



## Note

## High abundance of genetic *Bacteroidetes* markers for total fecal pollution in pristine alpine soils suggests lack in specificity for feces

Julia Vierheilig<sup>a,b</sup>, Andreas H. Farnleitner<sup>b,c</sup>, Denny Kollanur<sup>b</sup>, Günter Blöschl<sup>d</sup>, Georg H. Reischer<sup>b,\*</sup>

<sup>a</sup> Centre for Water Resource Systems (CWRS), Vienna University of Technology, Karlsplatz 13/222, A-1040 Vienna, Austria

<sup>b</sup> Institute of Chemical Engineering, Research Division Biotechnology and Microbiology, Research Group Environmental Microbiology and Molecular Ecology, Vienna University of Technology, Gumpendorfer Straße 1a/166-5-2, A-1060 Vienna, Austria

<sup>c</sup> InterUniversity Cooperation Centre Water and Health (ICC Water & Health), Gumpendorfer Straße 1a/166-5-2, A-1060 Vienna, Austria

<sup>d</sup> Institute of Hydraulic Engineering and Water Resources Management, Vienna University of Technology, Karlsplatz 13/222, A-1040 Vienna, Austria

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## ABSTRACT

Two frequently applied genetic *Bacteroidetes* markers for total fecal pollution (AllBac and BacUni) were found in high numbers in pristine soil samples of two alpine catchment areas casting doubt on their value as fecal indicators. This finding underlines the necessity to evaluate assays locally and against non-intestinal samples before application.

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The contamination of water by fecal pollution has enormous impacts on a global scale since fecal material frequently contains intestinal pathogens in significant numbers. The reliability of traditional indicators for fecal pollution (e.g. *E. coli*) has been brought into question in various studies during the last 20 years (e.g. Byappanahalli and Fujioka, 1998; Byappanahalli et al., 2006; Fujioka et al., 1999). State-of-the-art microbial hazard and risk assessment approaches increasingly demand comprehensive pollution analysis including the quantification of total microbial fecal pollution and a reliable identification of its major contributing sources (i.e. microbial source tracking – MST) (Farnleitner et al., 2011; Field and Samadpour, 2007). Numerous MST markers have recently been developed targeting source-specific abundant intestinal microbial populations (e.g. the phylum *Bacteroidetes*) (Santo Domingo et al., 2007). In addition genetic markers for total fecal pollution were proposed (e.g. Kildare et al., 2007; Layton et al., 2006; Okabe et al., 2007) and applied (Bae and Wuertz, 2009; Dick et al., 2010; Schriewer et al., 2010; Silkie and Nelson, 2009; Stapleton et al., 2009; Wuertz et al., 2011) as reference parameters relating source-specific pollution to a level of total fecal pollution. Like any fecal indicator parameter, these genetic markers should be absent in extra-intestinal habitats where no fecal pollution has occurred. While most MST markers

were tested for specificity using non-target fecal samples, studies investigating the specificity of markers for total fecal pollution against non-intestinal samples are very rare and cast considerable doubt on their reliability (Dick and Field, 2004; van der Wielen and Medema, 2010).

The aim of this study was to evaluate the reliability of two genetic *Bacteroidetes* quantitative PCR (qPCR) assays for total fecal pollution, AllBac (Layton et al., 2006) and BacUni (Kildare et al., 2007), by investigating their occurrence and concentration in pristine soil and fecal samples both collected from the same extensively studied alpine catchments. In addition we compared the amplicon sequences found with the two assays in soil and fecal samples, respectively.

**Sampling and analysis:** 42 soil and 20 fecal samples were concurrently collected in different vegetation zones (woodland, krummholz, alpine grassland) and at different altitudes (800–1800 m above sea level) in two well characterized alpine karst spring catchment areas in Eastern Austria. Detailed quantitative pollution source surveys (pollution source profiles) for the catchments showed that ruminant animals (cattle, red deer, roe deer, chamois) are by far the most important sources of fecal pollution (Farnleitner et al., 2011; Reischer et al., 2011). Soil sampling was restricted to sites with i) no visible signs of fecal pollution and ii) no indication of recent animal activity. Finally a sample was only considered “pristine” if it was negative for the MST marker BacR which is highly specific and sensitive for local ruminant fecal pollution sources (Reischer et al., 2006). Soil samples

\* Corresponding author. Tel.: +43 1 58801 166556; fax: +43 1 58801 17299.

E-mail address: [reischer@mail.tuwien.ac.at](mailto:reischer@mail.tuwien.ac.at) (G.H. Reischer).

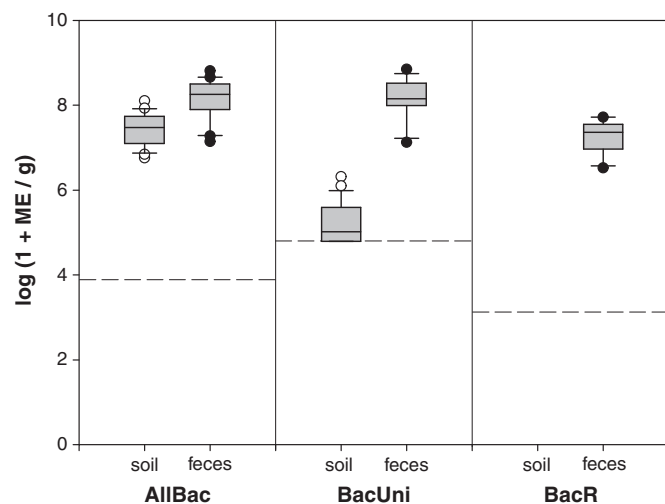
were taken from a depth of approximately 10 cm below the surface. The AllBac (Layton et al., 2006) and BacUni (Kildare et al., 2007) qPCR assays targeting fecal *Bacteroidetes* were implemented on our qPCR platforms, optimized for stringent marker detection and applied on the soil and fecal DNA. Marker copy numbers were quantified using plasmid standard dilution series and expressed as marker equivalents (ME) per g wet weight of soil or feces (Reischer et al., 2006). For a deeper comparison between the populations detected in soil and fecal samples, qPCR products of the two assays were cloned and sequenced to determine similarities to sequences of known origin in databases (Altschul et al., 1990; Cole et al., 2009). For a supplementary internal comparison between the sequences recovered from soil and fecal samples, the relatedness of the detected populations was determined by calculating the UniFrac metric (Lozupone and Knight, 2005; Lozupone et al., 2007), a phylogeny-based distance measure for the between-sample-diversity of bacterial communities. For methodical details refer to the supplementary material.

**Marker detection in fecal and soil samples:** The ruminant-specific BacR marker was not detectable in 29 of 42 soil samples which were hereafter considered “pristine”. Only these “pristine” samples were included in further analysis. All fecal samples found during the investigation were from ruminant animals confirming the assessment of the previous pollution source profile (Reischer et al., 2011). In all of these fecal samples BacR was present at similar levels (median  $2.3 \times 10^7$  ME g<sup>-1</sup>) (Fig. 1). The AllBac marker was found in all fecal samples, too (median  $1.8 \times 10^8$  ME g<sup>-1</sup>). AllBac could also be detected in all pristine soil samples at levels not even one order of magnitude lower than in feces and with little variation between the 29 samples (range from  $5.7 \times 10^6$  to  $1.3 \times 10^8$  ME g<sup>-1</sup>, median  $3.0 \times 10^7$  ME g<sup>-1</sup>). The BacUni marker showed levels very similar to AllBac in ruminant feces (median  $1.4 \times 10^8$  ME g<sup>-1</sup>) while levels in soil were lower but still detectable in 76% of all samples (median  $1.0 \times 10^5$  ME g<sup>-1</sup>) (Fig. 1).

The surprisingly high levels of the markers in soil motivated us to clone and sequence the qPCR amplicons of a subset of the soil and fecal samples in order to get an impression of the populations detected by the assays (two sequences per sample and assay). Comparison with the GenBank database showed that the sequences amplified from fecal samples showed high similarities with sequences derived from vertebrate fecal samples (average percentage

of best hits from fecal origin: 64% for AllBac and 88% for BacUni, see Supplementary Table 1). In soil samples on the other hand only an average of 21% of the best hits for the AllBac and an average of 15% for BacUni sequences showed highest sequence identities with sequences found in vertebrate animal feces. As an additional supporting analysis and visualization of the phylogenetic relatedness of the recovered sequences we applied cluster analysis on the UniFrac distance matrix. Sequence communities from the two assays were clearly distinct (Supplementary Fig. 1) and most of the resulting clusters were exclusively containing either fecal or soil communities.

**Applicability of markers for total fecal pollution:** The assays tested in this study are the two most widely applied genetic *Bacteroidetes* markers for total fecal pollution (Bae and Wuertz, 2009; Dick et al., 2010; Silkie and Nelson, 2009; Stapleton et al., 2009; Wuertz et al., 2011). However, the achieved results call the applicability of the proposed assays for the studied Austrian catchment areas into question. Considering their concentrations in pristine soils, neither of them seems to be highly specific for vertebrate intestinal *Bacteroidetes* populations at all. The quantitative level of BacUni marker in pristine soils is lower in comparison to AllBac. Nevertheless the sequences detected with this assay in soils were more closely related to sequences not derived from vertebrate feces. In general the retrieved sequence information suggests that the assays appear to detect autochthonous, non-intestinal *Bacteroidetes* populations in soils that are for the most part distinct from the intended fecal target populations. These findings are in accordance with the recently reported unaccountably high concentrations of the AllBac marker in ground and drinking water in the Netherlands (van der Wielen and Medema, 2010). In fact, members of the phylum *Bacteroidetes* are, in addition to their dominant role in intestinal microbiota, also well known as abundant members of soil microbiota (Lauber et al., 2009; Roesch et al., 2007). The broad application of the tested assays combined with the apparent lack of investigations of their fecal indication performance is even more surprising when considering how vividly and controversially the occurrence of conventional fecal indicators (such as *E. coli*) in non-intestinal habitats such as soils and sediments has been discussed in recent years (Byappanahalli et al., 2006; Fujioka et al., 1999). The only qPCR assay specifically addressing this issue was published by Dick and Field (2004) who actually amended their original *Bacteroidetes* assay primers in an addendum in proof in order to exclude non-intestinal targets. Unfortunately these primers also seemed to have performed poorly in the recent Dutch study (van der Wielen and Medema, 2010) and do not seem to be widely used (Wuertz et al., 2011). Scientific literature also lacks information about the potential persistence of fecal *Bacteroidetes* populations in soil or other extra-intestinal environments. Our results underline the need to develop improved molecular markers for total fecal pollution detection. It should be stated that most proposed assays have been based on a fragmentary puzzle of sequence information which was available at the time of development. To improve assay design more sequence data are necessary, especially about extra-intestinal habitats such as soil or sediment. This study emphasizes the need to test the specificity and sensitivity of qPCR-based assays for total fecal pollution on the local level and especially against non-intestinal environmental samples. Although there is a strong demand and pressure for marker-based detection techniques for total fecal pollution in water quality monitoring and risk assessment, currently none of the tested assays seems to meet one of the most basic requirements, which is being indicative of vertebrate fecal material.



**Fig. 1.** Concentrations of AllBac and BacUni markers for total fecal pollution in pristine soil samples ( $n=29$ ) and fecal samples ( $n=20$ ) in marker equivalents (ME) per g wet weight. BacR marker concentrations are given as a reference of fecal pollution levels. BacR was not detected in the 29 soil samples which were selected based on this property. Boxes, 25th and 75th percentile; lines within the boxes, median; whiskers, 10th and 90th percentile, respectively; data is  $\log^{+1}$ -transformed; dashed line represents assay limit of detection under the given circumstances.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.mimet.2012.01.009.

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