Testing the applicability of DNA based microbial faecal source tracking methods on a large scale in the River Danube and its important tributaries

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Introduction

Faecal pollution is an impairment to all kinds of water uses particularly for recreation and drinking water abstraction. The assessment of faecal influence on water is usually achieved by cultivating indicator organisms like *Escherichia coli*, *Enterococci* or faecal coliforms. These parameters are reliable indicators for faecal pollution. However, they give no indication whatsoever about the source of faecal input. These sources can range from human sources like treated or untreated waste water and agricultural sources like grazing or stabled livestock to wildlife sources like game or birds. The list of potential sources is unique for each catchment and it is the objective of microbial source tracking (MST) techniques to identify these sources of faecal pollution (Scott, et al., 2002, Simpson, et al., 2002). The information provided by these methods pinpoints faecal inputs and allows target oriented measures for catchment protection (Sinton, et al., 1998).

Application of microbial source tracking techniques has so far bee restricted to relatively small watersheds and coastal waters. Most of these studies used cultivation based methods which are extremely laborious and not applicable to larger geographical areas. Methods for the molecular-biological detection of source specific genetic markers are a promising alternative to these methods (Bernhard and Field, 2000, Bonjoch, et al., 2004). Our group recently developed two such methods for the detection and quantification of human (BacH marker) and ruminant (BacR marker) faecal DNA markers (Reischer, et al., 2006a, Reischer, et al., 2006b).

The developed methods are based on the detection of 16S rDNA markers carried by faecal members of the phylum *Bacteroidetes*. This group of bacteria comprises obligate anaerobic genera like *Bacteroides* which constitute a considerable fraction of the gut flora of mammals e.g. making up to 40% of the bacterial flora in human faeces with cell numbers up to 10^{11} per gram faeces (Sghir, et al., 2000). This is a much higher abundance than usual faecal indictors (human faeces contains an average of 10^8 CFU *E. coli* per gram) and makes this group of bacteria very sensitive indicators for faecal pollution (Allsop and Stickler, 1984, Allsop and Stickler, 1985). However, the difficult cultivation and enumeration of these organisms have so far hindered their use as water quality parameters. The detection and quantification of the marker sequences are achieved by quantitative real-time polymerase chain reaction (qPCR) and circumvent the need for cultivation (direct detection methods).

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The aim of this study was to test the applicability of direct DNA-based source tracking methods on a large river and its catchment.

Methods

Water samples. Sampling was performed during the Joint Danube Survey (JDS) project (13 August - 19 September 2001). Details about the project and the sampling locations can be found in the report of the International Commission for the Protection of the Danube River (ICPDR) (Literáthy, et al., 2002). All large capitals (Vienna, Budapest, Belgrade and Bucharest) and river inflows were sampled upstream and downstream in a frequency of every 20 to 40km. On a total of 98 stations, 50-150ml of water, collected 30cm below the water surface, were filtered through 0.22µm pore-size filters to collect the prokaryotic cells on the filters, representing the total in situ prokaryotic community. The filters were kept frozen until DNA extraction.

DNA Extraction. Precursor experiments have shown that it is essential for subsequent PCR amplification to avoid contamination of the DNA extracts by inhibiting agents such as humic acids, commonly found in water samples from the river Danube. Thus, extraction of the DNA from the filters were performed using the UltraClean Soil DNA Kit (MO BIO Laboratories) according to the manufacturer's instructions. The kit is efficiently separating DNA from contaminating substances and thus, is the most appropriate extraction method for our samples. After extraction all samples were analysed for the presence of amplifiable bacterial DNA as checked by applying a universal bacterial PCR assay (Teske, et al., 1996).

Quantitative real-time PCR. The human-specific BacH marker and the ruminant-specific BacR marker were detected and quantified according to the procedures described in previous publications (Reischer, et al., 2006a, Reischer, et al., 2006b). The procedure involves an amplification of the marker in a PCR reaction and the monitoring of the formed reaction product by measuring the fluorescence emitted by 5'nuclease digested hybridisation probes. Quantification is achieved by using a standard dilution series with known marker copy numbers in the same analysis. The results are calculated as marker equivalent numbers per filtered volume of water.

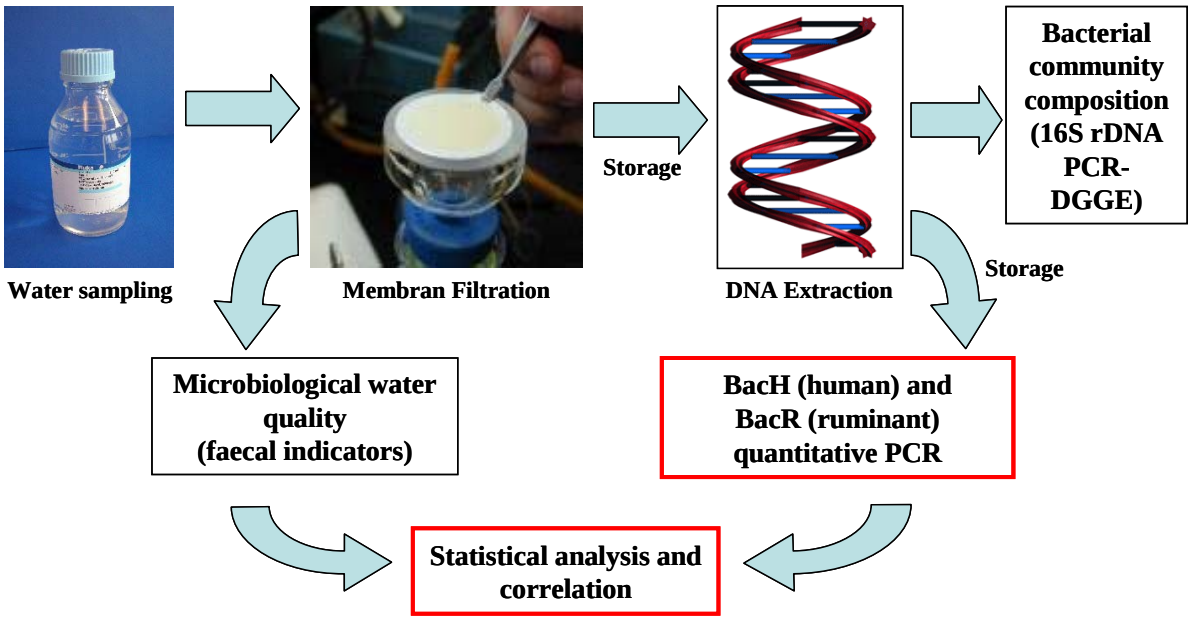

Fig. 1. Experimental framework for the application of BacH and BacR quantitative PCR assays on samples from the Danube river.

Results

Evaluation setup. This study was based on samples taken during the Joint Danube Survey of 2001 which included the determination of the microbiological water quality (Kavka and Poetsch, 2002). Additionally water samples were filtered for the investigation of the total bacterial community by 16S rDNA-denaturing gradient electrophoresis analysis (Farnleitner, et al., 2006, Winter, et al., 2006). The residual DNA samples were used to test two recently developed methods for bacterial source tracking BacH and BacR qPCR in order to assess the basic applicability of the methods on large catchments. Fig. 1 shows the study setup and the relation to the previous studies.

Marker detection results. Detection of the human specific BacH marker was possible in 14 of 24 samples from important tributaries to the Danube river. It was also detectable in 28 of 74 samples taken in the Danube river itself. Detection of the ruminant specific BacR marker was only achieved in 4 tributary samples and the levels were below the limit of quantification in all these cases.

The correlation of the log of BacH marker concentrations with log faecal coliforms counts (Kavka and Poetsch, 2002) in the tributary samples resulted in a linear correlation coefficient of 0.84 signifying that 70% of the total average faecal pollution detected by faecal coliforms can be attributed to human sources. The same correlation for the main river samples yielded a coefficient of 0.63 still allocating 39% of the average faecal pollution to human sources.

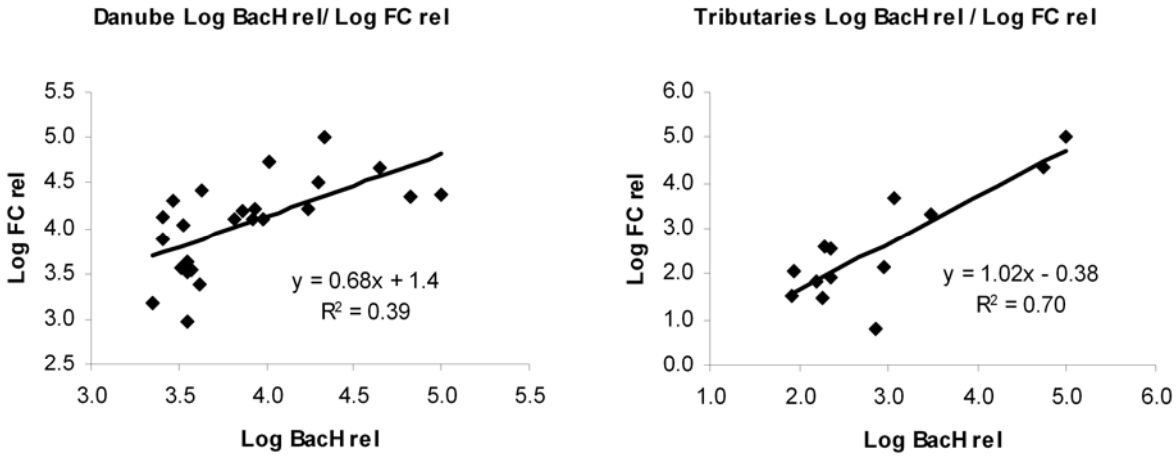

Fig. 2a and 2b. Correlation between BacH marker concentrations and faecal coliform counts in the Danube river samples (a) and the tributary samples (b) respectively (used values are the log+5 of the percentages to the respective maximum values).

Discussion

The presented study is a preliminary evaluation of the general applicability of the developed source tracking methods on a large river system. The samples used were available from a previous study, had been stored at -20°C for a considerable time span of over 4 years before the newly developed method allowed analysis by quantitative PCR. Unfortunately the detected levels for the ruminant specific BacR marker were not high enough for quantitative analysis. Because of possible degradation of DNA the quantitative results have to be considered with caution. However DNA degradation should affect all samples in a similar way. Despite our concerns about the data the correlation of the source tracking marker data and the faecal indicator counts was very strong. The fraction of faecal pollution attributable to human sources in the tributary samples was remarkably high albeit this fraction was lower in the Danube samples. This fact may be due to the direct and stronger faecal influence on the

tributaries, while the conditions in the main river are mainly influenced by hydrology (dilution effects) and ecological factors. The constant slope of the relationship between the human marker and the faecal coliforms (1,02) also indicates a more direct relationship between those two parameters in the smaller rivers as compared to the Danube where differences in persistence/survival of these target parameter seem to be obvious (ratio between parameters is dependent on concentration range i.e. slope smaller than 1). The gained results support the assumption of the applicability of marker based source tracking methods on large surface water systems and might point to a dominant role of human faecal influence on the investigated water sampling sites at the time of the sampling. With an adapted sampling and sample storage procedure it should be possible to conduct large-scale source tracking efforts and get reliable source-specific quantitative data of faecal influence.

To this end it will be necessary to investigate the ecology of the marker carrying organisms on this larger geographical scale. Persistence data of the marker and degradation rates in surface water of varying trophic levels have to be established. It might also be necessary to obtain additional DNA sequence data in order to adapt the methods to this larger scale. In addition measures for quality control in sample storage and analysis will improve reliability. With these measures it will be possible to collect large numbers of samples from different locations and during a prolonged period of time. In contrast to microbiological methods these collected samples can then be stored, transported and analysed at a convenient time and laboratory. An incorporation of the new methods in the course of the next Joint Danube Survey in 2007 has already been proposed. The methodology employed in this study is adaptable and might also be used to detect markers indicating other faecal sources like birds as soon as faecal genetic markers for those organisms are identified.

The results from quantitative source tracking methods can be brought in conjunction to classical microbiological parameters for water quality. The new methods do not distinguish between live and dead cells and give no indication of viability of organisms of faecal origin. As a consequence it will always be necessary to put their results in connection with cultivation based faecal indicator data. Source specific data should be very useful in catchment protection and the pinpointing of pollution sources. With the proposed methods it should also be possible to study and localise non-point sources of faecal pollution like surface run-off (Simpson, et al., 2002). They might also allow the monitoring of remediation efforts and the assessment of the achieved improvements. Direct source tracking methods might also provide additional qualitative information to the field of quantitative microbial risk assessment (QMRA) in the context of water safety plans as proposed by the WHO (WHO, 2004). Thus they might assist in the protection of river water as an important drinking water resource.

Summary

This study represents the first application of direct source tracking methods on a large river catchment. The results, especially the high levels of faecal pollution relatable to a specific source, are a positive indication for the applicability of the methods on a large geographical scale. Due to the simple and fast methodology the future evaluation and application might provide important new insights in the origin of faecal pollution in large catchment areas.

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