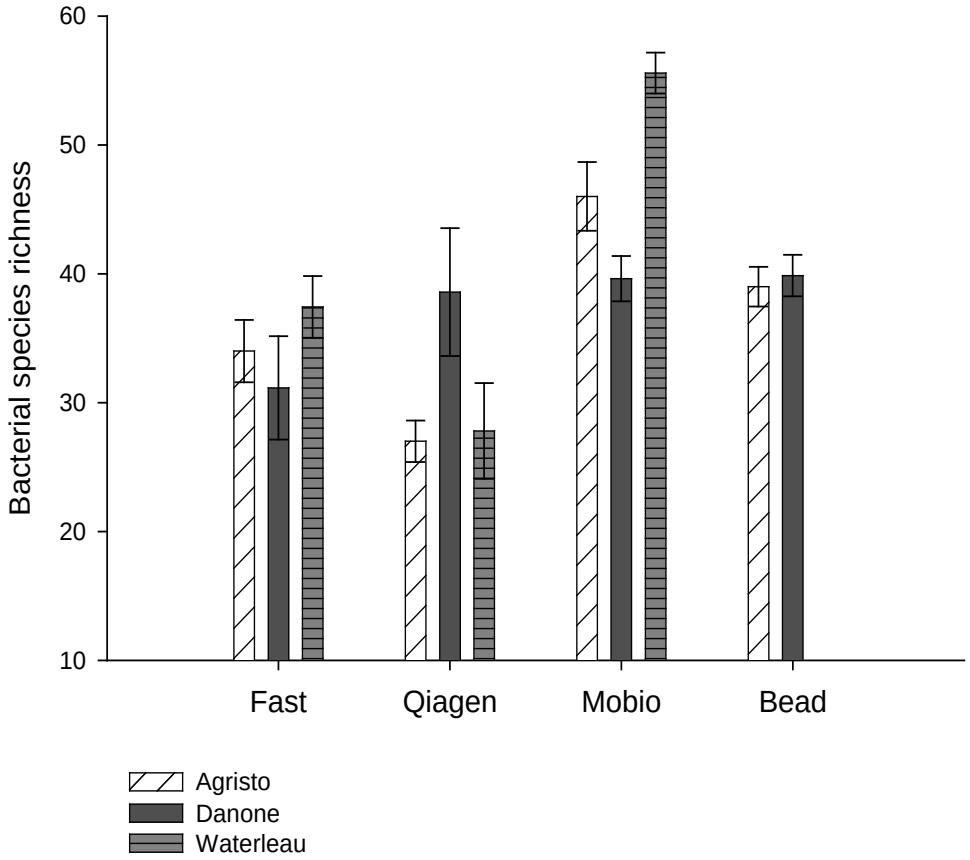

Fig 4: Average bacterial species richness observed upon application of four DNA extraction methods on three different sludge types. Bacterial richness is expressed as the total number of peaks (OTUs) within each ARISA electropherogram. Error bars represent standard errors (n=7). Results for the Bead method in the case of the Waterleau sludge are not available, due to repeated unsuccessful PCR amplification.

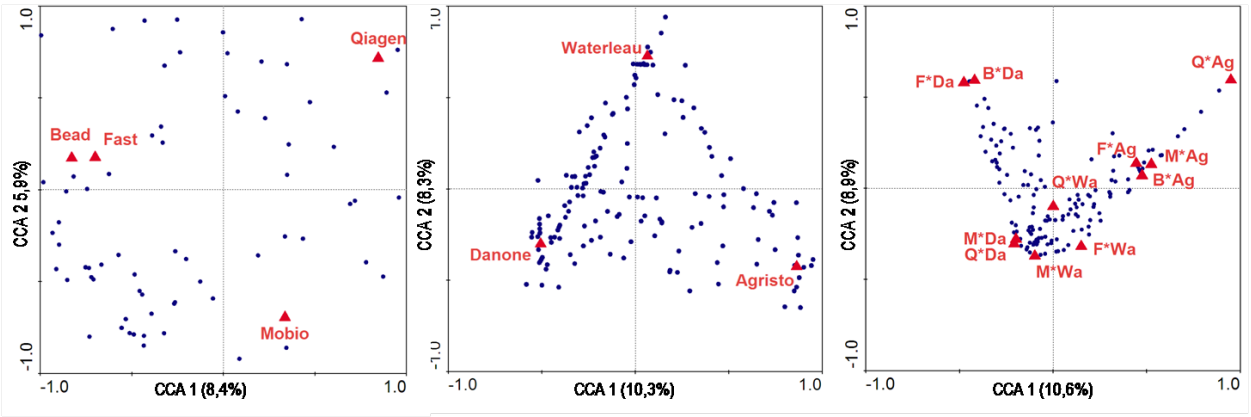

Fig 5: CCA ordination diagram based on the bacterial ARISA data, representing the effects of DNA extraction method (A), sludge type (B) and interactions between both predictor variables (C). Red triangles (centroids) represent the average position of replicates for each level of experimental factors. Blue circles represent individual OTUs (not labeled). There are no data available for the Bead method on Waterleau sludge due to repeated unsuccessful PCR amplification. B = Bead, F = Fast, M = Mobio, Q = Qiagen, Da = Danone, Ag = Agristo and Wa = Waterleau.