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Occurrence of human-associated *Bacteroidetes* genetic source tracking markers in raw and treated wastewater of municipal and domestic origin and comparison to standard and alternative indicators of faecal pollution

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ABSTRACT

This was a detailed investigation of the seasonal occurrence, dynamics, removal and resistance of human-associated genetic *Bacteroidetes* faecal markers (GeBaM) compared with ISO-based standard faecal indicator bacteria (SFIB), human-specific viral faecal markers and one human-associated *Bacteroidetes* phage in raw and treated wastewater of municipal and domestic origin. Characteristics of the selected activated sludge wastewater treatment plants (WWTPs) from Austria and Germany were studied in detail (WWTPs, n = 13, connected populations from 3 to 49000 individuals), supported by volume-proportional automated 24-h sampling and chemical water quality analysis. GeBaM were consistently detected in high concentrations in raw (median $\log_{10}$ 8.6 marker equivalents (ME) 100 ml^{-1}) and biologically treated wastewater samples (median $\log_{10}$ 6.2–6.5 ME 100 ml^{-1}), irrespective of plant size, type and time of the season (n = 53–65). GeBaM, *Escherichia coli*, and enterococci concentrations revealed the same range of statistical variability for raw (multiplicative standard deviations $s^* = 2.3–3.0$) and treated wastewater ($s^* = 3.7–4.5$), with increased variability after treatment. *Clostridium perfringens* spores revealed the lowest variability for raw wastewater ($s^* = 1.5$). In raw wastewater correlations among microbiological parameters were only detectable between GeBaM, *C. perfringens* and JC polyomaviruses. Statistical associations amongst microbial parameters increased during wastewater treatment. Two plants with advanced treatment were also investigated, revealing a minimum $\log_{10}$ 5.0 (10th percentile) reduction of GeBaM in the activated sludge membrane bioreactor, but no reduction of the genetic markers during UV irradiation (254 nm). This study highlights the potential of human-associated GeBaM to complement wastewater impact monitoring based on the determination of SFIB. In addition,

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human-specific JC polyomaviruses and adenoviruses seem to be a valuable support if highly specific markers are needed.

1. Introduction

Contamination of aquatic systems by wastewater of human origin can pose a serious threat to public health because it frequently contains high numbers of intestinal pathogens (Stevens et al., 2009). Appropriate disposal systems combined with efficient faecal pollution monitoring techniques for municipal and domestic wastewater are thus essential for safeguarding our water resources. Wastewater treatment plants (WWTPs) based on primary (mechanical), secondary (biological), and tertiary (enhanced biological and chemical) treatment are designed to remove organic carbon (C), nitrogen (N) and phosphorus (P) out of wastewater to a great extent. Although providing a first essential barrier, conventional WWTPs are not built to sufficiently remove microbial faecal loads to support the safe use of effluent wastewater for human related activities, such as recreational purposes or irrigation. Disinfection of wastewater effluents has not yet become a common standard in most regions of the world, and such advanced treatment is often restricted to the discharge of wastewater into sensitive aquatic areas. Rainfall events may also lead to a bypass of WWTPs (i.e. combined sewer overflows) and the contamination of water resources with raw wastewater (Molina et al., 2014; Shibata et al., 2014; Tryland et al., 2014).

Routine monitoring of microbial faecal pollution in the aquatic environment is still based on the selective cultivation of standard faecal indicator bacteria (SFIB), including *Escherichia coli* and intestinal enterococci (ISO, 2005). Without doubt, water quality testing based on the application of SFIB has contributed to a fundamental improvement in water safety management since the end of the 19th century (Tallon et al., 2005). However, the application of SFIB has also recently been subjected to increasing criticism (Ishii and Sadowsky, 2008). Several studies suggested that SFIB in aquatic habitats also originate from non-enteric compartments, such as soil, sediment and algae (Byappanahalli et al., 2012; Desmarais et al., 2001; Whitman et al., 2003). In addition, typing of isolated SFIB strains, to recover information about their origin, requires the formation of unrealistically large catchment-specific strain libraries and does currently not provide a feasible option for monitoring applications (Domingo et al., 2007). These limitations obviously call for additional indicators and tools to complement the existing standard methods to obtain a more detailed and certain view on the existing faecal pollution patterns to support MST and risk assessment (Harwood et al., 2014).

Amongst the vast number of alternative parameters (Hagedorn et al., 2011; Wuertz et al., 2011), PCR-based assays for the analysis of human-associated genetic *Bacteroidetes* faecal markers (GeBaM) have gained increasing popularity in the field of faecal pollution analysis and microbial source tracking (MST) during recent years (Harwood et al., 2014). Quantitative PCR (qPCR)-based GeBaM assays for general-, human-, wastewater-, or animal-associated faecal sources have been developed (Kildare et al., 2007; Layton et al., 2006; Reischer et al., 2006; Shanks et al., 2009). Several evaluation studies including various aquatic environments successfully demonstrated the value of GeBaM diagnostics (Boehm et al., 2009; Reischer et al., 2011; Ridley et al., 2014; Riedel et al., 2014; Sauer et al., 2011; Tambalo et al., 2012). However, the application of qPCR-based GeBaM assays is not yet standardized. It requires careful study design and background information on the catchment to create unbiased results and to recognize methodical limits (Boehm et al., 2013; Reischer et al., 2011).

A useful parameter for the analysis of general- or host associated microbial faecal pollution in water has to fulfil several basic performance criteria, including source-sensitivity and source-specificity (Wuertz et al., 2011). Considerable effort has been dedicated to sensitivity and specificity testing of GeBaM qPCR assays during recent years, most frequently based on individual sampling strategies covering various sources of animal and human excreta or wastewater (Ahmed et al., 2013; Boehm et al., 2013; Keity et al., 2012; Reischer et al., 2013, 2011; Riedel et al., 2014; Shanks et al., 2009). Emphasis has also been put on sampling techniques, DNA extraction, and PCR quantification procedures (Cankar et al., 2006; Karlen et al., 2007; Shanks et al., 2012; Siefring et al., 2008; Stoeckel et al., 2009). However, information on the occurrence of GeBaM in wastewater regarding the characteristics of the disposal system (combined and separate sewer systems), its seasonal variability, and its relationship to standard and alternative faecal indicators is scarce (Srinivasan et al., 2011).

The aim of this study was to investigate the prevalence and abundance of human-associated GeBaM by qPCR determination in raw and treated wastewater of well-characterized municipal wastewater treatment plants over one year. Emphasis was put on the selection of municipal WWTPs with primary, secondary, and tertiary treatment, as such systems are representative for the situation in Austria and the Central European Region (CER). Small domestic WWTP (dWWTPs) were also included in our investigation, as they are frequently implemented in remote areas, where the connection to municipal sewer systems is not possible. Although advanced treatment was not the main focus of this study, the investigation of UV disinfection at one selected WWTP was included, as such treatment is becoming increasingly important. The Taqman HF183 qPCR assay (Haugland et al., 2010) and the BacHUM qPCR assay (Kildare et al., 2007) were selected for the determination of human-associated GeBaM concentrations, following recommendations of recent evaluation studies (Boehm et al., 2013; Layton et al., 2013; Reischer et al., 2013). To support methodical cross-comparisons, cultivation-based SFIB using ISO standard methods and viral faecal markers for human-specific faecal pollution were simultaneously determined. Among these, JC polyomavirus (JCPyV) as well as human adenoviruses (HAdV), which have been used as human faecal viral indicators and highly specific MST tools (Bofill-Mas et al., 2000; Pina et al., 1998), and bacteriophages infecting *Bacteroides thetaiotaomicron,* which have been proposed as a human-associated faecal indicator, were tested. Raw and treated wastewater was also analysed by chemical standard methods to support treatment plant characterisation and comparison of elimination characteristics between microbial and chemical parameters.

2. Materials and methods

2.1. Selection criteria and parameters to characterize the sewer disposal systems and WWTPs

The Danube Region and other parts of the CER are defined as