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METHODS

How to Deal with PCR Contamination in Molecular Microbial Ecology

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Abstract Microbial ecology studies often use broad-range PCR primers to obtain community profiles. Contaminant microbial DNA present in PCR reagents may therefore be amplified together with template DNA, resulting in unrepeatable data which may be difficult to interpret, especially when template DNA is present at low levels. One possible decontamination method consists in pre-treating PCR mixes with restriction enzymes before heat-inactivating those enzymes prior to the start of the PCR. However, this method has given contrasting results, including a reduction in PCR sensitivity. In this study, we tested the efficiency of two different enzymes (DNase 1 and Sau3AI) as well as the effect of dithiothreitol (DTT), a strong reducing agent, in the decontamination procedure. Our results indicate that enzymatic treatment does reduce contamination levels. However, DNase 1 caused substantial reductions in the bacterial richness found in communities, which we interpret as a result of its incomplete inactivation by heat treatment. DTT did help maintain bacterial richness in mixes treated with DNase 1. No such issues arose when using Sau3AI, which therefore seems a more appropriate enzyme. In our study, four operational taxonomic units (OTU) decreased in frequency and relative abundance after treatment with Sau3AI and hence are likely to represent contaminant bacterial DNA. We found higher within-sample similarity in community structure after treatment with Sau3AI, probably better reflecting the initial bacterial communities. We argue that the presence of contaminant bacterial DNA may have consequences in the interpretation of ecological data, especially when using low levels of template DNA from highly diverse communities. We advocate the use of such decontaminating approaches as a standard procedure in microbial ecology.

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Introduction

Microbial ecology is the study of how microorganisms interact with their environment and how their communities vary according to environmental factors. The majority of studies within this field now adopt culture-independent, PCR-based approaches involving the extraction of DNA present in environmental samples followed by specific sequence amplification. The targeted sequences contain both conserved and highly variable parts, which allow for the amplification of DNA from a broad range of microorganisms while discriminating them down to the family or genus level. The most widely used PCR primers for studying bacterial communities target either the 16S ribosomal RNA (rRNA) bacterial gene or the 16S-23S ribosomal DNA (rDNA) intergenic spacer region, but other primers have also been used. The choice of appropriate primers depends on the aims of the study as well as the range of the phylogenetic groups being targeted [1].

Such PCR-based approaches are very powerful as they are less time- and space-consuming than culture-based techniques and allow for the detection of non-cultivable microorganisms which can represent more than 90 % of those present in environmental samples [1]. However, because they are based on the use of broad-range PCR primers, contamination with sources of bacterial DNA other than that present in environmental samples may become an issue. In particular,

contamination of commercially available PCR reagents with bacterial DNA has been suggested (e.g. [2]) and is now well acknowledged in other fields of research [3–5]. A simple way of detecting PCR contamination is the use of negative controls (i.e. to which no template DNA is added). Many studies in microbial ecology—probably the majority—include such controls; however, surprisingly, few of them explicitly report their use, and of those that do, very few report their actual outcome (whether negative controls were indeed blank after PCR amplification). For example, of 44 studies located by Web of Science™ that were published in 2013 and used automated ribosomal intergenic spacer analysis (ARISA)[1], only two mentioned negative controls [2, 6]. Only one of these reported putative contamination of negative controls by PCR reagents and provided a list of potential contaminating operational taxonomic units (OTU) [2]. As a result, it is difficult to accurately assess how common DNA contamination of PCR reagents is in microbial ecology.

The reason that contamination of PCR mixes is not more often reported may be varied. To start with, contamination might, in many cases, not be an issue, which is why authors simply do not report the use of negative controls. Contamination may however remain undetected unless negative PCR controls are genotyped along with study samples. Negative controls can indeed appear blank on agarose gels due to low amplification levels, while in fact revealing several amplicons if further genotyped. In contrast to other fields of research where the reliability of the presence/absence of bacteria is crucial (e.g. diagnostic microbiology or forensics), microbial ecology aims at understanding general patterns of community structure and comparing them across habitats or time periods, rather than focusing on one, or a few, bacterial types. This might be a reason why contamination of PCR reagents is not more often detected amongst the usually high bacterial diversity found in environmental samples.

In the simplest cases of contamination, where one or very few contaminating OTU are clearly identified, the temptation might be simply to remove them from the data, hoping to a posteriori solve the problem. This is inappropriate, especially when low levels of template DNA are used (e.g. when the level of template DNA is close to that of contaminant DNA): the presence of contaminant DNA might then hamper the amplification of bacterial phylotypes present at low level in the template and hence change the observed community structure [7].

Hence, proper decontamination (i.e. destruction of contaminant DNA prior to the start of PCR) must be preferred whenever possible. Several methods have been reported, including UV irradiation of PCR mixes or pre-treatment of reagents with either endonuclease restriction enzymes or DNase 1, followed by heat inactivation prior to the start of the PCR [5, 8–10]. Unfortunately, the first method (UV irradiation) has been shown to inactivate the highly UV-sensitive *Taq* polymerase enzyme, resulting in decreased PCR efficiency. As for the second type of method (enzymatic treatment), it has been associated with a reduction in PCR sensitivity [10], which might be problematic since most bacterial types in a community are present at low levels. This effect might be caused by incomplete inactivation leading to residual enzymatic activity and hence degradation of primers and/or template DNA during the PCR procedure [9, 10].

Another, rather elegant, method based on primer elongation, by which template DNA can be distinguished from contaminant DNA, was recently published [11] but has not been tested on environmental communities. Furthermore, it remains both quite expensive and time-consuming and might not be appropriate for large samples.

Efficient inactivation of restriction enzymes prior to the start of PCR would therefore help solving the issue of PCR contamination in microbial ecology. One study reported the successful use of dithiothreitol (DTT), a strong reducing agent, to irreversibly inactivate DNase 1 [12]. Based on this information, we conducted a study aimed at exploring how the use of restriction enzymes, with or without DTT, may affect bacterial community structure, using the ARISA (automated ribosomal intergenic spacer analysis) method. This allowed us to establish what the most appropriate combination of enzyme and DTT is in our study. We suggest that similar preparatory steps should be used in microbial ecology studies more often than they have been so far.

Methods

Sampling of Bacterial Communities and DNA Extraction

Three human bacterial communities (skin, oral and faecal samples, hereafter named "Sk", "Mo" and "Fe", respectively) were sampled using sterile swabs (Copan Italia S.p.A.). Two replicate samples were collected per sample type. Swabs were stored at −20 °C for a few days. Prior to DNA extraction, tubes, pipette tips, water samples and gloves were UV-irradiated for at least 20 min. Total bacterial DNA was extracted from each swab under a laminar flow hood located in a specifically dedicated laboratory room, using the DNeasy Blood & Tissue Kit following the manufacturer's protocol for Gram-positive bacteria (Qiagen). For each sample type, DNA extracts were pooled in the same microcentrifuge tube to reach a final volume of 200 μL.

[1] *Web of Science*™ search: TS=((automated AND ribosomal AND intergenic AND spacer) OR ARISA) AND PY=(2013)

Decontamination and Molecular Fingerprinting of Bacterial Communities

To describe the bacterial community structure and composition of our samples, we used the automated ribosomal intergenic spacer analysis (ARISA). This DNA-fingerprinting method, widely used in microbial ecology, is based on the amplification of the internal transcribed spacer (ITS) region lying between the 16S and 23S rRNA genes in the ribosomal operon. The ITS region is extremely variable in both sequence and length for different phylotypes, which will then appear as different bands (operational taxonomic units, OTU) in the amplification profile. We used the FAM-labelled S-D-Bact-1522-b-S-20 primer (5′-[6FAM] TGCGGCTGGA TCCCCTCCTT-3′) and the L-D-Bact-132-a-A-18 primer (5′-CCGGGTTTCCCCATTCGG-3′) to amplify the 16S-23S ITS [13].

Amplification was performed using PCR in 20 μL reactions containing 0.2 mM deoxyribonucleotide triphosphate (dNTPs), 1.25 mM $MgCl_2$, 0.5 μM of each primer, 5 % DMSO and 0.25 unit of GoTaq® DNA polymerase with 1× buffer (Promega). A standard volume of 5 μL of DNA extract was used. It was not possible to accurately measure the concentration of extracted bacterial DNA because of the likely co-extraction of host (human) DNA.

Work surfaces were bleached and UV-irradiated, and tubes, PCR plates, pipette tips and gloves were UV-irradiated for at least 15 min prior to the preparation of the PCR mix. Prior to the addition of dNTP, primers and template DNA into the PCR mix, we added either the Sau3AI restriction enzyme (Thermo Fisher Scientific Inc.) or DNase 1 (New England Biolabs Inc.) in various concentrations (0/0.06/0.125/0.25 units). Enzymatic mixes were incubated for 30 min at 37 °C to allow for degradation of contaminant DNA. DTT was then added (0/15/30 mM) and the mixes incubated for 4 min at 95 °C to inactivate the enzymes. The various concentrations of enzyme and DTT were combined in a full factorial design across the three sample types (12 combinations of enzyme and DTT concentrations per enzyme type, i.e. 24 combinations per sample type). The same combinations were applied to negative controls where 5 μL UltraPure distilled water (Invitrogen™) was added instead of DNA extract.

PCR was conducted using the following programme: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and a final elongation step at 72 °C for 10 min. The successful amplification of the samples was confirmed on a 1 % agarose gel, and those PCR products that appeared as highly concentrated were diluted by a ratio of 1:10.

PCR products were mixed with the GeneScan™ 1200 LIZ® Size Standard (Applied Biosystems) and separated in the POP-7 polymer using an ABI 3730xl capillary sequencer (Applied Biosystems). Outputs were analysed with the GeneMapper software (Applied Biosystems). Only the fragments ranging from 100 to 700 bp and with a peak height above 50 raw fluorescent units (RFU) were considered. The number of peaks >700 bp was negligible in our samples. To account for size calling imprecision, a 3-bp wide binning was applied to ARISA profiles. Of the three possible binning frames, we kept the one resulting in the highest within-sample similarity, as calculated using the Bray-Curtis index. Binning was done in R (R Core Team, 2013) and pairwise similarity was calculated in PRIMER 6 (PRIMER-E Ltd).

For each sample, we considered the number of OTUs as an index of bacterial richness. To investigate the effect of enzymatic treatment on bacterial richness, we used generalized linear models fitted with a Poisson distribution for each sample type, with enzymatic treatment (none, Sau3AI or DNase 1)

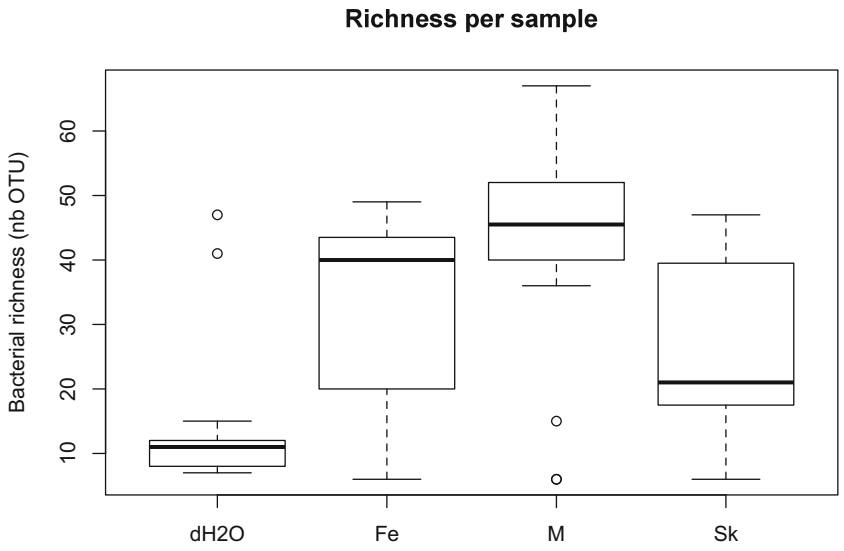

Fig. 1 Bacterial richness found after PCR (across all enzyme and DTT concentrations). *dH20* ultrapure water (negative control), *Fe* faecal sample, *M* mouth sample, *Sk* skin sample

Table 1 Summary statistics for bacterial richness in water controls and three positive samples (across combinations of enzyme and DTT concentrations)

Sample	Min	Max	Mean±SE	Number
dH_2O	7	47	12.7±1.9	24
Fe	6	49	33.2±3.1	24
M	6	67	43.9±3.3	24
Sk	6	47	26.0±2.7	24

as a factor. Post hoc comparison tests were performed using the multcomp package.

To investigate the effects of DTT on enzyme inactivation, we used generalized linear models fitted with a Poisson distribution, for each enzymatic treatment (no enzyme, Sau3AI or DNase 1). Initial models included sample type as a factor and both enzyme and DTT concentrations as covariates, as well as their interaction. Stepwise model selection was performed based on Akaike information criterion (AIC) values.

To test whether enzymatic treatment affected community structure, we ran an analysis of similarity (ANOSIM) based on the Bray-Curtis similarity matrix obtained from log-transformed data. To visualize those effects, we plotted our data on a multidimensional scaling (MDS) graph, where distance between dots represents dissimilarity between pairs of bacterial communities.

Finally, we separately compared OTUs from non-treated samples to those treated with Sau3AI both in frequency (with Fisher's exact tests) and in relative abundance (with non-parametric Kruskal-Wallis tests). All statistical analyses were done in R (R Core Team, 2013) except ANOSIM and MDS, which was done in PRIMER 6 (PRIMER-E Ltd).

Results

Contamination of Negative Controls

Despite all care taken during the PCR procedure, our 24 negative controls displayed values of bacterial richness ranging from 7 to 15 OTU, with two samples reaching 47 and 41 OTU, respectively. Most of these values are relatively low but nevertheless fall within the range of values obtained for our positive samples (Fig. 1, Table 1).

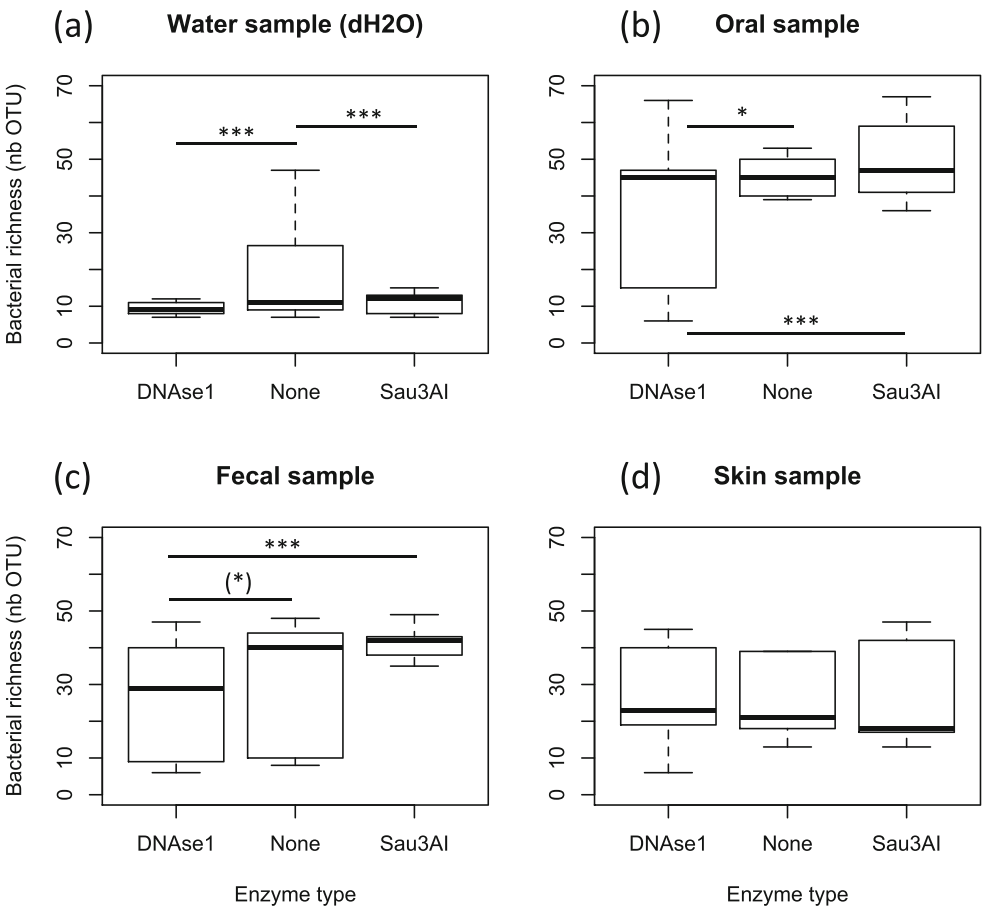

Fig. 2 Effect of pre-PCR enzymatic treatment on bacterial richness measured after amplification. Statistical results shown are from post hoc comparison tests based on generalized linear models (see "Methods"). (*)$P<0.1$; *$P<0.05$; ***$P<0.001$

Table 2 Effects of sample type, enzyme and DTT concentrations on bacterial richness, for each type of enzymatic pre-treatment of PCR mixes

	df	Deviance	*P* value
No enzymatic treatment (24)			
Sample type	3	259.0	$<10^{-4}$
With Sau3AI (36)			
Sample type	3	284.6	$<10^{-4}$
Enzyme concentration	2	4.7	0.03
With DNase 1 (36)			
Sample type	3	153.9	$<10^{-4}$
Enzyme concentration	2	40.3	$<10^{-4}$
DTT concentration	2	61.3	$<10^{-4}$
Enzyme×DTT	1	20.0	$<10^{-4}$

Sample sizes are indicated in parenthesis. Deviance and *P* values come from generalized linear models fitted using a Poisson distribution. Final models after AIC-based stepwise regression are shown

Effects of Enzymatic Treatment on Bacterial Richness

Enzymatic treatment (presence of either DNase 1 or Sau3AI) decreased contamination in our water controls (both $P<0.001$, Fig. 2a). For both oral and faecal samples, fewer OTU were amplified in PCR mixes pre-treated with DNase 1 than with Sau3AI (both $P<0.001$). Noticeably, fewer OTU were amplified after using DNase 1 than without any enzyme ($P=0.02$ and 0.09, respectively, Fig. 2b, c), which indicates residual DNase 1 activity during the PCR procedure. No significant effect of enzymatic treatments was observed for the skin sample, where bacterial richness was generally lower than in the two other samples (Table 2, Fig. 2d).

Effect of DTT on Enzyme Inactivation

DNase 1 concentration strongly affected bacterial richness, which was lowest at the intermediate concentration of

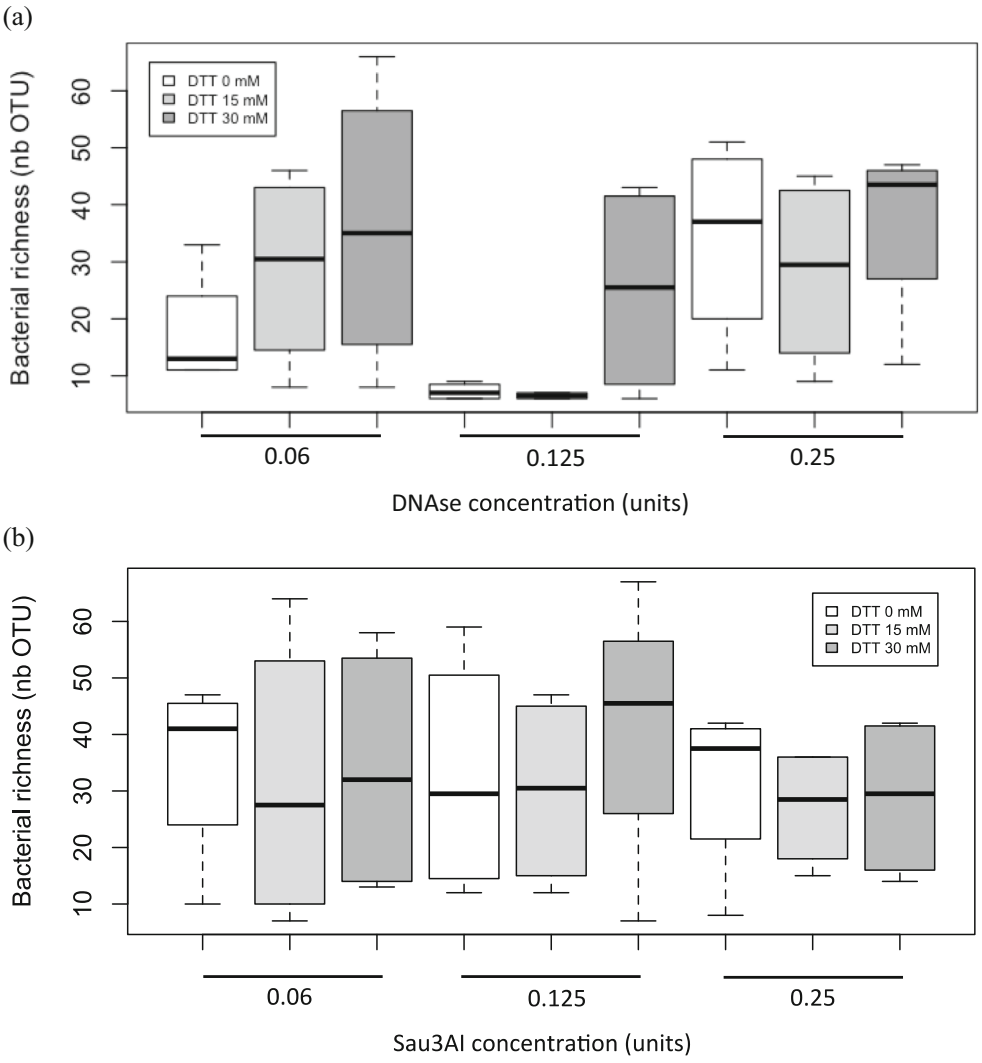

Fig. 3 Effect of the interaction between DTT and either DNase 1 (**a**) or Sau3AI (**b**) concentrations on bacterial richness (number of OTUs) measured after the PCR procedure

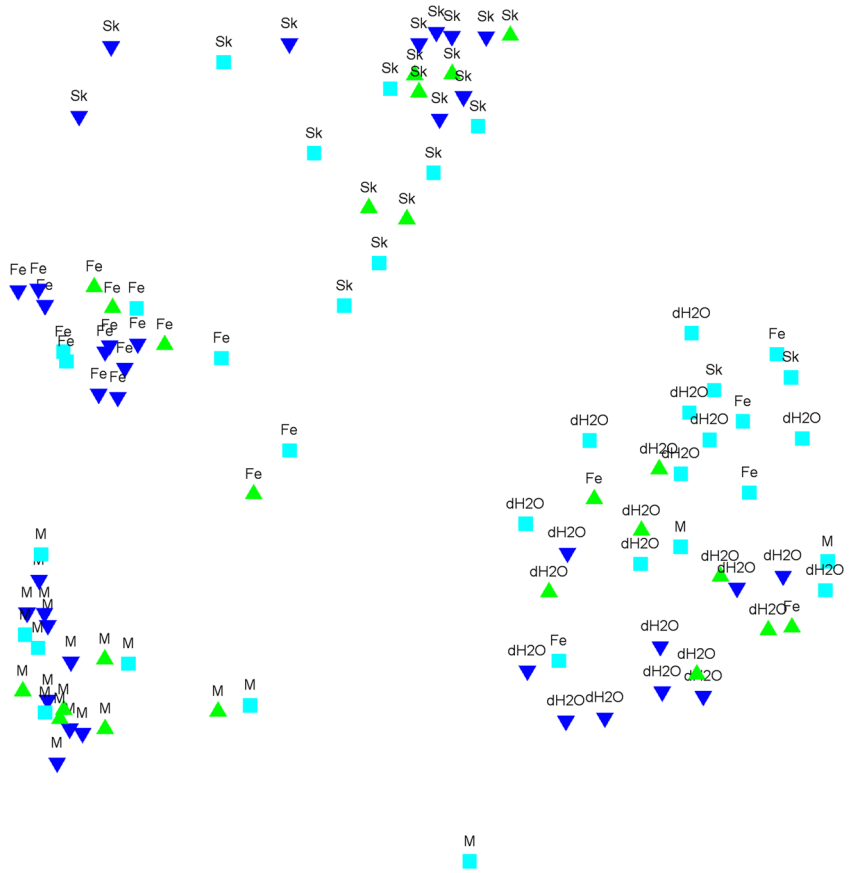

Fig. 4 Multidimensional scaling (MDS) graph showing the effects of sample type (*dH2O* ultrapure water control samples, *Fe* faecal samples, *M* oral samples, *Sk* skin samples) and enzymatic pre-PCR treatment (*green triangles* represent no treatment, *blue triangles* represent Sau3AI treatment, *blue squares* represent DNase 1 treatment) on bacterial community structure

0.125 unit ($P<10^{-4}$). In the presence of DNase 1, DTT concentration was positively related to bacterial richness ($P<10^{-4}$). Moreover, there was a significant interaction between DNase 1 and DTT concentrations ($P<10^{-4}$): at the lowest DNase 1 concentration, a DTT concentration of 15 mM resulted in increased bacterial richness, but at the intermediate DNase 1 concentration of 0.125 unit, bacterial richness was only increased when DTT was present at its highest concentration of 30 mM. Bacterial richness was neither reduced nor affected by DTT concentration for a DNase 1 concentration of 0.25 units (Table 2, Fig. 3a).

In contrast to DNase 1, Sau3AI only had a weakly significant negative effect on bacterial richness ($P=0.03$). DTT had no significant effect on bacterial richness in the presence of Sau3AI, and there was no significant interaction between the two (Table 2, Fig. 3b).

Effect of Enzymatic Treatment on Bacterial Community Structure

Enzymatic treatment (nested within sample type) significantly affected bacterial community structure (ANOSIM, $R=0.13$, $P=0.001$). Average within-sample similarity was 51 % with no enzymatic treatment, 64 % with Sau3AI and 32 % with DNase 1. Either non-treated samples or samples treated with

Table 3 OTUs whose frequency and relative abundance were reduced by pre-PCR enzymatic treatment with Sau3AI

Fragment length (bp)	Frequency (%)		*P* value	Relative abundance±SE (%)		*P* value
	No treatment	Sau3AI		No treatment	Sau3AI	
131	37.5	5.6	0.02	0.17±0.05	0.02±0.02	0.002
224	33.3	5.6	0.04	0.24±0.10	0.02±0.01	0.009
302	25.0	2.8	0.04	1.43±0.90	0.05±0.05	0.01
323	16.7	0.0	0.03	0.10±0.06	0.0±0.0	0.01

P values result from Fisher's exact tests and Kruskal-Wallis tests for frequency and relative abundance, respectively

DNase 1 appeared more spread out across the MDS graph than samples treated with Sau3AI (Fig. 4).

OTUs Affected by Treatment with Sau3AI

Four OTUs decreased significantly both in frequency and in relative abundance in samples treated with Sau3AI as compared to non-treated samples. Those OTUs had respective fragment lengths of 131 bp (frequency, from 38 to 6 %; relative abundance, from 0.17 to 0.02 %), 224 bp (frequency, from 33 to 6 %; relative abundance, from 0.24 to 0.02 %), 302 bp (frequency, from 25 to 3 %; relative abundance, from 1.43 to 0.05 %) and 323 bp (frequency, from 17 to 0 %; relative abundance, from 0.10 to 0 %) (Table 3).

Discussion

The non-negligible bacterial richness found in our water samples shows that despite all the care taken to ensure clean laboratory practice, contamination with bacterial DNA may remain difficult to tackle in studies relying on PCR using broad-range primers. Before running this study, we had performed a number of attempts to obtain negative controls in the context of another study and had therefore become very cautious about the cleanliness of lab surfaces and consumables. Despite all the care taken, water samples were rarely blank after PCR, which suggested that factors others than lab practice may participate in PCR contamination. As suggested in a number of other studies [3–5], it therefore seems to us that commercial PCR reagents are a likely source of contaminant bacterial DNA.

Our results also suggest that contamination can be dealt with. Restriction enzymes did reduce contamination: bacterial richness was significantly lower in water samples treated with either of the two enzymes than in non-treated water samples. In two out of three positive samples, however, we found lower bacterial richness after treatment with DNase 1 than either without treatment or after treatment with Sau3AI. This indicates that DNase 1 may remain partially active during the PCR procedure and hence degrade template DNA and/or rare amplicons. The addition of DTT prior to the inactivation step resulted in higher richness than without DTT, thereby confirming that DTT helps irreversibly heat-inactivate DNase 1 [12]. At relatively high DNase 1 concentrations, the enzyme seemed to lose its residual activity, as reflected in higher richness values. This was also noticed in a previous study [12]. The proposed explanation is that inactive dimers resulting from heat inactivation can reverse back to active monomers more easily when present in low than in high concentrations, thereby resulting in higher residual DNase 1 activity at low to intermediate concentrations.

No similar phenomenon was observed when using Sau3AI. Even though Sau3AI concentration affected bacterial richness, its effect was much weaker than that of DNase 1: a fourfold increase in Sau3AI concentration only resulted in a minor reduction in bacterial richness. This suggests effective inactivation of Sau3AI prior to the start of PCR. As a consequence, values of bacterial richness measured after Sau3AI treatment were quite consistent across the range of concentrations tested. Moreover, treatment with Sau3AI resulted in significantly higher within-sample similarity, as compared to both non-treated samples and samples treated with DNase 1. Sau3AI therefore seems a more reliable enzyme than DNase 1 for pre-PCR treatment.

Noticeably, those OTUs that were affected by Sau3AI (i.e. putative contaminant OTUs) were not amongst the most abundant in PCR products, although they were quite frequent. This, together with the observation that treatment with Sau3AI increased within-sample similarity, tends to confirm that in highly diverse environmental samples with low levels of template DNA, contamination may not affect community structure by simply adding "extra" OTUs to the final data, but rather by hampering the amplification of rare OTUs.

Addition of DTT did not seem to affect the Taq polymerase present in the mix: we found no significant relationship between DTT concentration and either bacterial richness or similarity in community structure, as might have been expected if DTT reduced Taq polymerase activity.

In this study, there were a few OTUs still present even in those negative controls that had been treated. This might represent residual contamination due to the presence of some bacterial DNA in either the dNTP or the primer mix, as those were added after the enzyme-inactivation step. We acknowledge that our study does not allow us to establish a detailed list of contaminant OTUs found in each of the PCR reagents separately; however, despite the quite small sample sizes used, both contamination and enzymatic treatments were found to strongly affect bacterial richness and community structure. Our results therefore indicate that the level of contaminant bacterial DNA as well as the conditions under which PCRs are run are likely to alter the perceived bacterial community structures and their subsequent analysis. In this case, we advocate that bacterial ecologists should systematically check for contamination in their PCR mixes and, whenever relevant, perform preliminary studies to establish which decontamination method is appropriate for their own studies. We are aware that many of them may already do so but also hope that this study will provide a starting point to improve the consistency of results when molecular microbial ecologists are confronted with contamination issues.

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References

1. Osborn AM, Smith CJ (2005) Molecular microbial ecology. Taylor & Francis, Abingdon
2. Van Dongen WFD, White J, Brandl HB et al (2013) Age-related differences in the cloacal microbiota of a wild bird species. BMC Ecol 13:11. doi:10.1186/1472-6785-13-11
3. Mühl H, Kochem A-J, Disqué C, Sakka SG (2010) Activity and DNA contamination of commercial polymerase chain reaction reagents for the universal 16S rDNA real-time polymerase chain reaction detection of bacterial pathogens in blood. Diagn Microbiol Infect Dis 66:41–9. doi:10.1016/j.diagmicrobio.2008.07.011
4. Spangler R, Goddard NL, Thaler DS (2009) Optimizing Taq polymerase concentration for improved signal-to-noise in the broad range detection of low abundance bacteria. PLoS One 4:e7010. doi:10.1371/journal.pone.0007010
5. Borst A, Box AT, Fluit AC (2004) False-positive results and contamination in nucleic acid amplification assays: suggestions for a prevent and destroy strategy. Eur J Clin Microbiol Infect Dis 23:289–99. doi:10.1007/s10096-004-1100-1
6. Or A, Comay O, Gophna U (2013) In situ transplant analysis of free-living bacteria in a lotic ecosystem. Res Microbiol 164:262–9. doi:10.1016/j.resmic.2012.12.004
7. Biesbroek G, Sanders EAM, Roeselers G et al (2012) Deep sequencing analyses of low density microbial communities: working at the boundary of accurate microbiota detection. PLoS One 7:e32942. doi:10.1371/journal.pone.0032942
8. Carroll NM, Adamson P, Okhravi N (1999) Elimination of bacterial DNA from Taq DNA polymerases by restriction endonuclease digestion. J Clin Microbiol 37:3402–4
9. Champlot S, Berthelot C, Pruvost M et al (2010) An efficient multistrategy DNA decontamination procedure of PCR reagents for hypersensitive PCR applications. PLoS One. doi:10.1371/journal.pone.0013042
10. Corless CE, Guiver M, Borrow R, Fox AJ (2000) Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. J Clin Microbiol 38:1747
11. Chang S-S, Hsu H-L, Cheng J-C, Tseng C-P (2011) An efficient strategy for broad-range detection of low abundance bacteria without DNA decontamination of PCR reagents. PLoS One 6:e20303. doi:10.1371/journal.pone.0020303
12. Silkie SS, Tolcher MP, Nelson KL (2008) Reagent decontamination to eliminate false-positives in Escherichia coli qPCR. J Microbiol Methods 72:275–82. doi:10.1016/j.mimet.2007.12.011
13. Ranjard L, Poly F, Lata J et al (2001) Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. Appl Environ Microbiol 67:4479–4487. doi:10.1128/AEM.67.10.4479