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Establishment of a new approach for determining hygiene standard values for fecal pollution based on the acceptable risk of pathogen infection

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Acknowledgements

Chapter 1

Introduction

1. Introduction

1.1. Background of this study

The 21st century is often called “the century of water,” and water shortage has become a serious problem over the world. More than two billion people live in highly water-stressed areas because of the uneven distribution of available freshwater resources in time and space. Global population will grow, at least for several decades, and water demand will increase as well (Oki and Kanae 2006).

Wastewater reclamation for providing potable water and irrigation water is promoted as one of the effective countermeasures for the increasing water demand. To evaluate the human health risk by the wastewater reclamation, it is necessary to identify and quantify contaminants in the reclaimed wastewater (Asano et al. 2007). Generally, the fecal contamination level by the pathogenic microorganism is evaluated and managed by using the quantity value of the alternative microorganism that is called an indicator of fecal pollution. Conventional fecal pollution indicators, including coliforms, fecal coliforms, *Escherichia coli*, and *Enterococcus* spp. have enormously contributed to the control and maintenance of public health (Savichtcheva and Okabe 2006; Scott et al. 2002). However, fundamental shortcomings of these conventional indicators are emphasized. Firstly, conventional indicators have an inability to identify sources of fecal contamination in water, because they are present in animal feces and soil. To identify sources of fecal contamination in water, diverse alternative indicators of fecal contamination have been developed (Field et al. 2007; Meays et al. 2004; Savichtcheva and Okabe 2006; Scott et al. 2002; Simpson et al. 2002). Especially, genetic markers of human enteric bacteria are being used as alternative indicators for identifying fecal pollution sources with a library- and culture-independent way. The quantitative PCR (qPCR) assay for 16S rRNA of *Bacteroides-Prevotella* has been constructed for that purpose and most widely used (Dick et al. 2005; Okabe et al. 2007; Seurinck et al. 2005). For application of *Bacteroides-Prevotella* 16S rRNA genetic marker as a fecal pollution indicator, the developments of specific detection method of each contamination sources such as human, cow, pig and bird feces are needed. Quantification method of these genetic markers should be standardized for practical use; those enable us to check whether the sample processing performed normally and to compare results obtained from different laboratories (Meays et al. 2004; Stewart et al. 2003; Harwood et al. 2009; Stoeckel et al. 2009).

The second fundamental shortcoming of conventional indicators is that the current water hygiene standards using conventional indicators were not determined based on the possible adverse effects on human health. In the other words, it is not clear that the density of indicator microorganisms below the current value of water hygiene standards is meaning the low risk of

infection by pathogenic microorganisms. In order to ensure the microbiologically safe water management, World Health Organization recommended that the monitoring parameters and the standard values are established according to forms of water utilization such as drinking water, wastewater and reclaimed water (WHO, 2001). For example, WHO Scientific group (2011) suggested that health-based targets should be set using quantitative risk assessment and should take into account local conditions and hazards. Therefore, it is very critical to establish evidence-based water hygiene standards based on the acceptable risk of infection.

In addition to it, very poor correlations have been found between the amount of bacterial indicator microorganisms and that of pathogenic microorganisms (World Health Organization 2001). Concentrations of bacterial indicators and pathogens are independently fluctuated, owing to the difference in biological and physicochemical properties of these microbiological entities. This makes it difficult occasionally to predict the presence of pathogens in water by detecting conventional indicators. For microbiologically safe water management, it is critical to understand the occurrence characteristics of pathogens in water and to establish the water hygiene standards in consideration of concentration fluctuation.

1.2. Objectives

The final goal of this study is to construct the framework to determine the hygiene standard values based on the acceptable risk of pathogen infection. To achieve this final goal, some small goals were set. Firstly, *Bacteroides-Prevotella* 16S rRNA genetic markers were developed for each contamination source. Secondly, to use these genetic markers for the detection and the monitoring of fecal contamination in environmental water, quantification method of them was improved. Thirdly, the acceptable risks of pathogen infection were calculated by the point estimation of quantitative microbial risk analysis. Fourthly, the quantitative relationships of indicators and pathogens were calculated by using surveillance data of pathogen and indicator concentrations in water. Finally, candidates of hygiene standard values were determined based on the acceptable risk of pathogen infection.

This thesis is composed of the following chapters:

Chapter 2 (Literature review) summarized literature findings regarding the fecal indicators, the standardization and normalization of quantification process, and the hygiene standard values.

Chapter 3 (Development of the Chicken- and duck-associated *Bacteroides-Prevotella* genetic

markers for detecting fecal contamination in environmental water) described the development of real-time PCR assays for chicken- and duck-associated *Bacteroides-Prevotella* 16S rRNA genetic markers in order to quantitatively evaluate avian fecal contamination in water.

Chapter 4 (Improvement of the quantification method of alternative indicator for fecal contamination “Genetic marker”) showed the development of genetically-engineered strain of *E. coli* (designated as strain MG1655 Δ lac::kan) as a sample process control (SPC) for qPCR assays using propidium monoazide treatment to accurately quantify *Bacteroides-Prevotella* gene markers in environmental water samples. In addition, an internal amplification control (IAC) was also constructed and applied to further improve the measurement accuracy.

Chapter 5 (Effects of temperature and predator on the persistence of host-specific *Bacteroides-Prevotella* genetic markers in water) showed the fate of some human-specific genetic markers in river water was compared with that of conventional indicator microorganisms at various water temperature conditions. In addition to that, the possible effect of predators on the persistency of bacterial genetic markers in natural water environment was also investigated.

Chapter 6 (Acceptable concentration of pathogens in environmental water derived by Quantitative Microbial Risk Analysis (QMRA)) showed the calculation of the acceptable concentration of pathogens for determination of the hygiene standard values based on the acceptable risk of pathogen infection.

Chapter 7 (Development of the hygiene standard value of conventional and alternative indicators based on the acceptable risk of pathogen infection) described the framework to determine the hygiene standard values based on the acceptable risk of pathogen infection. Firstly, concentrations of pathogens and indicators in environmental water are quantified in multiple sites for a period of time. Secondly, Bayesian model is used to estimate the concentration distribution of pathogens, indicators and the distribution of the ratio of a pathogen to an indicator. Thirdly, the quantitative relationship between indicator and pathogen is calculated by using the distribution of the ratio of a pathogen to an indicator. Finally, the quantity of indicator in water ensuring that the pathogen concentration is significantly below the acceptable concentration of pathogen is determined as the water quality standard value by using the quantitative relationship between indicator and pathogen and the acceptable concentration of pathogens in river water (Chapter 6).

Chapter 8 (Conclusions) summarized the findings of this study and provided future perspectives.

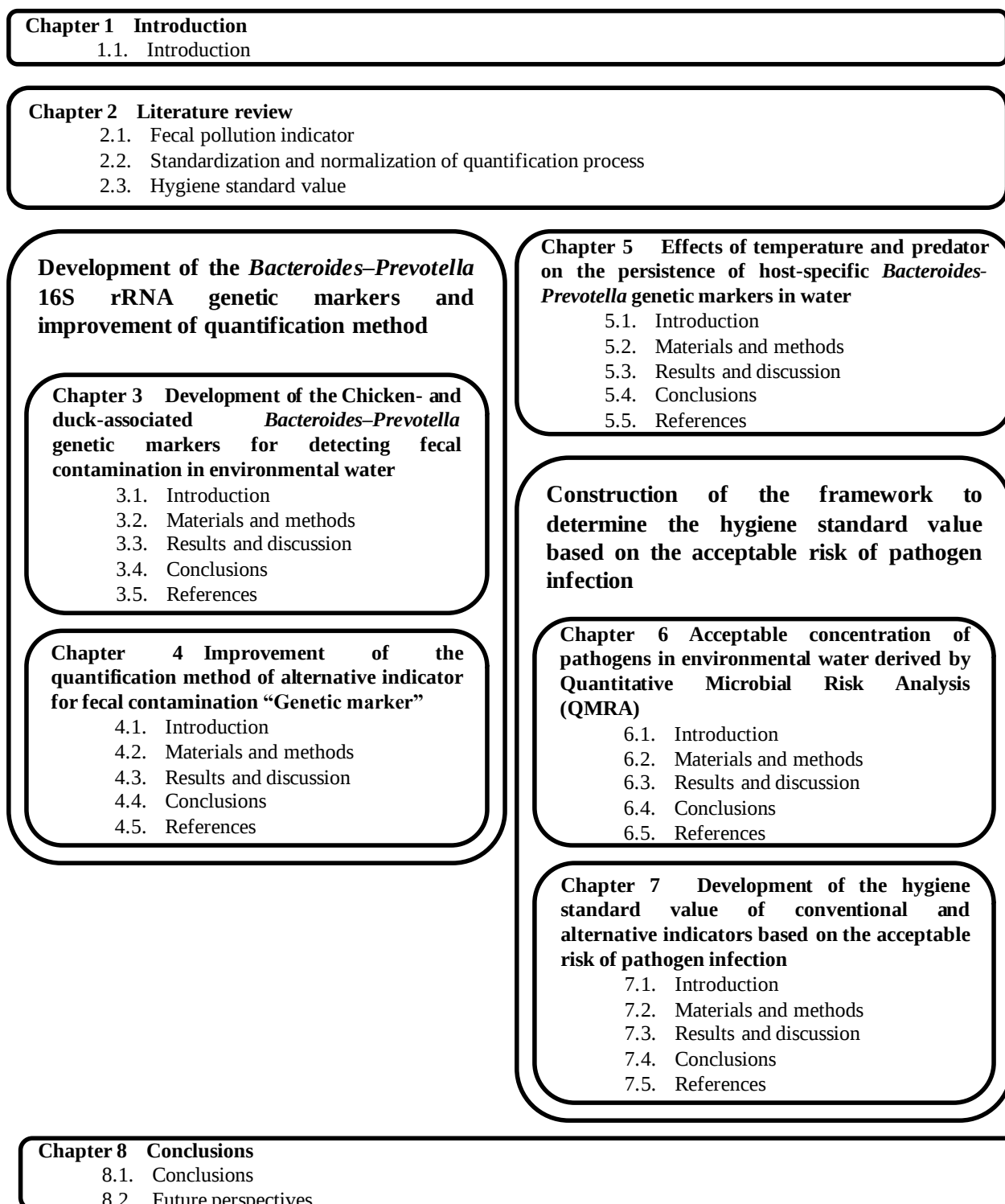


Figure 1.1 Composition of this thesis

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Chapter 2

Literature review

2.1. Fecal pollution indicator

The fecal contamination level is evaluated and managed by using the quantity value of the alternative microorganism that is called an indicator of fecal pollution. Conventional fecal pollution indicators, including coliforms, fecal coliforms, *Escherichia coli* and *Enterococcus* spp. have enormously contributed to the control and maintenance of public health (Savichtcheva and Okabe 2006; Scott et al. 2002).

Ideally, fecal pollution indicators are nonpathogenic, rapidly detected, easily enumerated, have survival characteristics that are similar to those of the pathogens of concern, and can be strongly associated with the presence of pathogenic microorganisms (Scott et al. 2002). However, the following problems are pointed out for conventional fecal pollution indicators; 1) replication in natural environment, 2) bacterial indicator originated from natural environment, 3) identification of the fecal pollution source is impossible, and 4) low correlation with pathogen density.

Thus, many of alternative fecal pollution indicators had been developed. Alternative fecal pollution indicators are typically divided into four methods; 1) culture-dependent and library-dependent, 2) culture-dependent and library-independent, 3) culture-independent and library-dependent, and 4) culture-independent and library-independent method. In this section, major fecal pollution indicators in each method are introduced.

2.1.1. Culture-based and Library-dependent method

Culture-based and library-dependent method is the technique of source tracking, which use the cultivation of indicator microorganism as detection method, and use the reference data for source identification. The reference data is the detection pattern result obtained by applying this technique to a known microbe or microbial community such as *E. coli*. This reference data is constructed beforehand, and then the contamination source is presumed by comparing with the detection pattern of an indicator microorganism. The disadvantages of this method are that a lot of work and expense are needed to cultivate, and source identification will be difficult because of small reference data. Culture-based and library-dependent methods include the antibiotic resistance analysis (Hagedorn et al. 1999; Harwood et al. 2000; Kaspar et al. 1999; Sayah et al. 2005; Whitlock et al. 2002; Wiggins 1996; Wiggins et al. 1999), the DNA fingerprinting such as ribotyping (Carson et al. 2001; Hartel et al. 2003; Jenkins et al. 2003; Scott et al. 2003; Stoeckel et al. 2004; Tynkkynen et al. 1999), REP-PCR (Dombek et al. 2000; Mohapatra and Mazumder 2008; Stoeckel et al. 2004) and pulse-field gel electrophoresis (PFGE) (Avery et al. 2004; Lasalde et al. 2005; Stoeckel et al. 2004; Tynkkynen et al. 1999), the carbon-source utilization profiling (CUP) (Carpenter-Boggs et al. 1998; Hagedorn et al. 2003; Hagedorn et al. 2011; Miller and Rhoden 1991; Stoeckel et al. 2004), fatty acid methyl ester

(FAME) analysis (Carpenter-Boggs et al. 1998; Genthner et al. 2005; Parveen et al. 2001) and so on. In this paragraph, typical culture-based and library-dependent methods are introduced.

a) Antibiotic resistance analysis (ARA)

ARA is one of the microbial source tracking method, which is based on the high probability of presence of antibiotic resistance bacteria because of high level exposure of antibiotics to human and livestock intestinal flora. The antibiotic resistance analysis uses the antibiotic resistance patterns of fecal *streptococci* (Hagedorn et al. 1999; Harwood et al. 2000; Wiggins 1996; Wiggins et al. 1999), *enterococci*, fecal coliforms (Harwood et al. 2000; Whitlock et al. 2002), or *E. coli* (Kaspar et al. 1999; Sayah et al. 2005) as an indicator bacteria pattern. To premise source of fecal pollution, the antibiotic resistance patterns from indicator bacteria in human feces are compared with that from indicator bacteria in animal feces. However, bacteria acquire the resistance to antibiotics by a plasmid, and lose the resistance to antibiotics plasmid, depending on the environmental condition. Moreover, the sensitivity of antibiotic resistance pattern may change specifically, if the kind of used antibiotics between human and livestock were different. Therefore, much reference data including the antibiotic analysis result from many kind of strain and from broad area may be needed.

b) DNA fingerprinting: ribotyping, REP-PCR, and Pulse-field gel electrophoresis (PFGE)

DNA fingerprinting methods include ribotyping (Carson et al. 2001; Hartel et al. 2003; Jenkins et al. 2003; Scott et al. 2003; Stoeckel et al. 2004; Tynkkynen et al. 1999), REP-PCR (Dombek et al. 2000; Mohapatra and Mazumder 2008; Stoeckel et al. 2004), Pulse-field gel electrophoresis (PFGE) (Avery et al. 2004; Lasalde et al. 2005; Stoeckel et al. 2004; Tynkkynen et al. 1999) and so on. In Ribotyping, genomic DNA is processed by the specific restriction enzyme and the pattern of the band is obtained using an oligonucleotide probe. This pattern is investigated about each host such as human and livestock, and they are accumulated as the reference data, then source identification is performed by comparing the electrophoresis pattern of unknown sample and reference data. Although it has been reported that ribotyping detects effectively the contamination source to humans or non-humans (Carson et al. 2001; Hartel et al. 2003; Jenkins et al. 2003; Scott et al. 2003; Stoeckel et al. 2004; Tynkkynen et al. 1999), essential problem remains. Since the analysis result from isolated unknown strain is compared, the size of the reference database of a known pollution source is large significance for this technique. Moreover, the disadvantages of this method are that we will spend a lot of work and expense to identify sources by the cultivation and isolation of target bacteria, DNA extraction, the electrophoresis, the southern blotting and the statistical analysis. Some factors such as the difference in the target area and the feeding habits of hosts may affect the verification of an analysis result.

Repetitive element PCR is the technique of using the primer which can amplify the dispersed repetitive sequence which exists in various positions on DNA, in order to perform DNA fingerprinting. Repetitive element PCR includes the repetitive extragenic palindromic sequence PCR (rep-PCR), PCR with extragenic repeating elements (BOX-PCR), and so on. The detected band patterns are analyzed by multivariate analysis of variance (MANOV) in order to construct the reference data for source identification. Then source identification is performed by comparing the band pattern of unknown sample and reference data. Like the case with ribotyping, the size of the reference database of a known pollution source is large significance for this technique. In addition to it, low reproducibility is also disputed (Scott et al. 2002; Seurinck et al. 2005).

Pulsed-field gel electrophoresis (PFGE) is a technique of performing DNA fingerprinting after the restriction enzyme treatment to bacterial DNA. Like the case with ribotyping, the number of the restriction site shows the degree of the variation of DNA, the band pattern of each strain is accumulated as the reference data for source identification. Since this technique had the high identification ability and the high reproducibility, it was used on the epidemiologic survey in many cases (Swaminathan et al. 2001). However, this technique has some problems; 1) electrophoresis tank to which the direction of an electric field can be changed is required, 2) in order to perform the restriction enzyme treatment, bacteria are embedded as a plug of gel, thus the time of an enzyme reaction will be long (about one week), and 3) the autolysis of bacteria sometimes occur and then it leads the unanalyzable band pattern.

c) Carbon-source utilization profiling (CUP)

Carbon source utilization profiling based on the ability of bacteria to metabolize numerous carbon and nitrogen substrates. The ability to metabolize numerous carbon and nitrogen substrates in many bacteria and community were tested by using specific system such as PhenePlate system (Bio Sys inova, Stockholm, Sweden) and Biolog™ Microstation System™ (Biolog, Inc., CA, USA) (Carpenter-Boggs et al. 1998; Hagedorn et al. 2003; Miller and Rhoden 1991). Advantages to this technique include rapid analysis time, less personnel training, relatively standardized operation, and geographical stability (Hagedorn et al. 2011). However, Stoeckel et al. (2004) reported that CUP would have made inaccurate reports of contributing sources. Since the size of the reference database of a known pollution source is large significance for this technique, additional information and not only geographical variability but also temporal stability will be needed.

2.1.2. Culture-based and Library-independent method

Culture-based and library-independent method is the technique of source tracking, which use the cultivation of indicators as detection and quantification method, and avoid the use of preliminarily-constructed reference data for source identification. The reason for not using reference data is that the quantitative values of indicator microorganisms directly show the contamination level. Culture-based and library-independent methods include the bacteriophage methods (Allwood et al. 2003; Griffith et al. 2003; Havelaar et al. 1986; Love and Sobsey 2007; Noble et al. 2003; Payan et al. 2005; Stewart-Pullaro et al. 2006; Tartera and Jofre 1987; Tartera et al. 1989), the bacteria methods (Blanch et al. 2006; Byamukama et al. 2005; Hill et al. 1996; Kuntz et al. 2004; Leclerc et al. 1996; Mara and Oragui 1983; McFeters et al. 1974; Mushi et al. 2010; Pourcher et al. 1991; Rhodes MW, and Kator 1999; Skanavis and Yanko 2001; Scott et al. 2002; Wheeler et al. 2002), and virus methods (Fong et al. 2010; Fong et al. 2005; Fong and Lipp 2005; Guttman-Bass and Nasser 1984; Jiang et al. 2001; Jiménez-Clavero et al. 2005; Ley et al. 2002; Pianetti et al. 2000; Pusch et al. 2005). In this paragraph, typical culture-based and library-independent methods are introduced.

a) Bacteriophage methods

In this method, bacteriophages derived from intestinal flora such as F-specific RNA coliphages (Allwood et al. 2003; Griffith et al. 2003; Havelaar et al. 1986; Love and Sobsey 2007; Noble et al. 2003; Stewart-Pullaro et al. 2006), and *Bacteroides fragilis* bacteriophages (Payan et al. 2005; Tartera and Jofre 1987; Tartera et al. 1989), are used as indicator microorganism. Coliphage is a virus which has the ability of infection to *E. coli*. Since the feces from animals or humans contain RNA coliphage from which serotype differs, the source identification and the monitoring of contamination level will be achieved by the quantification of each coliphage serotype. F-specific RNA coliphages were classified into four groups by serotyping. Havelaar et al. (1986) reported that all isolates from animal were belonging to either group I or IV. In contrast with animal isolates, isolates from hospital wastewater belonged to serogroups II or III. However, the number of coliphage which is presence in natural environment is quite low compared with that of the conventional fecal pollution indicators, thus the detection sensitivity and their concentrations are very important. Moreover, additional information of the persistence of these coliphages in environmental water and the gene signature of the other bacteriophages are needed, to prevent the false negative or false positive results (Brion et al. 2002; Schaper et al. 2002).

Bacteroides fragilis bacteriophage is a bacteriophage which has the ability of infection to *Bacteroides fragilis*. *Bacteroides* spp. are one of the most predominant species in human and animal intestinal flora. This technique uses *B. fragilis* HSP40, which is detected from human feces. Moreover, these bacteriophages could not proliferate in environment water, and the correlation between this bacteriophage and enteric virus has been suggested (Payan et al. 2005; Tartera and Jofre

1987; Tartera et al. 1989; Grabow et al. 1995). Therefore, it was thought that Bacteriophage infected with *B. fragilis* HSP40 may be able to use as an indicator of human fecal contamination. However, it has some problem such as geographical stability that they are not detected from sewage system sometimes (Payan et al. 2005; McLaughlin and Rose 2006).

b) Bacteria methods

Conventional fecal pollution indicators, including coliforms, fecal coliforms, *Escherichia coli* and *Enterococcus* spp. are classified into this method. However, a poor correlation between the amount of bacterial indicator microorganisms and that of pathogens is sometimes observed. Then alternative target bacteria have been focused on. Alternative target includes *Bifidobacterium* spp. (Mara and Oragui 1983; Mushi et al. 2010; Rhodes MW, and Kator 1999), *Clostridium perfringens* (Byamukama et al. 2005; Hill et al. 1996; Skanavis and Yanko 2001), *Enterococcus* spp. (Kuntz et al. 2004; Leclerc et al. 1996; Pourcher et al. 1991; Wheeler et al. 2002), a fecal coliform / fecal streptococcus ratio (FC / FS ratio) (Blanch et al. 2006; McFeters et al. 1974; Pourcher et al. 1991; Scott et al. 2002) and so on.

Bifidobacterium spp. are gram-positive anaerobes with high abundance in human feces (10^9 - 10^{10} cells/g, or 1-6% of human intestinal flora), and they are hardly detected from animal feces. Especially, isolates from human feces have an ability of sorbitol-fermenting and they are called sorbitol-fermenting bifidobacteria (SFB) (Mara and Oragui 1983; Mushi et al. 2010; Rhodes MW, and Kator 1999). By using the human bifid sorbitol agar (HBSA), the contamination by human feces and wastewater are found in distinction from animal feces. However, the detection of *Bifidobacterium* spp. needs the cultivation during 4 to 6 days and sampling method should be optimized because they rapidly die off.

The rationale behind fecal coliform / fecal streptococcus ratio (FC / FS ratio) was the observation that human feces contain higher fecal coliform counts, while animal feces contain higher levels of fecal streptococci (Scott et al. 2002). The ratio ratio of >4.0 would indicate human pollution and the ratio of ≤ 0.7 would indicate nonhuman pollution (Scott et al. 2002). The advantage of this method is the rapid detection and the minimal expertise. However, this approach has variable persistence of fecal streptococci species depending on environmental condition and water treatments.

c) Virus methods

Conventional fecal pollution indicators sometimes show a poor correlation between the amount of themselves and that of pathogenic virus. Virus indicators are alternative indicators which attracts attention recently compared with bacterial indicators. In this method, viruses derived from intestinal flora such as *Enterovirus*, *Adenovirus*, human *Polyomavirus* Torque teno virus, Picobirnavirus, Pepper mild mottle virus, Aichi virus and so on are used as indicator virus.

Enteroviruses have a single-stranded RNA without an envelope, and associated with several human and mammalian diseases. *Enteroviruses* includes some species; *Enterovirus*, *Rhinovirus*, *Coxsackievirus*, *Echovirus* and *Poliovirus*. Human-specific enterovirus species have been reported as an indicator of fecal pollution contamination from human feces (Guttman-Bass and Nasser 1984; Pianetti et al. 2000; Pusch et al. 2005). On the other hand, bovine enteroviruses (BEV) are expected as an indicator of fecal pollution from ruminant feces (Jiménez-Clavero et al. 2005; Ley et al. 2002). Adenoviruses have a double stranded DNA genome without an envelope. Pina et al. (1998) suggests that the presence of adenovirus may be able to monitor the viral contamination in natural water. And then, Adenoviruses, especially adenovirus type 40 and 41 have been used as an indicator of human fecal contamination (Fong et al. 2010; Fong et al. 2005; Fong and Lipp 2005; Jiang et al. 2001; Pusch et al. 2005). Many of virus methods are under development and consideration. These virus indicators have not been classified into the culture-based method, because RT-qPCR assay had been widely used, recently.

2.1.3. Culture-independent and Library-dependent method

Culture-independent and library-dependent method is the technique of source tracking, which avoid the use of cultivation as detection method, and use of preliminarily-constructed reference data for source identification. Advantage of this method detects indicators more quickly because of not using cultivation, meanwhile, disadvantages of this method are that source identification will be difficult because of small reference data. Culture-independent and library-dependent methods include terminal restriction fragment length polymorphism (T-RFLP) (Bernhard and Field 2000a; Dorai-Raj et al. 2009; Savichtcheva and Okabe 2009), Denaturing gradient gel electrophoresis (DGGE) (Buchan et al. 2001; Esseili et al. 2008), Matrix-Assisted Laser Desorption / Ionization Time-of-Flight Mass Spectroscopy (MALDI-TOF-MS) (Giebel et al. 2008; Giebel et al. 2010; Siegrist et al. 2007) and so on. In this paragraph, typical culture-independent and library-dependent methods are introduced.

a) Terminal restriction fragment length polymorphism (T-RFLP)

This technique needs to perform the fragmentation analysis of fragment degraded by restriction enzyme after PCR amplification by fluorescent labeled primer. This technique presumes the source of fecal contamination by comparing fragment length polymorphism of unknown samples with that of reference data. *Bacteroidals* is the most promising target in this context, because this group is one of the predominant enteric bacteria in warm-blooded animal feces, and has host or group-specific distributions (Bernhard and Field 2000a; Dorai-Raj et al. 2009; Savichtcheva and Okabe 2009). Advantage to this technique includes rapid analysis time. However, in order to

measure the intensity of the detection peak of fragment length polymorphism analysis, the accuracy of quantified value is quite low.

b) Denaturing gradient gel electrophoresis (DGGE)

This technique is a relatively new application in field of source tracking. This technique needs to perform electrophoresis which use chemical gradient to denature the sample. The gradients for electrophoresis are generally used urea and formamide. This technique presumes the source of fecal contamination by comparing the band patterns of unknown samples with that of reference data. The intergenic spacer region (ISR) of 16S-23S rRNA, *uidA*, *mdh*, *phoE* and *uidA-4* of *E. coli* are assessed as the target of DGGE for source tracking of fecal pollution (Buchan et al. 2001; Esseili et al. 2008). However, because of relatively new application in field of source tracking, the size of the reference database of a known pollution source is large significance for this technique.

c) Matrix-assisted laser desorption / ionization time-of-flight mass spectroscopy (MALDI-TOF-MS)

MALDI-TOF-MS is also a relatively new application in field of source tracking (Giebel et al. 2008; Giebel et al. 2010; Siegrist et al. 2007). Advantage to this technique includes rapid analysis time. However, because of relatively new application in field of source tracking, the size of the reference database of a known pollution source is large significance for this technique. Moreover, this technique is under development and consideration of analysis and reproducibility.

2.1.4. Culture-independent and Library-independent method

Culture-independent and library-independent method is the technique of source tracking, which avoid the use of cultivation as detection method, and the use of preliminarily-constructed reference data for source identification. Advantages of this method are that we will not spend a lot of work and expense because of cultivation, and source identification will be easy because of the lack of the need for reference data. Culture-independent and library-independent methods include the chemical methods such as stanols (Bull et al. 2002; Gourmelon et al. 2010; Grimalt et al. 1990; Leeming et al. 1996; Macdonald et al. 1983; Reeves and Patton 2005; Venkatesan and Kaplan 1990; Vikaskumar et al. 2007), caffeine (Chen et al. 2002; Buerge et al. 2003; Burkhardt et al. 1999) and pharmaceutical compounds (Cimenti et al. 2007; Gassera et al. 2011; Haack et al. 2009; Katz et al. 2009) and molecular methods such as genetic markers (Ballesté and Blanch 2011; Bonjoch et al. 2004; Dick et al. 2005; Matsuki et al. 2004; Savichtcheva and Okabe 2006; Scott et al. 2002; Scott et al. 2005; Wheeler et al. 2002). In this paragraph, typical culture-independent and library-independent methods are introduced.

a) Chemical methods: sterols, caffeine and pharmaceutical compounds

The main sterol contained in feces is cholesterol, epicoprostanol, coprostanol, and cholestanol. This technique uses the percentage of these stanols for source identification. Coprostanol accounts for 60% of all stanol in human feces (Macdonald et al. 1983), thus it was thought that 5β -stanol / (5α -stanol+ 5β -stanol) and coprostanol / (coprostanol+ 5β -stigmastanol) and so on may suggest the contamination of feces (Bull et al. 2002; Gourmelon et al. 2010; Grimalt et al. 1990; Leeming et al. 1996; Reeves and Patton 2005; Venkatesan and Kaplan 1990; Vikaskumar et al. 2007). Caffeine is contained in drinking water, pharmaceutical compounds, coffee and so on. Therefore, it is thought that the caffeine which exists in environment suggests human fecal contamination (Chen et al. 2002; Buerge et al. 2003; Burkhardt et al. 1999). Some pharmaceutical compounds such as carbamazepine is also a relatively new target in field of source tracking (Cimenti et al. 2007; Gassera et al. 2011; Haack et al. 2009; Katz et al. 2009). However, these chemical methods have little information of the fate of them in environmental water and the correlation between the concentration of them and that of pathogens, thus additional information such as geographical variability, temporal stability will be needed (Chen et al. 2002; Sauvé et al. 2012).

b) Molecular methods: genetic markers by using qPCR

Host-specific genetic markers targeting *Bifidobacterium* spp., *Enterococcus* spp., *Bacteroides* spp. and so on have been regarded as alternative indicators of water contamination with feces (Ballesté and Blanch 2011; Bonjoch et al. 2004; Dick et al. 2005; Matsuki et al. 2004; Savichtcheva and Okabe 2006; Scott et al. 2002; Scott et al. 2005; Wheeler et al. 2002). *Bacteroides-Prevotella* group is the most promising target in this context, because this group is one of the predominant enteric bacteria in warm-blooded animal feces, and has host or group-specific distributions (Bernhard and Field 2000a; Bernhard and Field 2000b). Host-specific real-time quantitative PCR (real-time PCR) primers targeting 16S rRNA genes of *Bacteroides* and *Prevotella* genera have been successfully developed to quantify genetic markers in water environments and to identify fecal sources from humans, cows, pigs, and dogs (Kildare et al. 2007; Okabe et al. 2007).

2.1.5. References

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2.2. Standardization and normalization of quantification process

Bacteroides-Prevotella group is the most promising target in alternative indicators (Bernhard and Field 2000a; Bernhard and Field 2000b), and host-specific real-time quantitative PCR (real-time PCR) primers targeting 16S rRNA genes of *Bacteroides* and *Prevotella* genera have been successfully developed to quantify genetic markers in water environments and to identify fecal sources from humans, cows, pigs, and dogs (Kildare et al. 2007; Okabe et al. 2007). Thus, in this section, bacterial genetic markers are focused.

The detection of these genetic markers from environmental water needs the concentration step, recovery step, DNA extraction step, PCR step and so on. However, previous reports are based on the results using each equipment and each reaction reagent in each laboratory. Thus, the quantification methods should be standardized; those enable us to check whether the sample processing performed without any problems and to compare results obtained from different

laboratories (Meays et al. 2004; Stewart et al. 2003; Harwood et al. 2009; Stoeckel et al. 2009).

In addition, the DNA recovery and PCR amplification efficiencies must be determined for individual measurements to correct or monitor the measured values, because DNA recovery and PCR amplification may vary depending on the water quality of samples, the efficiency of reaction reagents, or the personal error. To determine the DNA recovery and PCR amplification efficiencies, two controls are generally employed: a sample process control (SPC) and an internal amplification control (IAC). However, very few studies have used SPC and/or IAC, especially in quantification of genetic markers (Diez-Valcarce et al. 2011).

In this section, the sample processing of genetic markers, SPC and IAC for bacterial genetic indicators are introduced.

2.2.1 Sample processing

The detection of genetic markers from environmental water needs the concentration step, recovery step, DNA extraction step, PCR step and so on. In the concentration step, filtration concentration method and centrifuge concentration method are used depending on the type of samples (Bernhard and Field 2000a; Kildare et al. 2007; Savichtcheva et al. 2007; Seurinck et al. 2005). In the DNA extraction step, some methods and DNA extraction kits such as bead-beating method, FastDNA kit for Soils (Q-Biogene, Carlsbad, CA) and QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) are used depending on the type of samples (Bernhard and Field 2000a; Dick et al. 2005; Gawler et al. 2007; Kildare et al. 2007; Okabe et al. 2007; Savichtcheva et al. 2007; Seurinck et al. 2005). In the qPCR step, some reaction reagents such as TaqMan PCR master mix (Eurogentec, CA, USA), qPCR Mastermix Plus for SYBR Green I (Eurogentec, CA, USA), Premix Ex Taq (Takara Bio, Otsu, Japan) and Power SYBR Green PCR Master Mix (Applied Biosystems, Forster City, CA, USA) are used (Kildare et al. 2007; Okabe et al. 2007; Savichtcheva et al. 2007; Seurinck et al. 2005). Then, simple comparison between data from some laboratory and data from another laboratory is difficult (Meays et al. 2004; Stewart et al. 2003; Harwood et al. 2009; Stoeckel et al. 2009).

2.2.2 Amplification control

PCR inhibitor potentially leads the false-negative result in PCR assay, because PCR inhibition by substances in environmental samples often cannot be completely prevented (Gonzague et al. 2002; Hartman et al. 2005; Hoorfar et al. 2004). IAC is a nontarget genetic component for determining any inhibitory effects on the activity of DNA polymerase enzymes. A known amount of IAC (e.g., plasmid DNA) is spiked to DNA samples, and the PCR amplification efficiency is

determined based on the measured values of IACs in the DNA sample and in a pure water control. Some researchers have reported that IACs enable accurate quantification and prevent the false negative results (Klerks et al. 2004; Hoorfar et al. 2003; Hoorfar et al. 2004). In the field of fecal source tracking, a plasmid DNA construct was developed to function as an IAC DNA target that can be spiked into DNA extracts to monitor for PCR inhibition (Shanks et al. 2009). Whether PCR inhibitions of IAC are detected or not may depend on the sample component and condition. Thus, IAC are essential to monitor the reaction efficiency of measured values of genetic markers from environmental water samples (D'Agostino et al. 2011; Shanks et al. 2009).

2.2.3 Sample process control (SPC)

Recovery of bacterial cells or virus particles from environmental samples is inhibited by known component in samples, and it leads the false-negative result in next quantification step (Stewart et al. 2008). The SPC is used to evaluate the DNA loss during sample processing (e.g., cell recovery and DNA extraction). A known amount of SPC is spiked to samples, and the DNA recovery efficiency is determined based on the amount of SPC recovered before and after the sample processing, as measured by SPC-specific qPCR assay. The ideal SPC strain should meet the following criteria. Firstly, they should have a similar cellular structure to the target bacteria (e.g., genetic marker bacteria). Secondly, the SPC strain should not be detected in unspiked samples. Thirdly, the concentration and DNA recovery efficiency of the SPC strain should be similar to that of the genetic marker bacteria.

In the field of fecal source tracking, Lebuhn et al. (2004) used some type of SPCs, avirulent *poliovirus*, *Cryptosporidium* and so on to allow the sample-to-sample comparison. Koike et al. (2007) used the SPC, which is plasmid-based target originated from *E. coli*, to estimate recovery efficiency of tetracycline resistance genes from water samples. Siefring et al. (2008) used two types of SPCs, salmon sperm DNA and *Lactococcus lactis* cells prior to DNA extraction to correct for recovery efficiency of *enterococci* and *Bacteroidales*. Stoeckel et al. (2009) used two types of SPCs, the plant pathogen *Pantoea stewartii* carrying the chromosomal target gene *cpsD* and *E. coli* carrying the plasmid-borne target gene *DsRed2*, for evaluating the recovery efficiency of *Bacteroidales* 16S rRNA genes from river water. However, DNA extraction from bacterial cells may be different from that of DNA fragment. However, potential environmental occurrence of these bacteria may confound the usage of these two bacterial strains as SPCs. In addition, since the comparisons of DNA recovery efficiencies between the SPC strains and the genetic marker bacteria have not been conducted, the applicability of these SPCs must be further evaluated.

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2.3. Hygiene standard value

Fecal pollution had been managed and evaluated by conventional fecal pollution indicators, such as coliforms, fecal coliforms, *Escherichia coli* and *Enterococcus* spp., and have enormously contributed to the control of public health all over the world (Savichtcheva and Okabe 2006; Scott et al. 2002). However, hygiene standard values for managing microbiological water quality in many countries are not set based on the acceptable risk of pathogen concentration. In this section, the water quality standard value in the Japan and USA, and guideline for determination of water quality standard value are introduced.

2.3.1 Water quality standard value in Japan

In Japan, bacterial water quality standards have been used in the water quality standards for drinking water, the environmental quality standards for water pollution, the water quality criteria for bathing beaches, and so on (MOE, Water Quality Criteria for Bathing Beaches; MOE, Environmental Quality Standards for Water Pollution; MHLW, Drinking Water Quality Standards). The concentration of general bacteria, the concentration of heterotrophic bacteria, the concentration of *E. coli*, total coliforms, fecal coliforms and so on are used in these standards, and the detection method of these bacteria is based on the cultivation (MOE, Environmental Quality Standards for Water Pollution (In Japanese); MOE, Surveillance of Water Quality in Bathing Beaches (In Japanese); MHLW, Measurement method of water quality standards (In Japanese)). The water quality standard values were set as concentration of these bacteria.

Previously, although it is thought that these water quality standards are based on epidemiological information, the reason for the setup of standard concentration has not been clearly reported. However, in May 2003, the Drinking Water Quality Standards were revised in response to the amendment to the Guidelines for Drinking Water Quality of the World Health Organization (MHLW, Water quality; WHO 2008).

2.3.2 Water quality standard value in USA

In USA, U.S.EPA had set the bacterial water quality standards in the National Primary Drinking Water Regulations (NPDWRs), the Recreational Water Quality Criteria, the Nationwide Bacteria Standards Protect Swimmers at Beaches and so on (U.S.EPA, NPDWRs; U.S.EPA, Nationwide Bacteria Standards Protect Swimmers at Beaches; U.S.EPA, Recreational Water Quality Criteria). Especially, bacterial water quality standards such as the concentration of *Cryptosporidium*, total coliform, fecal coliform, the concentration of *E. coli*, the concentration of *Giardia lamblia*, Heterotrophic plate count, the concentration of *Legionella*, the concentration of viruses (enteric) are used in the National Primary Drinking Water Regulations (U.S.EPA, NPDWRs). The greatest characteristic of water quality standards in USA is the use of Maximum Contaminant Level Goal (MCLG), Maximum Contaminant Level (MCL) and Treatment Technique (TT) (U.S.EPA, NPDWRs). MCLG is the level of a contaminant in drinking water below which there is no known or expected risk to health. MCLGs allow for a margin of safety and are non-enforceable public health goals. MCL is the highest level of a contaminant that is allowed in drinking water. MCLs are set as close to MCLGs as feasible using the best available treatment technology and taking cost into consideration. MCLs are enforceable standards. TT is a required process intended to reduce the level of a contaminant in drinking water. For example, MCL or TT of total coliforms in NPDWRs was set as 5%. This means that total coliform-positive (TC-positive) samples in a month are not more than 5.0% (For water systems that collect fewer than 40 routine samples per month, no more than one sample can be total coliform-positive per month). Every sample that has total coliform must be analyzed for either fecal coliforms or *E. coli* if two consecutive TC-positive samples are detected.

In addition to it, more stringent water quality standards are set in each county. For example, it is said that Title 22 Regulations from California Department of Social Services is one of the severest water quality standards. NPDWRs need that either fecal coliforms or *E. coli* are analyzed, if two consecutive TC-positive samples are detected. Meanwhile Title 22 needs following things; if any routine sample is TC-positive, either fecal coliforms or *E. coli* should be analyzed. The water supplier shall notify the local health officer within 48 hours from the time the results are received and shall take corrective actions as directed by the local health officer to eliminate the cause of the positive samples (CDSS 2013).

2.3.3 WHO guideline

WHO had recommended the bacterial water quality standards in Guidelines for Drinking Water Quality, Water Quality: Guidelines, Standards and Health, Assessment of Risk and Risk Management for Water-related Infectious Diseases, Guidelines for safe recreational water environments, and so on (WHO, Guidelines for Drinking Water Quality 4th edition; WHO,

Guidelines for safe recreational water environments, Volume 1 and 2; WHO, Water Quality: Guidelines, Standards and Health, Assessment of Risk and Risk Management for Water-related Infectious Diseases). Especially, bacterial water quality standards such as total coliform, fecal coliform, and concentration of *E. coli* are used in the Guidelines for Drinking Water Quality (WHO, Guidelines for Drinking Water Quality 4th edition). The greatest characteristic of water quality standards guideline in WHO is that they recommended the use of the epidemiological information and the quantitative microbial risk analysis (QMRA). QMRA is a rapidly evolving field that systematically combines available information on exposure and dose–response to produce estimates of the disease burden associated with exposure to pathogens (WHO, Guidelines for Drinking Water Quality 3rd edition). Ideally, health-based targets should be set using quantitative risk assessment and should take into account local conditions and hazards. However, there are limitations in the available data and models for QMRA. Short-term fluctuations in water quality may have a major impact on overall health risks, including those associated with background rates of disease and outbreaks, and are a particular focus of concern in expanding application of QMRA. Thus WHO said that further developments in these fields will significantly enhance the applicability and usefulness of this approach (WHO, Guidelines for Drinking Water Quality 3rd edition). Until now some countries such as Japan and China had developed and improved the drinking water quality standards according to this guideline (MHLW, Water quality; Ministry of Health of the People's Republic of China, Safe Drinking Water).

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Chapter 3

Development of the Chicken- and duck-associated *Bacteroides–Prevotella* genetic markers for detecting fecal contamination in environmental water

3.1. Introduction

Water contamination with feces has been a crucial issue from the perspective of microbiologically safe water management (Blanch et al. 2006; Simpson et al. 2002). Historically, total coliforms or thermotolerant coliforms (fecal coliforms) have been used as fecal pollution indicators in water utilization (World Health Organization 2011). These microorganisms are mainly of fecal origin from mammals and birds, and their presence in water may indicate fecal contamination and a possible association with enteric pathogen occurrences. Although these microorganisms have been employed as environmental and recreational water standard indicators because of their easy quantification and non-pathogenicity, several critical limitations have been described, including inability to detect non-culturable bacteria, difficulty to identify fecal sources (Field and Samadpour 2007), poor correlation with the presence of enteric pathogens (Harwood et al. 2005), and growth in natural water environments (Ishii et al. 2007).

Host-specific genetic markers have been regarded as alternative indicators of water contamination with feces (Dick et al. 2005; Savichtcheva and Okabe 2006; Scott et al. 2002). *Bacteroides-Prevotella* group is the most promising target in this context, because this group is one of the predominant enteric bacteria in warm-blooded animal feces, and has host or group-specific distributions (Bernhard and Field 2000a; Bernhard and Field 2000b). Host-specific real-time quantitative PCR (real-time PCR) primers targeting 16S rRNA genes of *Bacteroides* and *Prevotella* genera have been successfully developed to quantify genetic markers in water environments and to identify fecal sources from humans, cows, pigs, and dogs (Kildare et al. 2007; Okabe et al. 2007). This real-time PCR-based assay is more rapid and specific than the conventional culture-dependent enumeration methods.

In this study, we paid attention to waterfowls as carriers of waterborne pathogens by the worldwide migration (Lu et al. 2008). Wild waterfowls such as wild ducks might be non-point sources of fecal pollution, while poultries including chickens could be point fecal sources from chicken farms in a watershed. Since the importance of waterfowls as carriers of infectious disease pathogens has been emphasized (Kassa et al. 2001; Khawaja et al. 2005; Himathongkham and Riemann 1999), the monitoring and management of water contamination with avian feces are critical from the perspective of public health. Although avian-specific genetic markers have been proposed (Hamilton et al. 2006; Devane et al. 2007; Champagne et al. 2011; Weidhaas et al. 2011; Ryu et al. 2012), real-time PCR assays targeting 16S rRNA genes of chicken- and duck-associated *Bacteroides* and *Prevotella* genera have not been established so far.

The objective of this study is, therefore, to develop real-time PCR assays for chicken- and duck-associated *Bacteroides-Prevotella* 16S rRNA genetic markers in order to quantitatively

evaluate avian fecal contamination in water. *Bacteroides-Prevotella* 16S rRNA gene sequences were acquired from feces of chickens, ducks, humans, cows, and pigs. Chicken/duck-, chicken-, and duck-associated genetic markers were identified by a comparative 16S rRNA gene analysis. The cross reactivity of the newly designed primer and probe sets was tested with feces from chickens, ducks, and other animal feces (humans, cows, and pigs) and single or mixed fecal suspensions in environmental water. The quantification limit of each genetic marker was also determined. The established real-time PCR assays were used to quantify genetic markers in environmental water. Based on these results of real-time PCR assays, its applicability to the quantitative evaluation of chicken and duck-associated fecal pollution in water environments was discussed. Based on these results and discussions, *Bacteroides-Prevotella* group bacteria may not be always predominant in avian feces, which may lead to false negative results regarding avian fecal contamination in water. Combinations with other fecal pollution indicators, including operational taxonomic unit (OUT)-based approach (e.g., Unno et al. 2010) and chemical markers, may be required to reliably detect avian fecal contamination in water environments.

3.2. Materials and methods

3.2.1 Water and fecal samples

River water samples were collected from Toyohira River and Kamogamo River in Sapporo city, Hokkaido, Japan. Pond water samples were collected from Oono Pond in Hokkaido University, and wastewater samples were collected from Sosei River wastewater treatment plant in Sapporo city, Hokkaido, Japan. Oono Pond and Kamogamo River are known as stopovers of wild ducks (*Anas platyrhynchos*). Total coliforms counts were measured in each water sample except wastewater samples by plating on desoxycholate agar as previously described (Savichtcheva et al. 2007).

Human fecal samples (n=12) were collected from healthy adults. Fresh feces from cows (n=13), pigs (n=12), and chickens (*Gallus gallus domesticus*; n=32, including 9 samples originated from cecum) were collected from farms in Hokkaido University. Fresh feces from wild ducks (*Anas platyrhynchos*; n=39) were collected from Oono Pond in Hokkaido University. Fresh feces from Tundra swan (*Cygnus columbianus*; n=25) and greater white-fronted goose (*Anser albifrons*; n=16) were collected from Miyajima pond in Hokkaido Japan. All fecal samples were collected in sterile 50 mL-tubes and stored at -80 °C until the DNA extraction was performed.

Fecal suspensions in unfiltered water were prepared as previously described (Okabe and Shimazu 2007) using Oono pond water. Briefly, feces were added to an unfiltered pond water sample to prepare a single fecal suspension (chicken, duck, or human) at final concentrations of 0.1 g and

0.01 g feces per 100 mL. Mixed fecal suspensions (chicken + duck, duck + human, and chicken + human) were also prepared by mixing the same amount of feces (0.1 g or 0.01 g) from two animals out of the three in 100 mL of the fresh pond water. Therefore, the total concentration in the mixed fecal suspension is 0.2 g or 0.02 g per 100mL.

3.2.2 Recovery of bacterial cells from water sample

Bacterial cells in river and pond water samples were collected by capturing on a 0.22- μ m-pore-size polyethersulfone membrane filter. Bacterial cells on a membrane filter were eluted by soaking in 50 ml of sterile STE buffer (0.1 M NaCl, 10 mM Tris and 1 mM EDTA, pH 7.6) and rigorously shaken by a vortex mixer, and then stored for 1 h at 4 °C (Cho and Kim 2000; Okabe et al. 2007). Suspended cells in the STE buffer were precipitated by centrifugation at 10,000 $\times$ g for 15 min at 4 °C, and the pellet was resuspended in distilled MilliQ water for washing after the supernatant was gently removed. This washing step was repeated three times. The harvested and washed cells were resuspended in 2 ml of distilled water and subjected to DNA extraction.

Bacterial cells in wastewater treatment plant (WWTP) influent was precipitated by centrifugation at 10,000 $\times$ g for 10 min at 4 °C, and the supernatant was gently removed. Harvested cells were resuspended in 2 ml of distilled MilliQ water and subjected to DNA extraction.

3.2.3 DNA extraction and PCR amplification

Total DNA was extracted from fecal samples (200 μ g) and concentrated water samples (200 μ l or 800 μ l) by QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany). The *Bacteroides-Prevotella* specific primer set (Bac32F-Bac708R, Bernhard and Field 2000a) was used for PCR amplification targeting 16S rRNA gene. PCR was carried out with ABI PRISM GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) as described previously (Okabe et al. 2007). PCR products were separated with 1% (w/v) agarose gel electrophoresis, and visualized with staining by SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) and ultraviolet illumination.

3.2.4 Cloning and sequencing of 16S rRNA gene and phylogenetic analysis

PCR products were purified by using QIAquick PCR Purification Kit (QIAGEN, HILDEN, GERMANY). Purified PCR products were ligated into TOPO XL PCR cloning system (Invitrogen, Carlsbad, CA, USA). One Shot TOP10 Electrocomp *E.coli* (Invitrogen, Carlsbad, CA, USA) was transformed with ligated products, and plasmids were extracted and purified from transformed cells with the QIAprep Spin Miniprep Kit (QIAGEN, HILDEN, GERMANY). The concentration of plasmid DNA was measured by using DyNA Quant 200 (Amersham Bioscience). Partial sequencing

(ca. 670 bp) was performed with an automatic sequencer (Prism 3100, Avant Genetic Analyzer, Applied Biosystems, Forster City, CA, USA). Sequences with 97% or greater similarity were grouped into one OTU by using SIMILARITY_MATRIX program from the RDP (Maidak et al., 1997). Partial sequences were compared with similar sequences available in public databases (GenBank and NCBI) with a BLAST search (Altschul et al. 1990). Representative sequences from each OTU were aligned with CLUSTAL W package (Thompson et al. 1994). A phylogenetic tree was constructed by the neighbor-joining method (Saito and Nei 1987). Bootstrap resampling analysis of 1,000 replicates was performed to estimate the confidence of tree topologies. The sequence of the 16S rRNA gene from *Flexibacter litoralis* (AB078056) was used as an outgroup of the phylogenetic tree.

3.2.5 Design of chicken- and duck-associated primers and fluorescence probes

Primer design and reaction optimization for real-time PCR assays were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Stephen et al. 2009). Multiple alignments of the target phylogenetic groups and reference organisms were performed using the program CLUSTAL W (Thompson et al. 1994) to identify chicken/duck-, chicken- and duck-associated clusters. Chicken/duck-, chicken- and duck-associated genetic markers (named as Chicken/Duck-Bac, Chicken-Bac, and Duck-Bac, respectively) were designed using chicken/duck-, chicken- and duck-associated cluster sequences in *Bacteroides-Prevotella* 16S rRNA gene. Primer sets specific to each marker were designed using Primer Express software (Applied Biosystems, Forster City, CA, USA). Minor groove binder (MGB) probes were designed for the Chicken/Duck-Bac and Duck-Bac markers using Primer Express software. The 6-carboxy-fluorescein (FAM) was conjugated at the 5' ends of the probes, and MGB were conjugated at the 3' ends. The specificity of designed primers and probes was verified using probeCheck (Loy et al., 2008). For all designed primer, real-time PCR assay was performed to examine the cross reactivity of the designed primer and probe sets by using DNA (0.25 ng and 2.5 ng) extracted from fecal samples (23 chickens, 26 wild ducks, 13 cows, 12 pigs and 12 humans) and 11 WWTP influent samples.

3.2.6 Real-time PCR assay

SYBR Green assay was performed with the primer sets specific to BacPre1 (Okabe et al. 2007) and Chicken-Bac (this study) markers, and the TaqMan assay was performed with the primer sets specific to Human-Bac1 (Okabe et al. 2007), Chicken/Duck-Bac and Duck-Bac (this study) markers. For the SYBR Green assays, PCR mixture (25 µl) was composed of 12.5 µl of 2× Power

SYBR Green PCR Master Mix (Applied Biosystems, Forster City, CA, USA), 300 nM each of forward and reverse primers and 2.5 µl of template DNA. For the TaqMan assays, PCR mixture (25 µl) was composed of 12.5 µl of 2× Premix Ex Taq (Takara Bio, Otsu, Japan), 200 nM each of forward and reverse primers, 200 nM of TaqMan probe, 100 µg/µl of bovine serum albumin (BSA, Sigma, Steinheim, Germany), and 2.0 µl of template DNA. All PCR reactions were performed in MicroAmp Optical 96-well reaction plates with ABI PRISM 7000 sequence detection system (Applied Biosystems, Forster City, CA, USA). PCR reactions were performed for duplicate sets of samples. For the SYBR Green assays, the reactions were carried out by incubation at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec and annealing and extension at 60 or 62 °C (as shown in Table 3.1) for 1 min. A melting curve analysis was performed after an amplification to examine the presence of non-target amplicons. For the TaqMan assays, the reactions were carried out by incubation at 95 °C for 30 sec, followed by 40 cycles of denaturation at 95 °C for 5 sec and annealing and extension at 60 or 62 °C (as shown in Table 3.1) for 31 sec.

Standard sequences for the real-time PCR assays were prepared using recombinant PCR-XLTOPO vector plasmids inserted with *Bacteroides-Prevotella* specific 16S rRNA genetic marker (Bac32F-Bac708R) sequences. The concentration of plasmid DNA was measured by using DyNA Quant 200 (Amersham Bioscience). The ratio of 4.0×10^{-18} g-DNA/copy, estimated by Okabe et al. (2007), was used to convert the weight of plasmid to the number of copies. The concentration of diluted plasmid DNA was plotted against mean threshold cycle values (Ct). In order to compare the PCR amplification efficiency and detection sensitivity between assays, the slope of the standard curves was calculated by performing a linear regression analysis with ABI PRISM 7000 sequence detection software (v1.0; Applied Biosystems, Forster City, CA, USA). Amplification efficiency (E) was calculated as $E = (10^{-1/\text{slope}}) - 1$ (a reaction with 100% efficiency will give a slope of -3.32). The ranges of the quantitative determination for the plasmid DNA of BacPre1 and Human-Bac1 were 6.3 to 6.3×10^7 and 50 to 5.0×10^7 copies/reaction, respectively (Okabe et al. 2007). The quantification limit was defined as the lowest concentration of the *Bacteroides-Prevotella* 16S rRNA genetic marker within the linear range of a quantification curve.

3.2.7 Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene sequences of the clones used in the phylogenetic analysis were AB666096-AB666157.

3.3. Results and discussion

Table 3.1 Primers and probes used in this study

Marker	Primer and probe name	Sequence (5'-3')	Product size (bp)	Annealing temperature (°C)	References
BacPre1	qBac560F	TTTATTGGGTTTAAAGGGAGCGTA	165	62	Okabe et al. 2007
	qBac725R	CAATCGGAGTTCCTCGTGATATCTA			
Human-Bac1	qHS601F	GTTGTGAAAGTTTGCGGCTCA	124	62	Okabe et al. 2007
	qBac725R	CAATCGGAGTTCCTCGTGATATCTA			
	qHS 624MGB	CGTAAAATTGCAGTTGA			
	(5'-FAM and 3'-MGB)				
Chicken/Duck-Bac	qCD362F-HU	AATATTGGTCAATGGGCGAGAG	102	60	this study
	qCD464R-HU	CACGTAGTGTCGTTATTCCTTA			
	qBac394 MGB-HU	TCCTTCACGCTACTTGG			
	(5'-FAM and 3'-MGB)				
Chicken-Bac	qC160F-HU	AAGGGAGATTAATACCCGATGATG	105	60	this study
	qBac265R-HU	CCGTTACCCCGCCTACTAC			
Duck-Bac	qBac366F-HU	TTGGTCAATGGGCGGAAG	108	60	this study
	qDuck474R-HU	GCACATTCACACGTGAGA			
	qBac394 MGB-HU	TCCTTCACGCTACTTGG			
	(5'-FAM and 3'-MGB)				

3.3.1 Phylogenetic analysis and primer and probe designing

Bacteroides-Prevotella 16S rRNA gene was amplified from human, cow, pig, chicken and wild duck feces by using *Bacteroides-Prevotella* specific primer set of Bac32F-Bac708R (Bernhard and Field 2000a). *Bacteroides-Prevotella* sequences were acquired from all human, cow, and pig feces, and 67% (26/39) of duck and 69% (22/32) of chicken feces in this study. The detection of *Bacteroides-Prevotella* sequences was observed from 78% of the cecal feces from chickens (data not shown). These results imply that the detection frequency of *Bacteroides-Prevotella* spp. in avian feces is lower than that in mammal feces, which is concordance with previous studies (Table 3.2).

Figure 3.1 shows a phylogenetic tree constructed based on the *Bacteroides-Prevotella* 16S rRNA gene sequences acquired in this study, including 93 and 87 clones from ducks and chickens, respectively. These clones were grouped into 27 and 38 OTUs, respectively, and three of the OTUs contained both duck and chicken clones. Chicken/duck, chicken, and duck specific clusters were identified in the phylogenetic tree (Figure 3.1). The duck specific cluster was completely overlapped with the chicken/duck specific cluster. These chicken/duck, chicken, and duck specific clusters were used to design primer and probe sets for the real-time PCR assay (Table 3.1).

3.3.2 Development of real-time PCR assays for chicken- and duck-associated markers

Figure 3.2 shows the standard curves of the real-time PCR assays targeting Chicken/Duck-

Table 3.2 Positive ratio of *Bacteroides-Prevotella* spp. in fecal samples with conventional PCR assays

Facal	No of fecal	% positive for			
Sorce	samples	Bacteroidales	Location of collection	Primer set	Reference
Duck	39	66.7 (26/39)	Sapporo (Hokkaido, Japan)	Bac32F-Bac708R ^a	this study
Seagull	14	0 (0/14)	Virginia	Bac32F-Bac708R ^a	Fogarty et al. 2005
Wild Bird	10	90 (9/10)	France	AllBac296-AllBac467R ^b	Mieszkin et al. 2009
Chicken	32	68.8 (22/32)	Sapporo (Hokkaido, Japan)	Bac32F-Bac708R ^a	this study
	22	22.7 (5/22)	Virginia, West verginia,	Bac32F-Bac708R ^a	Fogarty et al. 2005
Geese	36	38.9 (14/36)	Virginia, West verginia,	Bac32F-Bac708R ^a	Fogarty et al. 2005
Cow	13	100 (13/13)	Sapporo (Hokkaido, Japan)	Bac32F-Bac708R ^a	this study
	48	100 (48/48)	Virginia, West verginia, Indiana	Bac32F-Bac708R ^a	Fogarty et al. 2005
	10	100 (10/10)	France	AllBac296-AllBac467R ^b	Mieszkin et al. 2009
Pig	12	100 (12/12)	Sapporo (Hokkaido, Japan)	Bac32F-Bac708R ^a	this study
	19	100 (19/19)	West verginia,	Bac32F-Bac708R ^a	Fogarty et al. 2005
	10	100 (10/10)	France	AllBac296-AllBac467R ^b	Mieszkin et al. 2009
Human	12	100 (12/12)	Sapporo (Hokkaido, Japan)	Bac32F-Bac708R ^a	this study
	20	100 (20/20)	Virginia,	Bac32F-Bac708R ^a	Fogarty et al. 2005
	10	100 (10/10)	France	AllBac296-AllBac467R ^b	Mieszkin et al. 2009
Dog	20	100 (20/20)	Virginia, West verginia,	Bac32F-Bac708R ^a	Fogarty et al. 2005
Horse	23	100 (23/23)	Virginia, West verginia,	Bac32F-Bac708R ^a	Fogarty et al. 2005
	10	100 (10/10)	France	AllBac296-AllBac467R ^b	Mieszkin et al. 2009
Sheep	10	100 (10/10)	France	AllBac296-AllBac467R ^b	Mieszkin et al. 2009

a, Bernhard and Field 2000a; b, Layton et al. 2006

Bac, Chicken-Bac and Duck-Bac specific genetic markers. The quantification limits were 50, 63, and 5.0 copies/reaction for Chicken/Duck-Bac, Chicken-Bac and Duck-Bac markers, respectively (Table 3.3). The amplification efficiencies of each real-time PCR assay developed in this study were between 0.9 and 1.1. The slope of the standard curves varied between -2.77 and -3.56, and the determination coefficient (r^2) was always higher than 0.99. The corresponding melting curve analyses for Chicken-Bac showed peaks at appropriate melting temperatures, which were indicative for a positive and correct amplification (Figure 3.3).

3.3.3 Quantification of host-associated genetic markers in fecal and wastewater samples

Specificities of the primer and probe sets developed in this study were tested by using total DNA extracted from feces of the target and non-target hosts and the untreated domestic wastewater (Table 3.4). When using the primer set BacPre1, positive PCR results were obtained from about 70% of the chicken and duck feces, about 100% of the other host feces, and 100% from the untreated domestic wastewater. The quantified values (geometric averages $\pm$ standard deviation; SD) in positive samples varied from 2.9 ± 1.0 to 4.7 ± 0.3 log₁₀ copies of BacPre1 marker/ng-DNA.

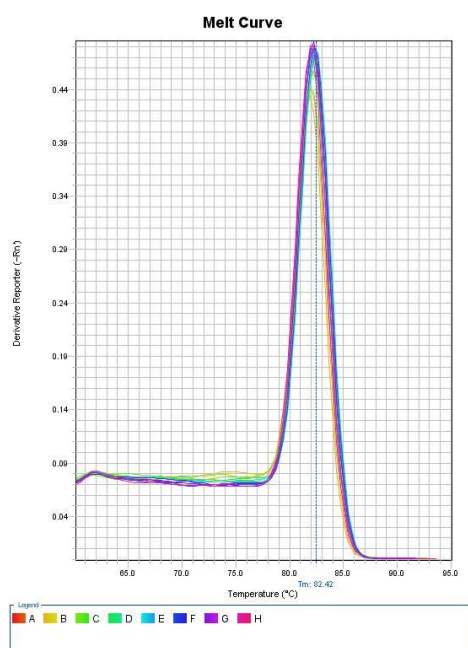
When using the primer and probe set for Chicken/Duck-Bac marker, 96% (25/26) and 61% (14/23) positive results were obtained from the feces of wild ducks and chickens, respectively. The quantified values among the positive samples were 3.7 ± 1.4 and 4.4 ± 0.8 log₁₀ copies/ng-DNA in wild duck and chicken feces, respectively. No positive samples were obtained from cow, pig, swan, goose, and human feces, and untreated domestic wastewater samples by using the primer and probe set for Chicken/Duck-Bac marker.

The primer set for Chicken-Bac marker gave positives in 70% (16/23) of the chicken feces, with the quantified values of 4.6 ± 1.0 log₁₀ copies/ng-DNA. No positive sample was obtained from duck feces in this assay. Positive results were obtained from 39% (5/13), 8.3% (1/12) and 12% (3/25) from the cow, pig, and swan feces, respectively, but the quantified values in positive samples of cow, pig, and swan feces were relatively low (2.3 ± 0.3 log₁₀ and below 1.8 log₁₀ copies/ng-DNA from cow, pig and swan feces, respectively).

The primer and probe set specific to Duck-Bac marker gave positives in 85% (22/26) of the wild duck feces, with quantified values of 3.1 ± 1.4 log₁₀ copies/ng-DNA. No positive sample was obtained from chicken feces in this assay. Although 31% (4/13) of cow fecal samples showed positive in real-time PCR using the primer set for Duck-Bac, the quantified value from the 4 positive samples was low (0.9 ± 0.0 log₁₀ copies/ng-DNA).

Amplification efficiencies and quantification limits of the newly-established primer and probe sets targeting the chicken and duck genetic markers were comparable with those previously

A) Plasmid



B) Environment water

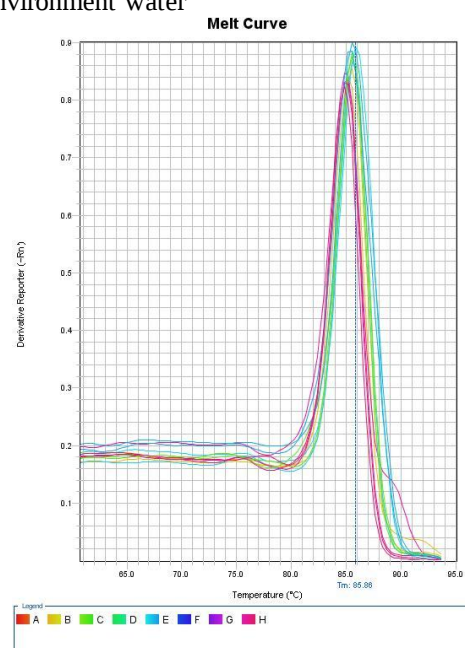


Figure 3.3 The plot of melting curve of standard plasmids and environment water samples using Chicken-associated primer.

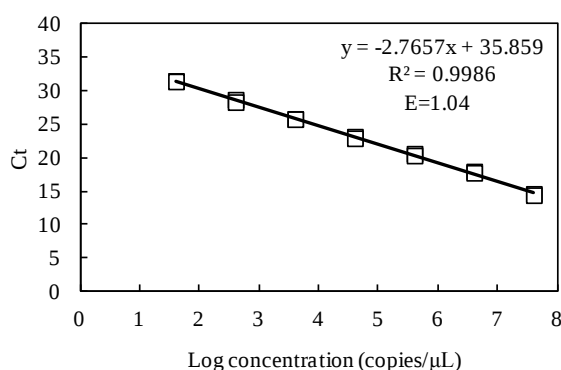
Table 3.4 Positive ratio and concentration of genetic markers (BacPre1, Chicken/Duck-Bac, Chicken-Bac and Duck-Bac) in fecal and wastewater samples

Fecal and water samples	BacPre1 ^a		Chicken/Duck-Bac		Chicken-Bac		Duck-Bac	
	Positive (%)	Concentration ^b	Positive (%)	Concentration	Positive (%)	Concentration	Positive (%)	Concentration
Duck	69 (18/26)	3.5 ± 1.4	96 (25/26)	3.7 ± 1.4	0 (0/26)	<1.8	85 (22/26)	3.1 ± 1.4
Chicken	74 (17/23)	4.0 ± 1.0	61 (14/23)	4.4 ± 0.8	70 (16/23)	4.6 ± 1.0	0 (0/23)	<0.7
Cow	100 (13/13)	4.7 ± 0.3	0 (0/13)	<1.7	39 (5/13)	2.3 ± 0.3	31 (4/13)	0.9 ± 0.0
Pig	100 (12/12)	4.5 ± 0.5	0 (0/12)	<1.7	8.3 (1/12)	1.8	0 (0/12)	<0.7
Human	100 (12/12)	4.6 ± 1.2	0 (0/12)	<1.7	0 (0/12)	<1.8	0 (0/12)	<0.7
WWTP ^c influent	100 (11/11)	2.9 ± 1.0	0 (0/11)	<1.7	0 (0/11)	<1.8	0 (0/11)	<0.7
Swan	96 (24/25)	4.4 ± 1.6	0 (0/25)	<1.7	12 (3/25)	1.8 ± 0.1	0 (0/25)	<0.7
White-fronted goose	100 (16/16)	3.7 ± 1.2	0 (0/16)	<1.7	0 (0/16)	<1.8	0 (0/16)	<0.7

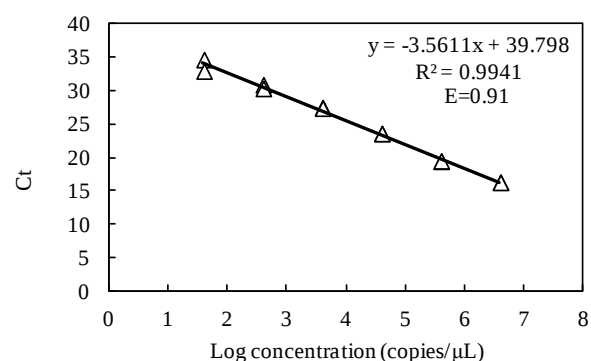
a, Okabe et al. 2007; b, concentrations are expressed in log10 copies per ng-DNA in feces and WWTP influent samples; c, wastewater treatment plant.

constructed targeting the genetic markers for the other animal hosts. Detection frequency of chicken and duck specific genetic markers with the primer and probe sets designed in this study was similar with or higher than those of previously published avian-specific markers. For example, Devane et al. (2007) demonstrated that a duck-specific marker targeting on an unknown bacterium (E2) was detected from 76% of duck feces (32/45), and cross-reacted with 20% of swan (2/10), 15% of Canada geese (3/20) and 13% of goat feces (2/15). Fremaux et al. (2010) showed that goose-specific markers of CGOF1-Bac and CGOF2-Bac gave positive ratios of 57 (58/101) and 50% (51/101), respectively. These results imply that *Bacteroides-Prevotella* group bacteria are not always

A) Chicken/Duck-Bac



B) Chicken-Bac



C) Duck-Bac

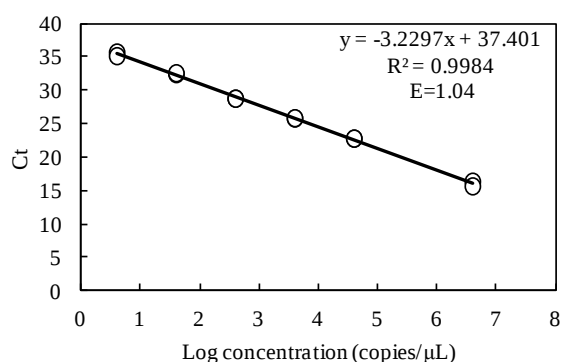


Figure 3.2 Standard curves generated using the real-time PCR assays targeting A) the Chicken/Duck-Bac markers, B) the Chicken-Bac marker, and C) the Duck-Bac marker. The known amounts of the standard plasmid DNA are plotted against mean threshold cycle values (Ct). The standard plasmids were generated by cloning the *Bacteroides-Prevotella* specific 16S rRNA genetic markers amplified from chicken and duck feces with Bac32F-Bac708R primers (Bernhard and Field 2000a) into TOPO XL PCR vector.

Table 3.3 The plot of melting curve of standard plasmids and environment water samples using Chicken-associated primer.

Marker	Standard curve	Amplification efficiency	Quantification limit (copies / reaction)
Chicken/Duck-Bac	$y = -3.04x + 39.22$	1.13	50
Chicken-Bac	$y = -2.81x + 33.94$	1.27	63
Duck-Bac	$y = -2.79x + 36.07$	1.28	5
BacPre1 ^a	$y = -2.74x + 31.48$	1.32	6.3
Human-Bac1 ^a	$y = -3.03x + 39.97$	1.14	50

^a Okabe et al. 2007

predominant in avian feces, and false negative results regarding avian fecal contamination in water might be obtained in the avian-specific *Bacteroides-Prevotella* genetic marker assays. Combinations with other fecal pollution indicators, including OTU-based approach (e.g., Unno et al. 2010) and chemical markers, may be required to reliably detect avian fecal contamination in water

environments.

3.3.4 Quantification of the host-associated genetic markers in feces suspended in environmental water

Quantification of the host-specific genetic markers was performed using environmental water samples suspended with fecal materials, in order to examine specificity and sensitivity of the assay. Table 3.5 shows quantified values of the assay for the host-specific genetic markers in fecal suspensions. BacPre1, a genetic marker for universal *Bacteroides-Prevotella*, was detected from all fecal suspensions from humans, chickens, and ducks, although the quantified values of BacPre1 were often slightly lower than those of chicken and duck-associated markers. Since ten-fold diluted samples (0.01 g feces/100mL) sometimes gave the same level of quantified values with the original samples (0.1 g feces/100mL) in the case of BacPre1, the amplification inhibition of this genetic marker at higher concentration was suspected. Avian feces may also contain avian-specific *Bacteroides* that cannot be amplified by BacPre1 assay, potentially causing the smaller quantification values obtained from BacPre1 assay than those obtained from chicken and duck-associated marker assays. The primer and probe set for Chicken/Duck-Bac was able to amplify the genetic marker only from chicken and duck feces, except the human fecal suspension used in the assay on Dec. 25th, 2009. Chicken-Bac and Duck-Bac markers were detected only when chicken and wild duck feces, respectively, were suspended.

The quantification limits of samples including inhibitory substances were examined (Table 3.6). Table 3.6 shows the quantification limit of newly-designed chicken and duck-associated *Bacteroides-Prevotella* assays and six published assays by using fecal suspensions in pond water. Quantification limits of the real-time PCR assays for *Bacteroides-Prevotella* genes in feces suspended in pond water were 54 copies/reaction for Chicken/Duck-Bac, 57 copies/reaction for Chicken-Bac, and 12 copies/reaction for Duck-Bac, respectively. These values were comparable with those in the real-time PCR assay for plasmid DNA (Table 3.3). The quantification limits of previously-reported genetic markers have been reported to be 10 copies of *Bacteroides* 16S rRNA genetic markers/reaction (Dick and field 2004), 47 copies of Human-specific 16S rRNA genetic marker/reaction (Seurinck et al. 2005), 120 copies of *Bacteroides-Prevotella* 16S rRNA genetic marker/reaction (Okabe and Shimazu 2007), and 21, 4.2, and 11 copies of human, cow, and pig-specific 16S rRNA genetic marker/reaction, respectively (Okabe and Shimazu 2007). Bernhard and Field (2000b) assumed that one cell of *Bacteroides* has five 16S rRNA operons. Although 16S rRNA operon number per cell may be different by genus or species (Pei et al. 2010), we used the same assumption to compare quantification limits obtained by different researchers. When this assumption

Table 3.5 Quantification of host-associated genetic markers (BacPre1, Human-Bac, Chicken/duck-Bac, Chicken-bac and Duck-Bac) in the fecal suspensions in pond water

Date	Fecal source	Concentration (g feces/ 100mL)	Target gene concentration $\pm$ standard deviation (log10 copies/100mL)			
			BacPre1 ^a	Chicken/Duck-Bac	Chicken-Bac	Duck-Bac
10-Dec-09	Duck	0.1	8.9 $\pm$ 0.2	9.7 $\pm$ 0.0	BQL	8.7 $\pm$ 0.0
		0.01	8.8 $\pm$ 0.1	8.7 $\pm$ 0.0	BQL	7.6 $\pm$ 0.2
	Human	0.1	8.2 $\pm$ 0.4	BQL ^b	BQL	BQL
		0.01	8.0 $\pm$ 0.2	BQL	BQL	BQL
	Duck :Human (1 : 1)	0.1	8.1 $\pm$ 0.1	9.0 $\pm$ 0.1	BQL	8.1 $\pm$ 0.1
		0.01	7.0 $\pm$ 0.6	7.9 $\pm$ 0.1	BQL	6.9 $\pm$ 0.0
25-Dec-09	Chicken	0.1	10.5 $\pm$ 0.1	10.3 $\pm$ 0.0	10.7 $\pm$ 0.04	BQL
		0.01	9.1 $\pm$ 0.1	9.1 $\pm$ 0.0	9.3 $\pm$ 0.15	BQL
	Duck	0.1	10.0 $\pm$ 0.1	10.0 $\pm$ 0.1	BQL	9.4 $\pm$ 0.1
		0.01	8.6 $\pm$ 0.1	8.9 $\pm$ 0.0	BQL	8.2 $\pm$ 0.0
	Human	0.1	10.0 $\pm$ 0.0	7.5 $\pm$ 0.1	BQL	BQL
		0.01	8.6 $\pm$ 0.1	6.4 $\pm$ 0.1	BQL	BQL
	Chicken :Duck (1 : 1)	0.1	10.2 $\pm$ 0.1	10.0 $\pm$ 0.0	9.9 $\pm$ 0.0	9.3 $\pm$ 0.0
		0.01	8.9 $\pm$ 0.0	9.0 $\pm$ 0.0	8.7 $\pm$ 0.0	8.1 $\pm$ 0.1
	Chicken :Human (1 : 1)	0.1	10.3 $\pm$ 0.0	9.8 $\pm$ 0.1	10.1 $\pm$ 0.0	BQL
		0.01	8.6 $\pm$ 0.1	8.9 $\pm$ 0.2	8.9 $\pm$ 0.0	BQL

^adata from Okabe et al. 2007; ^bBQL, below the quantification limit.

was employed, the result of Seurinck et al. (2005) can be recalculated as being 9.4×10^4 cells/L. When the same assumption was employed in this study, the quantification limits are turned to be 1.1×10^4 cells/L for Chicken/Duck-Bac, 1.1×10^4 cells/L for Chicken-Bac, and 2.4×10^3 cells/L for Duck-Bac (Table 3.6). These results indicate that the quantification limits of the established genetic makers in this study are comparable to those of published markers.

3.3.5 Quantification of genetic markers in environmental water

In order to confirm the applicability of the newly-constructed chicken and duck-associated assays, real-time PCR was performed to detect the avian-associated genetic markers in environmental water samples (Table 3.6). As for water samples from Oono Pond where wild duck stopover was observed, the Chicken/Duck-Bac and the Duck-Bac genetic markers were detected with the concentrations of 10^3 to 10^5 copies/100 mL; whereas the Chicken-Bac marker was not detected (i.e., below quantification limit) from the same samples. Total coliforms were not tested for Oono pond water. Similarly, the Chicken/Duck-Bac and the Duck-Bac markers were positive in water samples from Kamogamo River where wild duck stopover was observed as well. In the case of

Table 3.6 Quantification limit of the real-time quantitative PCR assays for 16S rRNA of Bacteroidales in feces suspended in pond water

Marker	Quantification Limit (copies / reaction)	Quantification Limit (cell / L) ^a	reference
Chicken/Duck-Bac	54	1.1×10^4	this study
Chicken-Bac	57	1.1×10^4	this study
Duck-Bac	12	2.4×10^3	this study
Total <i>Bacteroidales</i>	10		Dick et al., 2004
HF183	47	9.4×10^4	Seurinck et al., 2005
BacPre1	120		Okabe and Shimazu, 2007
Human-Bac1	21		Okabe and Shimazu, 2007
Cow-Bac2	4.2		Okabe and Shimazu, 2007
Pig-Bac2	11		Okabe and Shimazu, 2007

^a The number of 16S rRNA operons is assumed to be five (Bernhard and Field 2000b).

Kamogamo River samples, however, total coliforms and the Human-Bac marker were also positive at the concentration of 10^2 to 10^3 CFU/100mL and 10^3 copies/100mL, respectively. In contrast, water samples from Toyohira River, which receives treated wastewater as one of arterial rivers in Sapporo city, gave negative results for all three chicken and duck-genetic markers. Unlike Oono pond and Kamogamo River, only anthropogenic fecal pollution was implicated in Toyohira River since the Human-Bac1 marker was positive in two individual samples.

However, practical problems including unknown inhibitory effects arose in the application of the real-time PCR assays to environmental water. A process control marker (Stoeckel et al. 2009) and an amplification control (Shanks, et al. 2009) have to be employed in each assay to ensure the accuracy of quantified values of genetic markers. These quantified values of genetic markers and the recovery ratio of a process control marker should be stochastically treated, because all the values of marker quantity and recovery ratio are regarded as randomly sampled ones from each true population.

Further issues regarding the detection of avian fecal contamination in water environments include the survival/persistence of genetic markers and relationships between quantities of genetic markers and enteric pathogens in environmental water (Savichtcheva et al. 2007). For the application of host-specific genetic markers as alternative fecal indicators, the fate of genetic markers under various environmental conditions including temperature, salinity, chlorination and sunlight, and furthermore, relationships between occurrence of pathogens and genetic markers have to be studied. These issues of environmental fate, correlation with real pathogens, and live cell counts are the current challenges in the field of microbial source tracking with host-associated genetic markers.

3.4. Conclusions

It is important to identify the sources of fecal contamination and to quantify the relative contribution of each fecal source when infectious risks in water utilization are considered. Once the major fecal sources are identified, appropriate management and remediation efforts could be applied in a more time- and cost-effective manner. For this purpose, specific genetic markers for human and animals (e.g., cows, pigs, dogs, horses, and sheep) were identified, and real-time PCR assays were developed (Fogarty and Voytek et al. 2005; Mieszkin et al. 2009; Okabe et al. 2007). In addition, since avian feces are thought to be potential sources of pathogens (Fogarty et al. 2003), genetic markers were proposed for ducks (Devane et al. 2007), geese (Hamilton et al. 2006), cranes (Ryu et al. 2012), and poultry (Champagne et al. 2011; Weidhaas et al. 2011). However, real-time PCR assays targeting chicken and duck markers have not been well established yet. Therefore, in this study, additional genetic markers for ducks and chickens were proposed and real-time PCR assays were developed to quantify these markers.

In conclusion, we identified chicken and duck-associated genetic markers (Chicken/Duck-Bac, Chicken-Bac and Duck-Bac) in the 16S rRNA genes of the *Bacteroides* and *Prevotella* genera, and developed real-time PCR assays to quantify these markers in environment water. The quantification limits of the assays for Chicken/Duck-Bac, Chicken-Bac and Duck-Bac markers were 54, 57 and 12 copies/reaction, respectively. The specificity of chicken and duck-associated genetic marker assays developed in this study was similar or superior to that of the assays previously reported. Combined applications with other fecal pollution indicators may add more reliability to assure avian fecal contamination in water environments. Since avian may carry potential human pathogens, detection and quantification of avian fecal contamination allow us to evaluate potential human health risks associated with the water activities.

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Chapter 4

Improvement of the quantification method of
alternative indicator for fecal contamination
“Genetic marker”

4.1. Introduction

Many potential fecal contamination sources in water environments such as humans (Savichtcheva et al. 2007; Mieszkin et al. 2009a), farm animals (Mieszkin et al. 2009a; Savichtcheva et al. 2007), and wild animals and birds (Kassa et al. 2001; Kobayashi et al. 2013) impose significant risks to human health; therefore, it is necessary to identify the fecal contamination sources to allocate better management and mitigation efforts in a more time- and cost-effective manner. Traditionally, fecal contamination in water environments has been assessed and managed by cultivation-based enumeration of indicator microorganisms such as coliforms, fecal coliforms, *Escherichia coli*, and *Enterococcus* spp.; however, these methods cannot be used to identify the source of fecal contamination (Field et al. 2003; Scott et al. 2002). Many alternative indicators have been developed to identify sources of fecal contamination in water environments (Meays et al. 2004; Savichtcheva and Okabe 2006; Scott et al. 2002; Simpson et al. 2002). Among these indicators, the genetic markers of 16S rRNA of *Bacteroides-Prevotella* have been regarded as the most promising alternative (Field and Samadpour 2007), and quantitative PCR (qPCR) assays of *Bacteroides* genetic markers for targeting humans (Kildare et al. 2007; Okabe et al. 2007; Mieszkin et al. 2009b; Seurinck et al. 2005), cows (Kildare et al. 2007; Okabe et al. 2007; Mieszkin et al. 2009b), pigs (Okabe et al. 2007; Mieszkin et al. 2009a), and birds (Fremaux et al. 2010; Kobayashi et al. 2013) have been developed.

To promote practical use, the quantification methods should be standardized; those enable us to check whether the sample processing performed normally and to compare results obtained from different laboratories (Meays et al. 2004; Stewart et al. 2003; Harwood et al. 2009; Stoeckel et al. 2009). In addition, the DNA recovery and PCR amplification efficiencies must be determined for individual measurements to correct the measured values. To determine the DNA recovery and PCR amplification efficiencies, two controls are generally employed: a sample process control (SPC) and an internal amplification control (IAC) (Diez-Valcarce et al. 2011). The SPC is used to evaluate the DNA loss during sample processing (e.g., cell recovery and DNA extraction). A known amount of SPC is spiked to samples, and the DNA recovery efficiency is determined based on the amount of SPC recovered before and after the sample processing, as measured by SPC-specific qPCR assay. Meanwhile, an IAC is a nontarget genetic component for determining any inhibitory effects on the activity of DNA polymerase enzymes (Shanks et al. 2009). A known amount of IAC (e.g., plasmid DNA) is spiked to DNA samples, and the PCR amplification efficiency is determined based on the measured values of IACs in the DNA sample and in a pure water control. Both the SPC and the IAC are, therefore, essential to correct the measured values of genetic markers in environmental water samples (D'Agostino et al. 2011). However, very few studies have used SPC and/or IAC in

quantification of genetic markers so far (Diez-Valcarce et al. 2011).

The ideal SPC strain should meet the following criteria. Firstly, they should have a similar cellular structure to the target bacteria (e.g., genetic marker bacteria). Secondly, the SPC strain should not be detected in unspiked samples. Thirdly, the concentration and DNA recovery efficiency of the SPC strain should be similar to that of the genetic marker bacteria. Stoeckel et al. (2009) used two types of SPCs, the plant pathogen *Pantoea stewartii* carrying the chromosomal target gene *cpsD* and *E. coli* carrying the plasmid-borne target gene *DsRed2*, for evaluating the recovery efficiency of *Bacteroidales* 16S rRNA genes from river water. However, potential environmental occurrence of these bacteria may confound the usage of these two bacterial strains as SPCs. In addition, since the comparisons of DNA recovery efficiencies between the SPC strains and the genetic marker bacteria have not been conducted, the applicability of these SPCs must be further evaluated.

One of the drawbacks of qPCR-based quantification methods is a disability to distinguish DNA derived from live and dead bacterial cells, since DNA in the environment can be very stable and can persist for extended periods of time (more than 3 weeks) after cell death (Nielsen et al. 2007). Thus, propidium monoazide (PMA) treatment followed by qPCR has been proposed to discriminate DNA derived from dead cells in quantification of *Bacteroidales* gene markers (Bae and Wuertz 2009; Taskin et al. 2011; Varma et al. 2009). The PMA is a cell membrane-impermeable chemical but can selectively penetrate membrane-disrupted cells (i.e., dead cells) and intercalate into the DNA, which strongly inhibits PCR amplification (Nocker et al. 2006). By using qPCR after PMA treatment, only genetic markers of live target bacteria can theoretically be quantified in natural environmental samples. However, the effect of PMA treatment on DNA recovery efficiency is not well known to date. Therefore, the DNA recovery efficiency during an entire sample preparation including PMA treatment must be evaluated using an appropriate SPC.

The objective of this study was, therefore, to develop a genetically-engineered strain of *E. coli* (designated as strain MG1655 Δ lac::kan) as a sample process control (SPC) for qPCR assays with PMA treatment to accurately quantify *Bacteroides-Prevotella* gene markers in environmental water samples. In addition, an IAC was also constructed and applied to further improve the measurement accuracy.

4.2. Materials and methods

4.2.1 Constructions of SPC and IAC

Bacterial strains and plasmids used in this study are listed in Table 4.1. *E. coli* K12 strain MG1655 were grown in Luria-Bertani (LB) medium or SOC medium at 37°C (Sambrook et al. 1989;

Table 4.1 Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype	Source
<i>E. coli</i> K12 strains		
MG1655	F ⁻ λ <i>ilvG rfb-50 rph-1</i>	Blattner et al. (1997)
MG1655 Δ lac::kan	MG1655 Δ lac::Km ^r	This study
<i>Bacteroides fragilis</i>		
JCM 11019		Japan Collection of Microorganisms
Plasmids		
pKD13	Km ^r ; template for mutant construction	Datsenko and Wanner (2000)
pKD46	Amp ^r ; temperature-sensitive λ red recombinase expression vector	Datsenko and Wanner (2000)
pUC19	Amp ^r	Yanisch-Perron et al. (1985)

Datsenko and Wanner 2000). Antibiotics were used at the following concentrations: ampicillin, 100 μ g/ml; kanamycin, 50 μ g/ml. *Bacteroides fragilis* JCM 11019 were grown in Brain Heart Infusion liquid medium (BHI broth) containing hemin at 5 μ g/ml under anaerobic condition at 37°C for 48h.

A genetically-engineered *E. coli* strain was constructed by a homologous recombination technique using the one-step inactivation method previously described by Datsenko and Wanner (2000). Briefly, the FRT-flanked kanamycin resistance gene (*kan*) was PCR-amplified from pKD13 plasmid DNA by using del_lacZ_F and del_lacZ_R primers (Table 4.2), purified using QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), and mixed with strain MG1655 carrying pKD46. The P_{T5-lac} promoter was induced by the addition of 100 μ M isopropyl β -D-1-thiogalactopyranoside (IPTG). Cells were cultivated in SOC medium for 1 hour, followed by cultivation in LB agar medium supplemented with kanamycin and IPTG. After incubation, a white colony was picked up. As a result, *E. coli* K12 strain MG1655 Δ lac::kan (hereafter called strain MG1655 Δ lac::kan), which lacks a *lacZ* function by insertion of *kan* was obtained (Figure 4.1). A primer set of Kan-res-F and DS-Kan-R (Table 4.2) was designed to specifically quantify strain MG1655 Δ lac::kan by locating a primer annealing site in the junction region of *kan* and *mhpR* in strain MG1655 Δ lac::kan (Figure 4.1). As for the IAC, we constructed a pCR2.1-TOPO vector inserted with a chicken-specific *Bacteroides-Prevotella* gene marker (Chicken-Bac), and used its specific primers for the quantification of Chicken-Bac gene marker previously described (Kobayashi et al. 2013).

River water samples were collected from Toyohira River, Atsubetsu River, Nopporo River and Motsukisamu River in Sapporo, Hokkaido, Japan. Pond water samples were collected from Oono Pond in Hokkaido University, Sapporo, Hokkaido, Japan. Wastewater influent samples were collected from a domestic wastewater treatment plant (WWTP) in Sapporo, Hokkaido, Japan. Water

Table 4.2 Primers and probes used in this study

Target	Primer name	Sequence (5'→3')	AT (°C) ^a	Reference
FRT ^b -flanked kanamycin resistance gene	del_lacZ_F	ACGGGCAGACATGGCCTGCCCGTTAT TATTATTTTGGACACCAGACCAATGTAG GCTGGAGCTGCTTCG	74	This study
	del_lacZ_R	GTTGTGTGGAATTGTGAGCGGATAACA ATTTCACACAGGAAACAGCTATGATTG CGGGGATCCGTCGACC		
kan ^c	kt	CGGCCACAGTCGATGAATCC	60	Datsenko and Wanner (2000)
	k1	CAGTCATAGCCGAATAGCCT		
	k2	CGGTGCCCTGAATGAACTGC		
Junction region of kan and mhpR ^d	Kan-res-F	ATCGCCTTCTTGACGAGTTC	60	This study
	DS-Kan-R	TTTGCACCACTACGTTTTC		
Human specific <i>Bacteroides-Prevotella</i> (16s rRNA)	qHS601F	GTTGTGAAAGTTTGCGGCTCA	62	Okabe et al. (2007)
	qBac725R	CAATCGGAGTTCTTCGTGATATCTA		
	qHS624MGB	(FAM) -CGTAAAATTGCAGTTGA - (MGB)		
Universal <i>Bacteroidales</i> (BacPre1)	qBac560F	TTTATTGGGTTTAAAGGGAGCGTA	62	Okabe et al. (2007)
	qBac725R	CAATCGGAGTTCTTCGTGATATCTA		

^a, annealing temperature.^b, flippase recognition target.^c, kanamycin resistance gene.^d, gene encoding a DNA-binding transcriptional activator.

Figure 4.1 Region of the homologously-recombined site on the chromosome of *E. coli* strain MG1655 Δ lac::kan. *cynX* and *mhpR* encodes a cyanate transporter and a DNA-binding transcriptional activator, respectively, and are not essential for the *E. coli* cells to live. FRT is a flippase recognition target. Primer annealing sites (Kan-res-F and DS-Kan-R) are also shown in this figure.

quality parameters, including water temperature, pH, electric conductivity (EC), biochemical oxygen demand (BOD), suspended solids (SS), total coliforms, *E. coli*, total nitrogen (T-N) and total phosphorous (T-P) were measured for each water sample according to the standard methods (APHA et al. 2005).

4.2.2 Water and fecal samples

Human fecal samples (n=7) were collected from healthy adults. Fresh feces from cows (n=4), pigs (n=4), and chickens (n=4) were collected from Field Science Center for Northern Biosphere,

Experiment Farm in Hokkaido University. All fecal samples were collected in sterile 50 mL-tubes and stored at -80°C until used for DNA extraction. Fecal suspensions in pure water were prepared by adding fecal samples to MilliQ water at a final concentration of 0.1 g feces per 100 mL (Okabe and Shimazu 2007).

4.2.3 Recovery of bacterial cells from river water

Bacterial cells suspended in 3L of pond water or 5L of river water were collected by pressure filtration with a 0.22- μ m-pore-size polyethersulfone membrane filter. Bacterial cells on the membrane filter were eluted by soaking in 30 ml of sterile STE buffer (0.1 M NaCl, 10 mM Tris and 1 mM EDTA, pH 7.6) and rigorously shaken by a vortex mixer (Cho and Kim 2000; Okabe et al. 2007). Suspended cells in the STE buffer were collected by centrifugation at 10,000 $\times g$ for 15 min at 4°C. The harvested cells were resuspended in 0.5 ml and 0.8 ml of distilled MilliQ water for pond and river water samples, respectively. Bacterial cells recovered from influent samples of WWTP were harvested by centrifugation at 10,000 $\times g$ for 10 min at 4°C, and the supernatant was gently removed. The harvested cells were resuspended in 2 ml of distilled MilliQ water. Resulting cell suspensions were subjected to DNA extraction and propidium monoazide (PMA) treatment as described below.

4.2.4 Treatment of bacterial cells with PMA

One half of the resuspended cells recovered from water samples were subjected to DNA extraction and the other half was subjected to PMA treatment according to Bae and Wuertz (2009). PMA was dissolved in distilled MilliQ water to prepare 2 mM stock solution and stored at -20°C in dark. The PMA solution was added to 200 μ l or 400 μ l of resuspended bacterial cells in 1.5 ml centrifuge tube at final concentrations of 100 μ M, and incubated for 5 min in dark. Then, the tube was horizontally placed on ice and exposed to 500-W halogen light, which was placed 20 cm from the tube, for 10 min. The PMA-treated cells were then subjected to DNA extraction as described below.

In order to examine the effect of PMA on dead cells, heat-killed cells were prepared by heating the cells of strain MG1655 Δ lac::kan *E. coli* and *B. fragilis* JCM 11019 at 85°C for 20 min. And these samples were quantified by three kinds of processes; PMA treatment is not performed (w/o PMA w/o treat), PMA treatment is performed with PMA solution (w PMA w treat), or PMA treatment is performed without PMA solution (w/o PMA w treat).

4.2.5 DNA extraction and PCR amplification

Total DNA was extracted from fecal samples (200 µg) and cell suspensions (200 µl) obtained from water samples by using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) and PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), respectively. PCR was carried out with ABI PRISM GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) as described previously (Okabe et al. 2007). Annealing temperature was shown in Table 4.2. PCR products were separated with agarose gel electrophoresis, and visualized by staining with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) under ultraviolet illumination.

4.2.6 Quantitative PCR Assays

The qPCR assays were performed using SYBR Green chemistry to quantify the SPC strain MG1655 Δ lac::kan and Chicken-Bac (IAC). TaqMan qPCR assay was also performed to quantify Human-Bac1, a *Bacteroides-Prevotella* 16S rRNA genetic marker (Okabe et al. 2007). In SYBR Green assays, each PCR mixture (25 µl) composed of 1x SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan), 1x ROX Reference Dye (Takara Bio, Otsu, Japan), 400 nM each of forward and reverse primers, and 2 µl of template DNA. In the TaqMan qPCR assay, each PCR mixture (25 µl) composed of 1x Premix Ex Taq (Takara Bio, Otsu, Japan), 200 nM each of forward and reverse primers, 200 nM of fluorogenic probe, and 2.0 µl of template DNA. PCR reactions were performed in MicroAmp Optical 96-well reaction plates with Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction was carried out by heating at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing and extension at 60°C for 34 sec. Following to the amplification step, a melting curve analysis was performed for SYBR Green assays to confirm that no unexpected PCR products were obtained.

For the standard plasmid construction for the quantification of strain MG1655 Δ lac::kan, a pUC19 vector carrying the PCR amplicon generated from strain MG1655 Δ lac::kan with the primer set of Kan-res-F and DS-Kan-R was used. The standard plasmids for the quantification of Chicken-Bac and Human Bac1 markers were prepared as described previously (Kobayashi et al. 2013; Okabe et al. 2007). The ligated products were transformed into *E. coli* TOP10 competent cells (Invitrogen, Carlsbad, CA, USA). Plasmids were extracted and purified from *E. coli* cells by using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). The concentrations of plasmid DNA was adjusted from 10^{-1} to 10^{-8} ng per µL and used to generate standard curves. Standard curves were generated by linear regression analysis between threshold cycles (C_T) and the concentration of the plasmid DNA by using Applied Biosystems 7500 Real-Time PCR System software ver. 2.0.4 (Applied Biosystems, Foster City, CA, USA). Amplification efficiency of standard curve (E) was calculated as $E = (10^{-1/\text{slope}}) - 1$ (a reaction with 100% amplification efficiency will give a slope value of -3.32). The

quantification limit was defined as the lowest concentration of plasmid DNA that was amplified within the linear range of the standard curve. Levels of PCR inhibition were evaluated by the addition of IACs (Chicken-Bac plasmid) to all samples DNA prior to qPCR. Amplification efficiencies of the IAC were calculated as the quantified values of the IAC in environmental DNA samples divided by the quantified value of IACs in pure plasmid solution, thus 100% can be obtained when no PCR inhibition occurs.

4.2.7 DNA recovery efficiencies of *B. fragilis* and strain MG1655 Δ lac::kan from water samples

The DNA recovery efficiencies of *B. fragilis* JCM 11019 and the genetically-engineered *E. coli* strain MG1655 Δ lac::kan were compared to examine the applicability of the strain MG1655 Δ lac::kan as the SPC to the quantification of the bacterial genetic markers.

Quantification process of the bacterial genetic markers as described above can be divided into the following steps: (A) water filtration; (B) cell condensation by centrifugation, (C) PMA treatment, and (D) DNA extraction. The known amount of strain MG1655 Δ lac::kan and *B. fragilis* JCM 11019 were added to water samples ($n = 3$) before step A, B, or D, at a final concentration of 10^7 cells/mL, and the spiked samples were processed with or without PMA treatment (step C) as described above. A total of 18 samples (three samples $\times$ three steps for the addition of bacteria $\times$ two treatments (with or without step C)) were individually processed, and target genes were quantified by the qPCR assays as described above.

The concentrations of strain MG1655 Δ lac::kan and *B. fragilis* JCM 11019 were obtained by measuring optical density at 600 nm wave length (OD_{600}) and direct cell counting after 4',6-diamidino-2-phenylindole (DAPI) staining. The conversion of DNA concentrations to cell numbers were done based on the assumptions that a single cell of *B. fragilis* has six 16S rRNA operons (Klappenbach et al. 2000) and a single cell of *E. coli* strain MG1655 Δ lac::kan has one homologous recombinant region.

4.3. Results and discussion

4.3.1 Development of a qPCR assay for *E. coli* strain MG1655 Δ lac::kan

A standard curve was generated based on the qPCR assay targeting the junction region of *kan* and *mhpR* in the genome of *E. coli* MG1655 strain Δ lac::kan, which is a pUC19 vector carrying the PCR amplicon generated from strain MG1655 Δ lac::kan with the primer set of Kan-res-F and DS-Kan-R (Figure 4.2). The amplification efficiencies were around 1.0 (± 0.16), with the determination

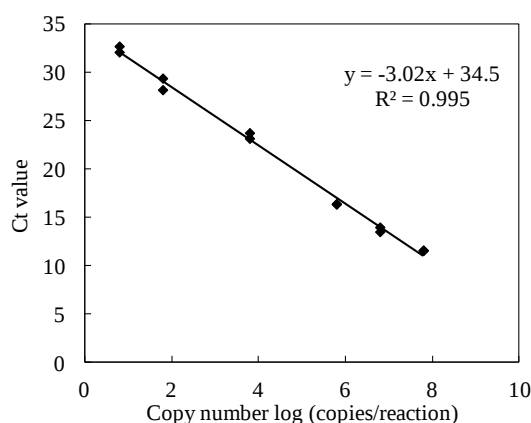


Figure 4.2 A standard curve generated based on the quantitative PCR assay using the primer set specific to strain MG1655 Δ lac::kan. The known amounts of standard plasmid (copies/reaction) are plotted against mean threshold cycle (C_T) values.

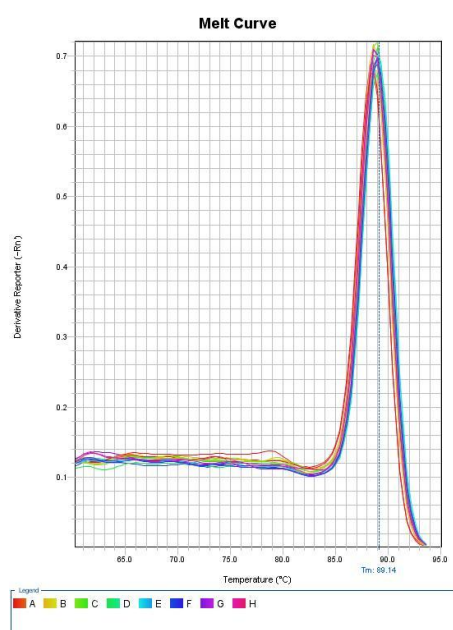


Figure 4.3 Melting curves in the amplification of the junction region of *kan* and *mhpR* in the plasmid by using a specific primer set (Kan-res-F and DS-Kan-R). Graph legends show each concentration of plasmid DNA (A: 10^{-8} ng/ μ L, B: 10^{-7} ng/ μ L, C: 10^{-6} ng/ μ L, D: 10^{-5} ng/ μ L, E: 10^{-4} ng/ μ L, F: 10^{-3} ng/ μ L, G: 10^{-2} ng/ μ L, H: 10^{-1} ng/ μ L).

coefficient (r^2) of >0.99 . The concentration of 6.2 copies/reaction was sometimes quantified, and the 62 copies/reaction ($n = 12$) was considered as quantification limit. Melting curve analysis revealed that only single peaks appeared at the appropriate melting temperature (89°C), suggesting that the specific amplification was obtained (Figure 4.3).

4.3.2 Quantification of strain MG1655 Δ lac::kan in fecal and wastewater samples

Specificity of the primer set (Kan-res-F and DS-Kan-R) designed in this study was examined by using DNA extracted from animal and bird feces, untreated domestic wastewater, and fecal

Table 4.3 Cross reaction of qPCR using a specific primer set for strain MG1655 Δ lac::kan.

Samples	UDL ^a (%)	UQL ^b (%)	Positive rate (%)	Concentration (log copies/ml)
Human feces	0 (0/7)	100 (7/7)	0 (0/7)	
Fecal suspension	29 (2/7)	71 (5/7)	0 (0/7)	
Fecal suspension with strain MG1655 Δ lac::kan ^c	0 (0/7)	0 (0/7)	100 (7/7)	6.6 $\pm$ 0.2
Cow feces	0 (0/4)	100 (4/4)	0 (0/4)	
Pig feces	50 (2/4)	50 (2/4)	0 (0/4)	
Chicken feces	75 (3/4)	25 (1/4)	0 (0/4)	
WWTP influent	75 (3/4)	25 (1/4)	0 (0/4)	

^a, under detection limit^b, under quantification limit ($10^{1.8}$ copies/reaction)^c, final concentration of added strain MG1655 Δ lac::kan cells was $10^{7.6}$ copies/ml.

suspension with or without addition of strain MG1655 Δ lac::kan (Table 4.3). In this experiment, the quantification limit was 62 copies/reaction. No positive amplification results were obtained from human, cow, pig, and chicken feces, untreated domestic wastewater, and fecal suspensions without the addition of strain MG1655 Δ lac::kan. Meanwhile, when strain MG1655 Δ lac::kan was spiked to the fecal suspensions ($n = 7$) at a final concentration of $10^{7.6}$ cells/mL, all seven fecal suspensions showed positive results with quantity measured as $10^{6.6 \pm 0.2}$ copies/mL. These results suggested that 90% of the DNA was lost during cell collection and DNA extraction process, or PCR reaction was inhibited by some inhibitory substances present in the fecal suspension samples.

The qPCR assay for the strain MG1655 Δ lac::kan was developed by using a primer set targeting the junction region of *kan* and its flanking gene (*mhpR*) both located on the chromosome. Therefore, we can exclude the amplification of the DNA derived from indigenous *E. coli* strains or kanamycin-resistant microbes that are potentially present in environmental water and fecal samples (Smalla et al. 1993; Kobashi et al. 2005). Based on the results of spiked experiments (Table 4.3), it was confirmed that the specific qPCR assay for the SPC strain MG1655 Δ lac::kan was developed. PCR amplification from strain MG1655 Δ lac::kan was stable because the primer annealing sites were located on the chromosome, not on a plasmid. In addition, we can easily convert copy numbers to cell numbers because the target sequence is present one copy per genome (Ishii et al. 2013). This strain lacks the lactose digestion capability because of the insertion of *kan* in the lactose metabolism genes, and therefore, this strain cannot grow well in lactose-based media such as desoxycolate medium. Therefore, the addition of the strain MG1655 Δ lac::kan does not influence coliform counts by conventional cultivation-dependent enumeration methods. The presence of *kan* in the chromosome of strain MG1655 Δ lac::kan is also advantageous in the preparation of SPCs because we can exclude the potential contamination of kanamycin susceptible exogenous bacteria.

4.3.3 Effect of PMA treatment on DNA recovery efficiency

PMA treatment was performed before qPCR assay to selectively quantify the DNA derived only from live bacterial cells. The heat-killed cells (*Bacteroides* spp. and *E. coli* strain MG1655 Δ lac::kan) were prepared and spiked to 3L pond water before filtration. Target gene was originated from samples processed by three kinds of treatment; PMA treatment is not performed (w/o PMA w/o treat), PMA treatment is performed with PMA solution (w PMA w treat), or PMA treatment is performed without PMA solution (w/o PMA w treat) (Figure 4.4). A total of 9 samples (three samples $\times$ three processes) were individually processed and quantified. The quantified values of w/o PMA w/o treat sample of strain MG1655 Δ lac::kan and *B. fragilis* JCM 11019 were $10^{5.2 \pm 0.1}$ copies/mL and $10^{4.4 \pm 0.04}$ copies/mL, respectively. The quantified values of w PMA w treat sample of strain MG1655 Δ lac::kan and *B. fragilis* JCM 11019 were $10^{3.8 \pm 0.4}$ copies/mL and $10^{2.7 \pm 0.05}$ copies/mL, respectively. The quantified values of w/o PMA w treat sample of strain MG1655 Δ lac::kan and *B. fragilis* JCM 11019 were $10^{5.3 \pm 0.02}$ copies/mL and $10^{4.3 \pm 0.02}$ copies/mL, respectively. The recovery efficiencies of both target genes in w PMA w treat sample were not statistically different from 100% (white bars in Figure 4.4), indicating that the DNA loss during PMA treatment was negligible. In contrast, when PMA treatment was applied to the heat-killed cells, the recovery efficiencies greatly decreased to 5.0 and 1.6% for the strain MG1655 Δ lac::kan and *B. fragilis* JCM 11019, respectively. These results suggested that the decrease in the recovery efficiencies were due to the effect of the PMA treatment.

It was clearly shown that the PMA treatment was effective to exclude, or at least greatly decrease, the PCR amplification from dead cells (Figure 4.4), similar to the previous reports (Bae and Wuertz 2009; Nocker et al. 2006; Taskin et al. 2011). In addition, PMA treatment had no significant influence on the DNA extraction and qPCR amplification for dead cells from strain MG1655 Δ lac::kan and *B. fragilis* JCM 11019 (white bars in Figure 4.4). Therefore, qPCR assays with PMA treatment is effective to selectively quantify the host-specific genetic markers derived only from live target cells. It should be noted that the sources of recent fresh fecal contamination could be detected and/or identified by using the qPCR assay with PMA treatment, since *Bacteroides*-*Prevotella* bacteria are believed to quickly lose viability in the environmental waters (Okabe and Shimazu 2007).

4.3.4 Quantitative relationship between strain MG1655 Δ lac::kan and *B. fragilis* JCM 11019

The quantitative relationship between of *B. fragilis* JCM 11019 and strain MG1655 Δ lac::kan spiked together to pond water samples were determined and compared (Figure 4.5). The

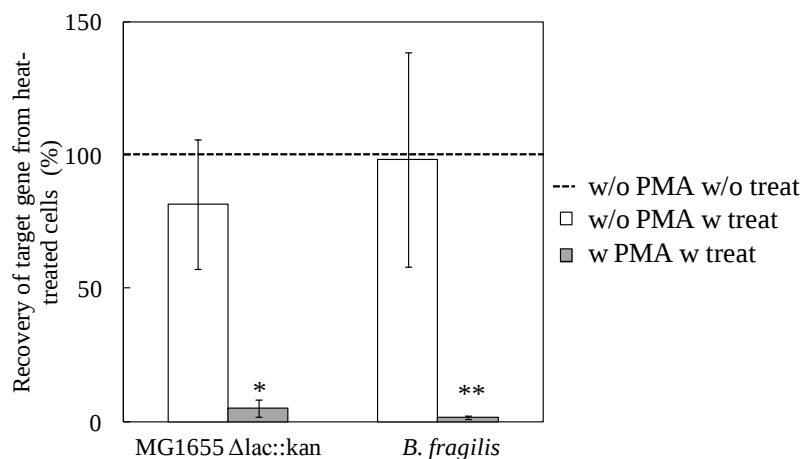


Figure 4.4 The recovery efficiencies of the target gene from heat-killed cells of strain MG1655 $\Delta lac::kan$ and *B. fragilis* JCM 11019 quantified by three kinds of processes; PMA treatment is not performed (w/o PMA w/o treat, $n=3$), PMA treatment is performed with PMA solution (w PMA w treat, $n=3$), or PMA treatment is performed without PMA solution (w/o PMA w treat, $n=3$). *, $p < 0.05$; **, $p < 0.01$ by ANOVA F -test between the quantities obtained with and without PMA treatment.

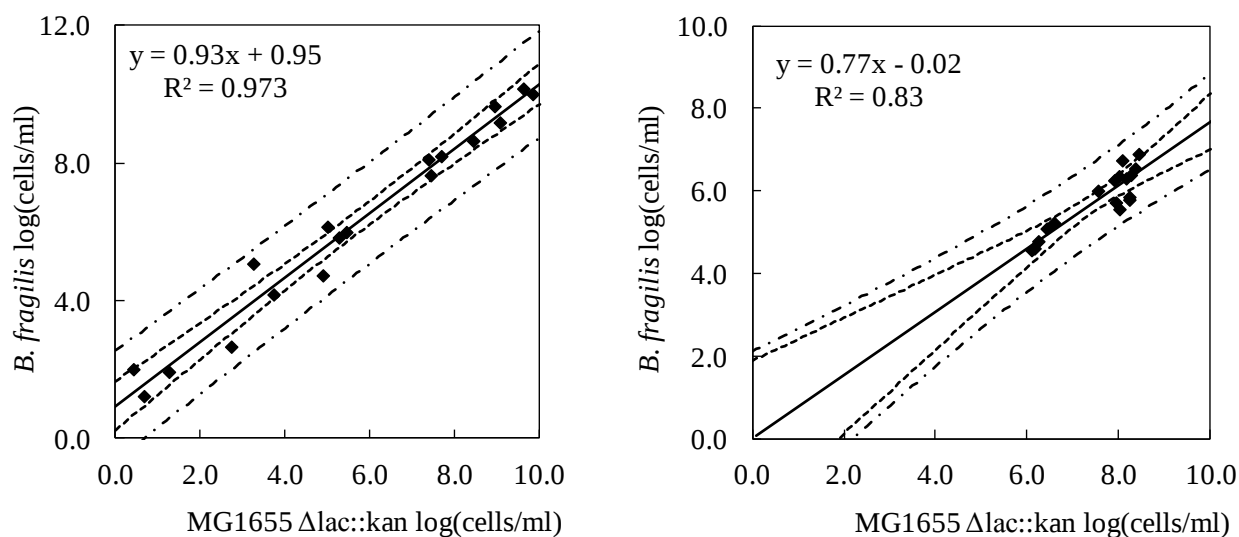


Figure 4.5 Quantitative relationships between strain MG1655 $\Delta lac::kan$ and *B. fragilis* JCM 11019 during the filtration, concentration and DNA extraction processes performed with A) the QIAamp DNA Stool Mini Kit and B) the PowerSoil DNA Isolation Kit. Dotted lines indicate 99% confidence interval, and dashed lines indicate 99% prediction interval.

quantification limits of *B. fragilis* JCM 11019 and strain MG1655 $\Delta lac::kan$ using Human-Bac1 (Okabe et al. 2007) and strain MG1655 $\Delta lac::kan$ primer sets were 4.5 and 62 copies/reaction, respectively. Strong positive correlation was obtained between the quantified values of *B. fragilis* JCM 11019 and those of strain MG1655 $\Delta lac::kan$ when the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) was used for DNA extraction (Figure 4.5A). The slope, intercept, determination coefficient and p -value of Figure 4.5A are 0.93, 0.95, 0.97 and 4.5×10^{-14} , respectively. Although the intercept value of 0.95 means that the quantified value of *B. fragilis* JCM 11019 is

always 10 times larger than that of strain MG1655 $\Delta\text{lac}::\text{kan}$, the slope value of 0.93 indicates that the recovery efficiency of strain MG1655 $\Delta\text{lac}::\text{kan}$ is proportional to that of *B. fragilis* JCM 11019 when the QIAamp DNA Stool Mini Kit was used. The coordinated recovery efficiency must be one of critical properties of SPC.

On the other hand, the slope, intercept, determination coefficient and p-value are 0.77, -0.02, 0.83 and 1.38×10^{-7} , respectively, when the PowerSoil DNA Isolation Kit (MoBio, Laboratories, Carlsbad, CA, USA) was used (Figure 4.5B). The slope value of 0.77 is not significantly different from 1.0, tested by F-test at $\alpha = 0.05$, which means that the recovery efficiency of strain MG1655 $\Delta\text{lac}::\text{kan}$ is statistically regarded to be identical to that of *B. fragilis* JCM 11019 when the PowerSoil DNA Isolation Kit was used. These results indicated in Figure 4.5A and 4.5B mean that the strain MG1655 $\Delta\text{lac}::\text{kan}$ as SPC may be used for multiple types of DNA extraction kit. The apparent difference between Figures 4.5A and 4.5B is that the recovery efficiencies of strain MG1655 $\Delta\text{lac}::\text{kan}$ and *B. fragilis* are more stable when the PowerSoil DNA Isolation Kit was used. That's why the dots in Figure 4.5B are concentrated in the range of 10^6 to 10^8 cells/mL, while dots are dispersed between 10^1 to 10^{10} cells/mL in Figure 4.5A. In the following experiments, we used the PowerSoil DNA Isolation Kit.

4.3.5 Inhibition factors of the recovery of strain MG1655 $\Delta\text{lac}::\text{kan}$

We also examined the PCR inhibitory effects by using Chicken-Bac plasmid (IAC). The amplification efficiency of Chicken-Bac IAC added in concentrated environmental water samples ranged from 47.0 and 103.4% (17%; coefficient of variation) (Figure 4.6). The variation in the quantified values of the IAC was smaller than that of the bacterial cell recovery as evaluated by using the SPC (106%; coefficient of variation). This means that impact of PCR inhibition was smaller than DNA loss during the bacterial cell recovery and DNA extraction processes.

Regarding the correction of qPCR results, previous reports mostly focused on the PCR inhibitory effects by using an IAC (Haugland et al. 2010; D'Agostino et al. 2011). However, the present study demonstrates that DNA loss during the bacterial cell recovery and DNA extraction processes influenced more on the qPCR results than the PCR inhibitory effects. This justifies that the inclusion of SPC is essential for accurate quantification of bacterial genetic markers, and should be included in the standardized qPCR protocol. If the recovery efficiency of SPC is found to be very low (e.g., less than 1%), it is suggested that DNA recovery is severely inhibited, and reanalysis from bacterial cell recovery may be required. It should be specified in the protocol that how low recovery efficiency of SPC is accepted.

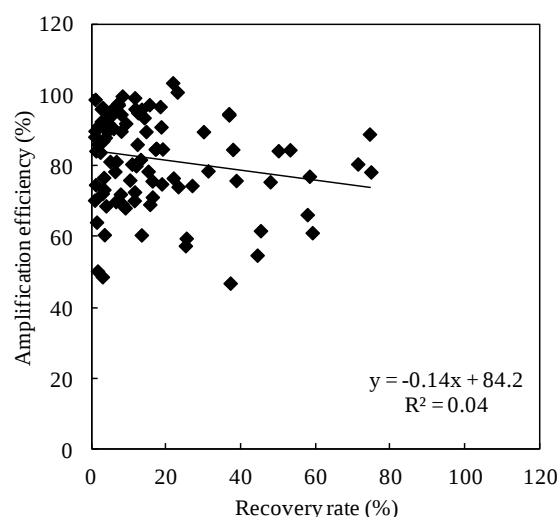
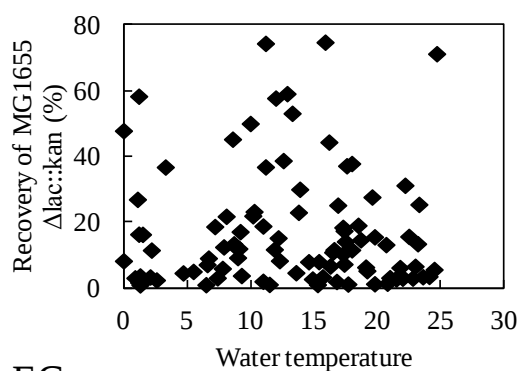


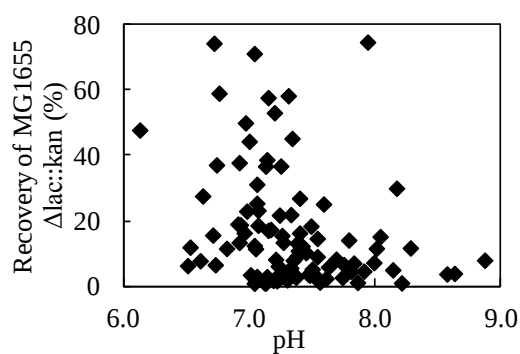
Figure 4.6 A simple correlation between the recovery of *Escherichia coli* K12 MG1655 Δ lac::kan and the amplification efficiency of Chicken-Bac Plasmid (IAC) in concentrated environmental water samples. An outlier removed in this figure by using test of rejection of Smirnoff-Grubbs' outlier test ($\alpha=0.05$).

In order to identify factors influencing the recovery of strain MG1655 Δ lac::kan from water samples, single correlations between the bacterial cell recovery and water quality parameters, including water temperature, pH, electrical conductivity (EC), dissolved oxygen (DO), biological oxygen demand (BOD), suspended solids (SS), total nitrogen (T-N), and total phosphorous (T-P) were assessed (Figure 4.7). As a result, no particular parameter was found to show a statistically significant correlation with the recovery of strain MG1655 Δ lac::kan. However, small variations of the recovery efficiencies were observed when SPC was added to identical samples, while large variations of the recovery efficiencies were detected when SPC was recovered from different samples. These results indicate that unknown inhibitory factors of DNA recovery present in these water samples were successfully detected by SPC.

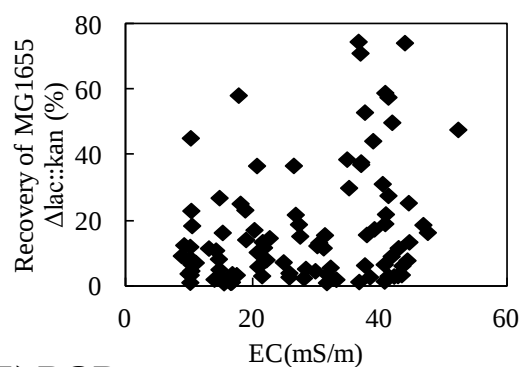
A) Water temperature



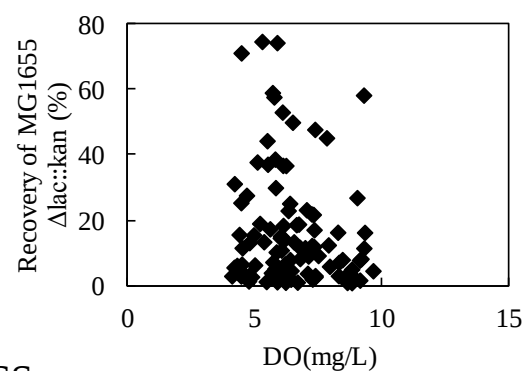
B) pH



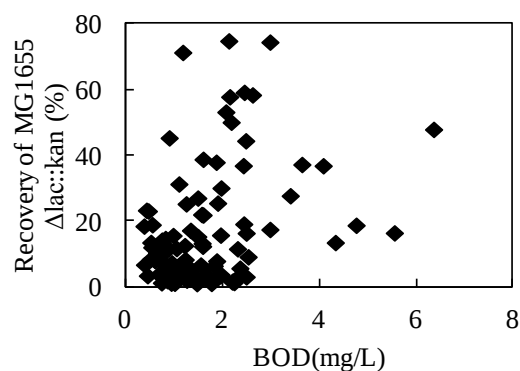
C) EC



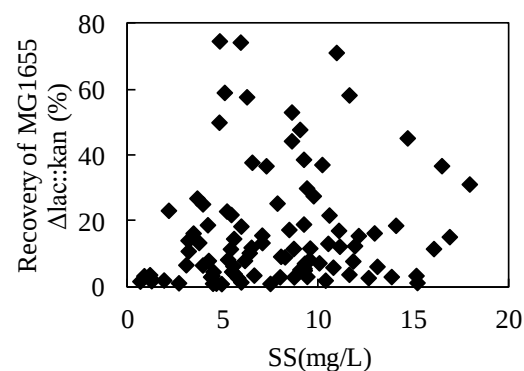
D) DO



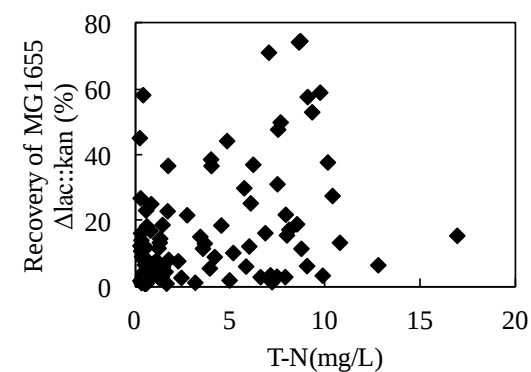
E) BOD



F) SS



G) T-N



H) T-P

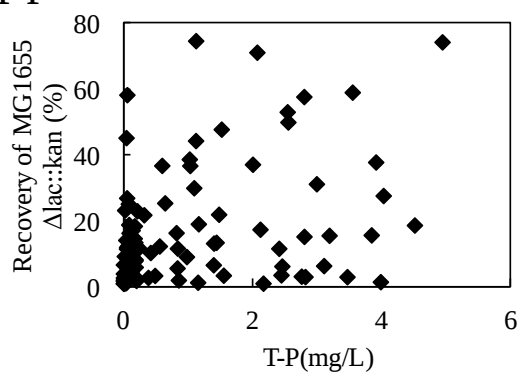


Figure 4.7 A simple correlation between the recovery of *Escherichia coli* K12 MG1655 $\Delta\text{lac}::\text{kan}$ and the amplification efficiency of Chicken-Bac Plasmid (IAC) in concentrated environmental water samples. An outlier removed in this figure by using test of rejection of Smirnov-Grubbs' outlier test ($\alpha=0.05$).

4.4. Conclusions

The host-specific *Bacteroides-Prevotella* 16S rRNA genetic markers are regarded as the most promising tool to determine the source of fecal contamination in environmental waters in addition to the levels of fecal contamination (Field and Samadpour 2007; Fremaux et al. 2010; Kildare et al. 2007; Kobayashi et al. 2012; Mieszkin et al. 2009a; Mieszkin et al. 2009b; Okabe et al. 2007; Seurinck et al. 2005). For more accurate assessment of human health risk based on monitoring these genetic markers in environmental waters, careful attentions should be paid to the DNA loss during sample preparation (e.g., bacterial cell recovery and DNA extraction) and inhibition of PCR amplification, since various dissolved and particulate substances that hamper the DNA recovery and inhibit PCR amplification are usually present in environmental waters (Green and Field 2012). It can be also assumed that substantial proportions of microbial populations have lost viability (i.e., dead cells). Therefore, the DNA recovery efficiency and PCR amplification efficiency in qPCR assays targeting live microbial populations must be used to check of the error in sample processing and to correct the quantified values.

In conclusion, we established a genetically-engineered *Escherichia coli* K12 strain (designated as strain MG1655 Δ lac::kan) as a sample process control (SPC) for the qPCR assays to more accurately quantify the host specific *Bacteroides-Prevotella* 16S rRNA genetic markers in environmental water samples. The inclusion of the SPC and IAC in qPCR assays with PMA treatment provides more reliable quantitative information on recent fecal contamination levels and sources, which would lead to better allocation of management and mitigation efforts in a more time- and cost-effective manner.

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Chapter 5

Effects of temperature and predator on the persistence of host-specific *Bacteroides-Prevotella* genetic markers in water

5.1. Introduction

The accurate quantification of pathogens is required to precisely evaluate human health risks in water utilization (Asano et al. 2007). Fecal contamination of water with pathogenic microorganisms has been evaluated and managed by fecal indicator microorganisms, including coliforms, fecal coliforms, *Escherichia coli* and *Enterococcus*. Although these indicator microorganisms have been used extensively for many years as indicators for determining the sanitary quality of surface, recreational, and shellfish growing waters (Scott et al. 2002), seminal drawbacks of these conventional indicators are emphasized especially in their inability to identify sources of fecal contamination in water (Harwood et al. 2005; Savichtcheva and Okabe 2006).

Diverse alternative indicators for identifying fecal contamination sources have been proposed (Blanch et al. 2006). Especially, genetic markers originated from enteric bacteria are being used as promising indicators for identifying fecal pollution sources library- and culture-independently (Okabe et al. 2007). Particularly, the quantitative PCR (qPCR) assays for 16S rRNA of Bacteroidales spp. have been constructed and widely accepted (Bernhard and Field, 2000; Field and Samadpour, 2007; Sylvie et al. 2005).

Since the decay rate of these bacterial genetic markers are expected to be heavily dependent on environmental conditions, the persistency of PCR-detectable DNA fragments from bacterial cells in environmental water has to be correctly figured out to discuss the applicability of bacterial genetic markers as fecal source identifiers (Field et al. 2003). For example, Okabe and Shimazu (2007) reported that indigenous microorganisms or protozoa are the major factors influencing the fate of host-specific *Bacteroides-Prevotella* 16S rRNA genetic markers in river and seawater. However, the fate of PCR-detectable 16S rRNA genetic markers in various environmental water conditions has been little investigated, and in addition, the differences in persistence characteristics among fecal pollution indicators (e.g. *Bacteroides-Prevotella*, *Bifidobacterium*, *Enterococcus*, human *norovirus*, and so on) have not been well analyzed.

In this study, the fate of some human-specific genetic markers in river water was compared with that of conventional indicator microorganisms at various water temperature conditions. In addition to that, the possible effect of predators on the persistency of bacterial genetic markers in natural water environment was also investigated.

5.2. Materials and methods

5.2.1 Sample collection and experimental design

River water was collected from Toyohira River in Sapporo, Hokkaido, Japan. Human fecal

samples were collected from healthy adults (n=3) in Sapporo City. Two grams of each fresh human feces were suspended in 5 L river water (1.2 mg-feces/mL). In addition, a fecal sample (5ml) from a *norovirus*-infected patient was added to the suspended fecal sample. The sample solution was incubated aerobically at 4, 10, 20 and 30 °C. Subsamples (50ml) were collected at 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 10th, 15th and 20th days after the incubation start for real-time qPCR assay and total coliforms enumeration.

Two samples were prepared for detection of predators that can prey target bacteria. One is concentrated river water. River water (10 L) was filtered with 0.2- μ m-pore-size polyethersulfone membrane (Millipore Corporation, Billerica, MA, USA) filters. The filters were soaked with sterile 50-mL disposable centrifuge tube containing 50 mL of sterile STE buffer (0.1 M NaCl, 10 mM Tris, and 1 mM EDTA (pH 7.6) and rigorously shaken by a vortex mixer. The organisms and suspended solid were scrubbed off the filter and then centrifuged at 10,000 x g for 10 min, and the supernatant was removed gently till 2 mL (final volume) remained. Another sample is a pre-cultured sample. The pre-cultured sample is a fecal suspension that contains human feces (1.2 mg/ml) in river water. Fecal suspension was prepared as described above, and the fecal suspension was incubated aerobically at 20 °C for 3-week. This pre-cultured sample was used for detecting protozoa that exist at very low concentration.

5.2.2 Genome extraction and complementary DNA (cDNA) synthesis

Subsamples were centrifuged at 10,000 x g for 10 min to recover bacterial cells as precipitate. The precipitate was suspended in 500 μ L of MilliQ water. Bacterial DNA from resuspended subsamples, concentrated river water sample and pre-cultured sample were extracted with QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany). Virus particles were recovered from subsamples with polyethylene glycol precipitation method (Lewis and Metcalf, 1988). RNA was extracted from the concentrate with QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). The cDNA (cDNA) was synthesized from extracted viral RNA with PrimeScripy RT reagent Kit (Takara Bio, Otsu, Japan).

5.2.3 Real-time PCR assay

The primers and probe used for real-time PCR assay are shown in Table 5.1. *Bacteroides-Prevotella*, *Bacteroides*, *Bifidobacterium* and *Enterococcus* were chosen as target bacteria, because these bacteria are included in intestinal flora and are well draw attention as genetic markers for source tracking. Human *norovirus* was also used as a target, in order to compare its persistence characteristic with bacterial genetic markers.

Table 5.1 The primers and probe used for this study

Target	Primer	Sequence (5'-3')	Reference
<i>Bacteroides</i>	qHS601F	GTTGTGAAAGTTTGC GGCTCA	Okabe S et al. 2007
- <i>Prevotella</i>	qBac725R	CAATCGGAGTTCTTCGTGATATCTA	
(16s rRNA)	qHS624MGB	(FAM)-CGTAAAATTGCAGTTGA-(MGB)	
<i>Bacteroides</i>	HF183F	ATCATGAGTTCACATGTCCG	Sylvie S et al. 2005
(16S rRNA)	Bac242R	TACCCCGCCTACTATCTAATG	
<i>Bifidobacterium</i>	Bi-ADO 1	CTCCAGTTGGATGCATGT	Bonjoch X et al. 2004
(16S rRNA)	Bi-ADO 2	CGAAGGCTTGCTCCCACT	
<i>Enterococcus</i>	<i>E. faecium</i> esp gene F	TATGAAAGCAACAGCACAAGTT	Scott TM et al. 2005
(esp gene)	<i>E. faecium</i> esp gene R	ACGTCGAAAGTTCGATTTCC	
<i>Enterococcus</i>	<i>E. faecalis</i> _F	CGCTTCTTTCCTCCCGAGT	Wheeler AL et al. 2002
(16S rRNA)	<i>E. faecalis</i> _R	GCCATGCGGCATAAACTG	
	<i>E. faecalis</i> _P	CAATTGGAAAGAGGAGTGCGGACG	
<i>Norovirus</i>	QNIF2d	ATGTTCAAGRTGGATGAGRTTCTCWGA	Allegra KS et al. 2007
(ORF1-ORF2	COG2R	TCGACGCCATCTTCATTACACA	
junction)	QNIFS	(FAM)-AGCACGTGGGAGGGGATCG-(TAMRA)	
18S rRNA gene	Euk-82F	AAACTGCGA ATGGCTC	Skillman LC et al. 2006
(Universal primer)	Medlin B	TGATCCTTCTGCAGGTTACCTAC	Medlin L et al. 1988
Predator (primer walking)	Seq1-F	CGGGTAACGGAGAATTAG	this study
	Seq1-R	CACAGACCTGTTATTGCC	this study
	Seq1-R S4	GACATCTAAGGGCATCAC	this study
	Seq2-F D3	GTTTCAGGCAGGTTTAAG	this study
	Seq2-F S5	GTTTCAGGCAGGTTTATAGC	this study
	Seq2-F S4	GAGTGTTCAAAGCAGGC	this study
	Seq2-R	CCCCTAACTTTCGTTCTTG	this study

A SYBR Green assay was performed with HF183-Bac242 and Bi-ADO1-Bi-ADO2 primer sets. In SYBR Green assay, each PCR mixture (30 µl) was composed of 15 µl of SYBR® Premix Ex Taq II (Takara Bio, Otsu, Japan), 0.6 µl of ROX Reference Dye, 9.6 µl of dH₂O, 400 nM of forward and reverse primer each, and 2 µl of template DNA. In the TaqMan assay, each PCR mixture (30 µl) was composed of 15 µl of 1x Premix Ex Taq (Takara Bio, Otsu, Japan), 200 nM of forward and reverse primer each, 200 nM of fluorogenic probe, and 2.0 µl of template DNA. PCR reactions were performed in MicroAmp Optical 96-well reaction plates with ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). For primer set of qHS601-qBac725-qHS624MGB, HF183-Bac242 and QNIF2d-COG2R-QNIFS, the reactions were carried out by incubation for 30 sec at 95 °C, followed by 40 cycles of denaturation at 95 °C for 5 sec and annealing and extension at 60 °C for 31 sec. For the primer set of Bi-ADO1-Bi-ADO2, the reaction was carried out by incubation for 30 sec at 95 °C, followed by 40 cycles of denaturation at 94 °C for 20 sec,

annealing at 55 °C for 20 sec and extension at 72 °C for 31 sec. A melting curve analysis for the SYBR Green assay was performed after amplification to distinguish the target PCR products from unspecific PCR products.

PCR standards were prepared using TOPO® TA PCR cloning system (Invitrogen, Carlsbad, CA, USA). The ligated products were transformed into *E. coli* TOP10 competent cells (Invitrogen, Carlsbad, CA, USA). Plasmids were extracted from transformed cells and purified with the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). The concentration of plasmid DNA was between 10^{-1} and 10^{-8} ng DNA per μL for the standard curve.

5.2.4 Total coliforms

Enumeration of total coliforms in water was carried out using desoxycholate agar according to Wastewater Examination Methods (Japan Sewage Works Association, 1997).

5.2.5 Decay rate

Decay rates of genetic makers and total coliforms at each temperature were estimated. The decay rate k was calculated with the following equation based on Chick model:

$$k = [\ln (N_t) - \ln (N_0)] / t \quad (\text{Eq. 5.1})$$

where k = decay rate, N_t = $\log_{10}$ (copies or CFU/100 ml) at time t , N_0 = $\log_{10}$ (copies or CFU/100 ml) at time zero, and t = time (in days).

5.2.6 PCR and Cloning of the 18S rRNA gene

Universal primer of 18S rRNA gene (Euk82F-Medlin-B, Skillman et al. 2006; Medlin et al. 1988) was used to amplify 18S rRNA gene (Table 5.1). Thermal conditions were 94 °C for 10 min, followed by 6 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 45 sec and extension at 72 °C for 90 sec, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 60 sec and extension at 72 °C for 8 min.

The PCR product was purified by using QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The purified PCR products were ligated into the TOPO® TA PCR cloning system (Invitrogen, Carlsbad, CA, USA). The ligated products were transformed into *E. coli* TOP10 competent cells (Invitrogen, Carlsbad, CA, USA). Plasmids were extracted from transformed cells and purified with the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany).

5.2.7 Sequencing and phylogenetic analysis of the 18S rRNA gene

The Sequence of PCR product was determined with Bigdye terminator method. Thermal conditions in the PCR were 95 °C for 4 min, followed by 35 cycles of denaturation at 96 °C for 10 sec, annealing at 50 °C for 5 sec and extension at 60 °C for 4 min and extension at 60 °C for 10 min. Then, the PCR product was purified by using BigDye Xterminator Kit (Applied Biosystems, Forster City, CA, USA). Partial sequencing was performed with an automatic sequencer (Prism 3100, Avant Genetic Analyzer, Applied Biosystems, Forster City, CA, USA). The primer walking approach was employed to analyze the total length of 18S rRNA gene. The primer used in the sequencing was listed in Table 5.1. Determined sequences were compared with similar sequences available in public database (NCBI) with a BLAST search. The representative sequences were aligned with CLUSTAL W package. A phylogenetic tree was constructed by the neighbor-joining method.

5.3. Results and discussion

5.3.1 The fate of genetic markers in river water

Time-dependent changes in copy numbers of human-specific genetic markers were monitored by real-time PCR, and at the same time, total coliforms were quantified. The decay pattern of each human-specific genetic marker was shown in Figures 5.1(a), (b) and (c), and that of total coliforms were shown in Figure 5.1(d). The initial concentrations of *Bacteroides-Prevotella* (qHS601-qBac725-qHS624MGB), *Bacteroides* (HF183-Bac242) and *Norovirus* genetic markers and total coliforms are about 10^8 copies/L, 10^{10} copies/L, 10^4 copies/L and 10^5 - 10^8 CFU/L, respectively. The decay rate of genetic makers and total coliforms at each temperature was shown in Table 5.2.

Fate of fecal indicators derived from *Bacteroides* (HF183-Bac242) was not indicated in Figure 5.1 (b), because *Bacteroides* genetic markers (HF183-Bac242) were not detected or less than quantification limit at 20 and 30 °C (the quantification limit of *Bacteroides* genetic markers (HF183-Bac242) is 4.5 copies/reaction). *Bacteroides* genetic marker (HF183-Bac242) was not detected from some human fecal samples in this study. These results imply that *Bacteroides* genetic marker (HF183-Bac242) may have an unidentified regional specificity, and there is a practical limitation in the solo application of this marker as a fecal indicator. These results indicate that seasonal and geographical variations in persistence of genetic markers must be considered when we applied them in environmental waters.

The genetic markers of *Enterococcus* were not detected from all fecal and water samples. Thus, PCR standards were not able to prepare and the quantification by using qPCR was not performed. The use of genetic marker of *Enterococcus faecalis* and *Enterococcus faecium* for routine

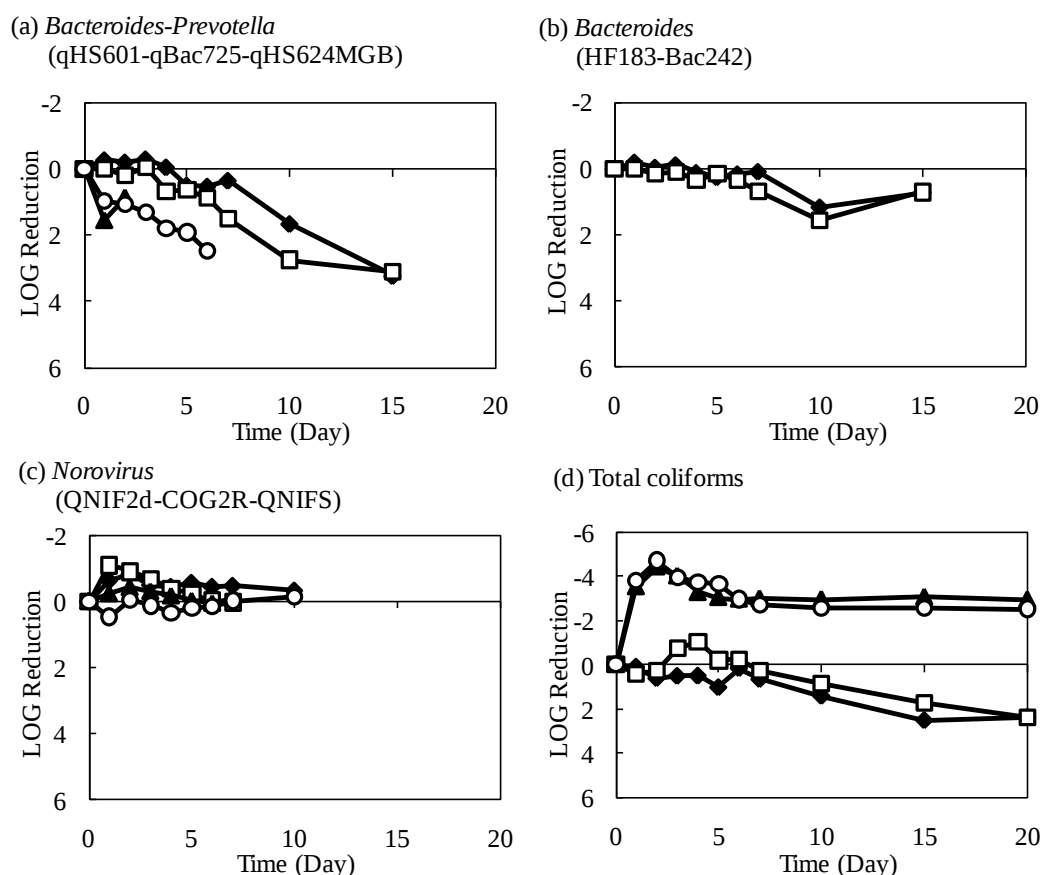


Figure 5.1 Fate of fecal indicators derived from (a) *Bacteroides-Prevotella* (qHS601-qBac725-qHS624MGB), (b) *Bacteroides* (HF183-Bac242), (c) human norovirus and (d) total coliforms in river water incubated at 4 °C (black diamond), 10 °C (white square), 20 °C (black triangle) and 30 °C (white circle). The data below the quantification limit was not shown.

Table 5.2 The decay rate of genetic markers and total coliforms at each temperature

	4°C	10°C	20°C	30°C
	k (1/Day)	k (1/Day)	k (1/Day)	k (1/Day)
<i>Bacteroides-Prevotella</i>	-0.17	-0.27	-0.24	-0.37
<i>Bacteroides</i>	-0.09	-0.09		
<i>Norovirus</i>	-2.2×10^{-6}	-0.09	-0.03	-0.03
Total coliform	-0.13	-0.14	0.01	-0.03

monitoring might be difficult because of the detection efficiency from samples and the number of these bacteria in feces.

The genetic marker of *Bifidobacterium* could not be detected under all conditions (the detection limit of *Bifidobacterium* marker is 4.3 copies/reaction). Since the conventional PCR also did not give positive results (data not shown), it seemed that the concentration of *Bifidobacterium* genetic marker in fecal samples used in this study was too low to be detected. If the genetic marker derived from *Bifidobacterium* is employed to find fecal contamination in environmental waters, extremely high efficiency in recovery and detection from environmental water is required, which

might not allow us to use this *Bifidobacterium* genetic marker in routine monitoring.

The amount of the genetic marker originated from *Bacteroides-Prevotella* spp. was decreased by 3-log or more at each temperature within 2 weeks. Sooner decay was observed at higher water temperature. Huge difference in the decay pattern of genetic marker was observed when the target area was different. The amount of the genetic marker of *Bacteroides* spp. detected by the primer set of HF183-Bac242 was constant during 1 week, and then decreased by 1-log or more (Figure 5.1(b)). No difference was observed between the amount of the genetic marker of *Bacteroides* spp. at 4 °C and 10 °C during 3-week incubation. This results is contrary to the report of Sylvie et al. (2005), in which the amount of the *Bacteroides* spp. genetic marker in freshwater was not reduced significantly at 4 °C throughout the incubation period of 24 days, but 1 and 2-log reductions were observed after 10 days of incubation at 12 °C and 6 days of incubation at 28 °C, respectively. These facts indicate that not only water temperature but also water quality affects the decay of genetic markers.

There was a substantial difference in the decay rates between *Bacteroides-Prevotella* and *Bacteroides* genetic markers, and the decay rates of these bacterial genetic markers were increased with temperature (Figures 5.1(a) and (b)). ANOVA test detected that there was a statistically significant difference ($\alpha = 5\%$) between the decay patterns of *Bacteroides-Prevotella* and *Bacteroides* genetic markers. This result indicates that the decay rate of genetic marker depends on bacterial species. The species-dependent decay pattern has been reported by other researchers. For example, Ballesté & Blanch (2010) reported that the survival patterns of *B. fragilis* and *B. thetaiotaomicron* over the seasons were different, although they belong to the same genus.

The amount of human *norovirus* gene was almost constant at each temperature within 1 week (Figure 5.1(c)), which means that *norovirus* gene is more persistent than bacterial genetic markers. ANOVA test detected that there was a statistically significant difference ($\alpha = 5\%$) between decay patterns of *Bacteroides-Prevotella* genetic marker and *Norovirus*. It has been reported that *norovirus* particles are stable under various conditions (Lee et al. 2008), thus the amount of human *norovirus* gene in environmental water might be available as a persistent genetic marker indicating human fecal contamination in water. However, the seasonal variation of human *norovirus* occurrence (Kitajima et al. 2010; Sano et al. 2006) must give a significant weakness in the application of human *norovirus* gene as a stable indicator for fecal contamination.

Total coliforms counts were gradually decreased at 4 °C and 10 °C (Figure 5.1(d)). However, total coliforms were proliferated and then gradually decreased at 20 °C and 30 °C. It has been shown that *E. coli* can grow in environmental water at high temperature (Desmarais et al. 2002), and this is also the case in total coliforms. The decay rate of total coliforms was bigger at high temperature, but decay rates at 20 °C and 30 °C were smaller than that at 4 °C and 10 °C because of proliferation. The

proliferation may confound the use of total coliforms as a reliable indicator of fecal contamination, as pointed out by many researchers.

5.3.2 The presence of predators that can prey target bacteria of genetic markers

Eukaryotic 18S rRNA gene in river water was detected for indentifying predators, and as a result, four clones were acquired. These clones were attributed to *Nassula* sp. and the diatoms. Diatom is photoautotrophic organism and dose not pray bacteria. *Nassula* sp. is likely to pray blue-green algae or green algae (Kojima et al. 1995).

On the other hand, three clones of 18S rRNA gene were acquired from the pre-cultured samples. These clones (D3, S4, and S5) exhibited highest identities with *Glaucoma scintillans* (98.3%), *Spumella-like flagellate* (99.5%), and *Colpidium campylum* (98.9%), respectively. Figure 5.2 shows the phylogenetic tree of *Ochromonadales* spp. and *Hymenostomatida* spp. *Glaucoma scintillans* can be cultured by using bacteria such as *Alcaligenes feacalis* (Kojima et al., 1995). Also *Colpidium* can pray bacteria. The heterotrophic species (*Spumella*, *Paraphysomonas*) prey bacteria, other algae, protists and so on (Kojima et al. 1995). These results imply that these protozoans are possible to pray *Bacteroidales* spp, but further study is needed to show the significant impact of these species detected in the present study on the fate of genetic markers.

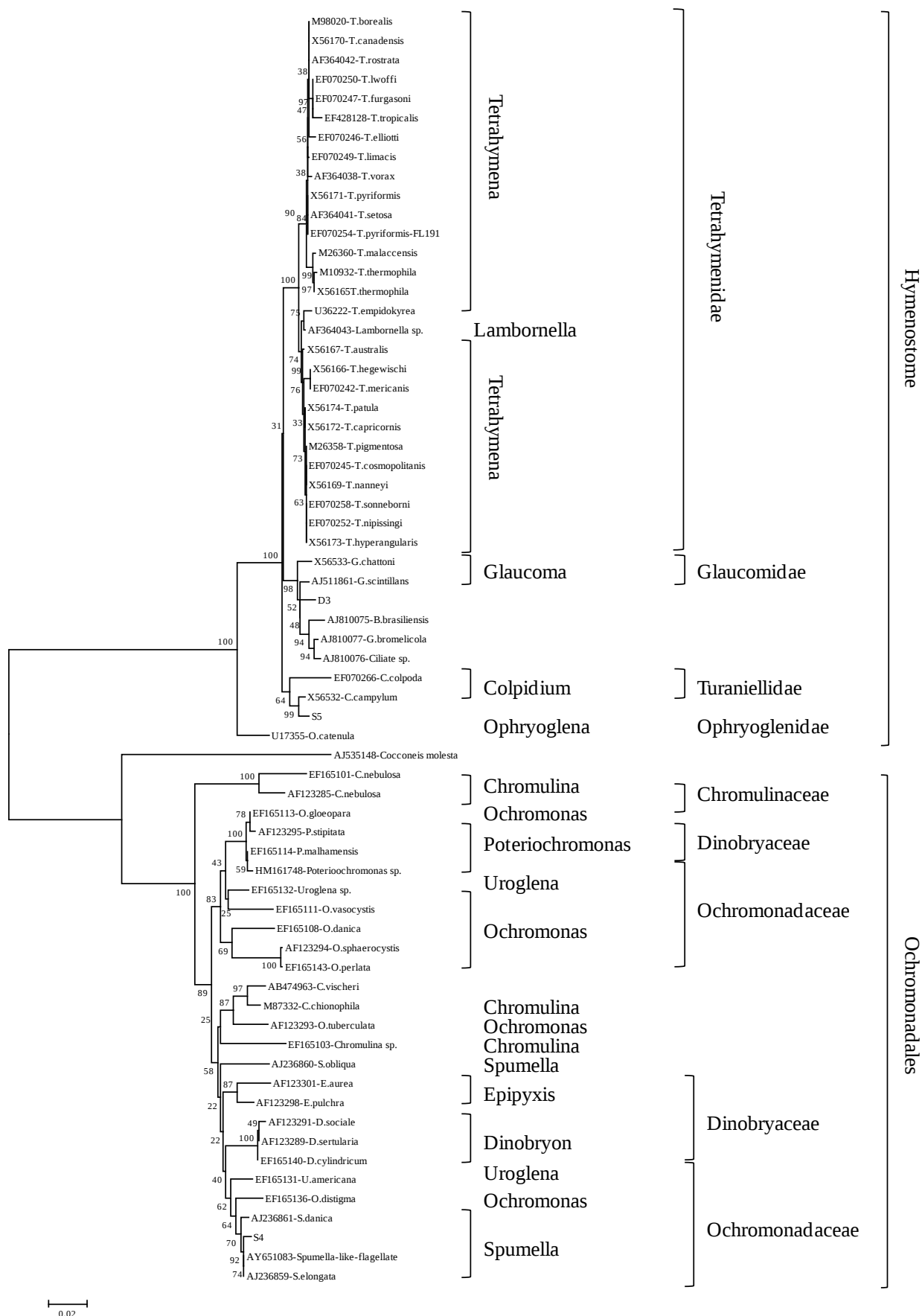


Figure 5.2 Phylogenetic tree of *Ochromonadales* and *Hymenostomatida*. The tree was generated by using neighbor-joining method. Scale bar indicates 5% sequence divergence. The values at the nodes are bootstrap values (100 times resampling analysis).

5.4. Conclusions

Genetic markers derived from *Bacteroidales* spp. have been proposed as promising indicators for fecal contamination in water environment. However, little is known about the persistency of *Bacteroidales* spp. 16S rRNA genetic markers in natural environment, which hampers the precise identification of fecal contamination sources. In this study, the persistency of some bacterial genetic markers, including *Bacteroides* and *Bifidobacterium*, in river water were investigated during a 3-week agitation at various water temperature conditions. The amount of the genetic marker of *Bacteroides-Prevotella* spp. detected by the primer set of qHS601-qBac725-qHS624MGB was decreased by 3-log or more at each temperature within 3 weeks. On the other hand, the amount of the genetic marker of *Bacteroides* detected by the primer set of HF183-Bac242 was constant during 1 week, and then decreased by 1 log or more. The genetic marker derived from *Bifidobacterium* could not be detected at all experiment.

Eukaryotic 18S rRNA gene originated from some bacteria predators were identified from incubated river water samples. Three clones were acquired in total, which have high identities with *Glaucoma scintillans* (98.3%), *Spumella-like flagellate* (99.5%), and *Colpidium campylum* (98.9%), respectively. These protozoans could affect the fate of *Bactericidales* genetic markers in environmental waters.

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Chapter 6

Acceptable concentration of pathogens in environmental water derived by Quantitative Microbial Risk Analysis (QMRA)

6.1. Introduction

The 21st century is often called “the century of water,” and water shortage has become a serious problem over the world. More than two billion people live in highly water-stressed areas because of the uneven distribution of available freshwater resources in time and space. Global population will grow, at least for several decades, and water demand will increase as well (Oki and Kanae 2006). Wastewater reclamation for providing potable water and irrigation water is promoted as one of the effective countermeasures for the increasing water demand.

The absence of suitable indicators in drinking water does not provide sufficient guarantee for microbial safety (Smeets et al. 2010). For microbiological safety management of wastewater reclamation, it is necessary to clearly determine how much pathogens have to be removed. Quantitative microbial risk assessment (QMRA) is a golden standard tool to assess pathogen reduction requirements in wastewater treatment for reclamation based on the acceptable risk of pathogen infection (Barker et al. 2013; Bichai and Smeets 2013; WHO, 2006). QMRA includes four steps; hazard identification, exposure assessment, dose-response relationship, and risk characterization (Asano et al. 2007; Haas et al. 1999), and is possible to derive how much pathogens are allowed to exist in treated and untreated wastewater.

In this chapter, the acceptable concentrations of pathogens in river water were derived by the standard protocol of QMRA for determining the hygiene standard value in the following chapter (Chapter 7).

6.2. Materials and methods

6.2.1 Scenario description

Firstly, the scenario for QMRA was determined in exposure assessment step. In chapter 7, the hygiene standard value will be determined by using surveillance data and the acceptable concentration calculated in this chapter. The surveillance of the concentration of hygiene indicators and pathogens are performed at Class B area in chapter 7. In environmental quality standards for conservation of the living environment in Japan, the water taken from Class B area is able to be used for water supply class 3, fishery class 2, and uses listed in Class C, D and E. In this study, the water supply class 3 was focused for scenario of QMRA. The use of water classified into water supply class 3 is needed to the purification by using the pre-treatment and other advanced methods.

6.2.2 Assumptions in QMRA

In this study, the conventional treatment (flocculation and rapid filtration), ozonation (CT=1.2 mg.min/L), granular activated carbon treatment and chlorination (CT=3.0 mg.min/L) were assumed in the advanced water treatment. The elimination capacities of the conventional treatment, the ozonation, the granular activated carbon treatment and the chlorination for bacteria were set 2.1-log (Hijnen and Medema 2010), 4-log, 0.7-log (Hijnen and Medema 2010) and 3-log, respectively. The information of elimination capacities of the ozonation and chlorination for bacteria on the drinking water purification were very few. Thus, we had an assumption that 10-log reduction of bacteria by the advanced water treatment was assured in this study. The elimination capacities of the conventional treatment, the ozonation, the granular activated carbon treatment and the chlorination for viruses were set 3.0-log (Hijnen and Medema 2010), 3-log (Lim et al. 2010), 0.4-log (Hijnen and Medema 2010) and 1.69-log (Kitajima et al. 2010), respectively. The consumption of unheated drinking-water in the target area was assumed as 1L/day (WHO, Guidelines for Drinking Water Quality 4th edition). The acceptable infection risk of 10^{-4} infection/person/year is employed, which is generally used by U.S.EPA (U.S.EPA 2004).

In hazard identification of this study, waterborne pathogens including *Norovirus*, *Sapovirus*, pathogenic *E. coli*, *Salmonella* spp. and *Campylobacter* spp. were selected as target pathogens. In the calculation of the acceptable concentration of these pathogens, the end point of disease was assumed to be diarrhea. As described above, the hygiene standard value will be determined in chapter 7 by using the acceptable concentration calculated in this chapter, thus the infection efficiency of pathogens were assumed to be safe side, id est, one cell or particle of pathogen has one copy of pathogenesis factor gene, and one copy is considered an infectious unit.

Some dose-response models have been developed to estimate the probability of infection (Haas et al. 1999). In dose-response relationship of this study, Beta-Poisson model was frequently used to calculate the acceptable concentration of pathogens in river water,

$$P_{inf} = 1 - \left(1 + \frac{d}{\beta}\right)^{-\alpha} \quad \text{Eq 6.1}$$

where P_{inf} ; the probability of infection, d ; the mean dose, and α and β ; the Beta-Poisson parameters. Beta-Poisson parameters in the dose-response analysis were collected from previous reports (Haas et al. 1999; Medema et al. 1996; Teunis et al. 2008a; Teunis et al., 2008b). We assumed that the Beta-Poisson parameters collected from previous reports (Haas et al. 1999; Medema et al. 1996; Teunis et al. 2008a; Teunis et al., 2008b) were applicable to *Norovirus*, *Sapovirus*, pathogenic *E. coli*, *Salmonella* spp. and *Campylobacter* spp.

Table 6.1 Beta-Poisson parameters used for this study

Pathogens	Beta-Poisson parameter		Reference
	N_{50}	α	
<i>Norovirus</i>	1.3×10^6	0.631	Teunis et al. 2008a
<i>Salmonella</i> spp.	2.4×10^4	0.313	Haas et al. 1999
<i>E. coli</i> O157:H7	2.1×10^2	0.373	Teunis et al. 2008b
<i>Campylobacter</i> spp.	9.0×10^2	0.145	Medema et al. 1996

Thus, the following five assumptions were employed: 1) The river water is going to be used as a drinking water source; 2) advanced water treatment processes are employed; 3) in the advanced water treatment processes, 8-log reduction of pathogenic viruses and 10-log reduction of pathogenic bacteria are expected; 4) one litter of drinking water per person is consumed everyday; and 5) the acceptable infection risk of 10^{-4} infection/person/year is employed. Beta-Poisson parameters in the dose-response analysis were collected from previous reports (Table 6.1).

6.3. Results and discussion

6.3.1 Acceptable risk of pathogen infection

Based on the assumptions of QMRA, the acceptable concentration of pathogens in environmental water were calculated (Table 6.2). For example, the acceptable concentration of *Norovirus* GII and *Sapovirus* were calculated to be 2.8×10^7 copies/L. This means that the concentration of *Norovirus* GII and *Sapovirus* should not exceed 2.8×10^7 copies/L in river water for keeping the acceptable infection risk of 10^{-4} per person per year. These calculated acceptable concentrations are very high concentration, because the advanced water treatment processes were assumed in QMRA.

In this study, the point estimation of QMRA was performed. However, this approach is not enough to obtain the accurate acceptable concentration of pathogens, because the individual variability was not concerned in this calculation. If you want to obtain the more accurate information, you should perform the distribution estimation of QMRA. However, many of information such as the removal capacity of advanced water treatment and the persistence of pathogens in environmental water are not enough to perform the distribution estimation. Thus, additional experiments and reports will be needed.

Table 6.2 The acceptable concentration of pathogens in environmental water

Pathogens	Risk of illness (ill/person/ year)	Incidence (%)	Infectious risk (infection/person/ year)	Probability of infection	Dose	Concentration at exposure (copies/L)	Acceptable concentration in river water (copies/L)
<i>Norovirus</i> GII, <i>Sapovirus</i>	10^{-4}	100%	10^{-4}	2.7×10^{-7}	2.8×10^{-1}	2.8×10^{-1}	2.8×10^7
<i>Salmonella</i> spp.					2.5×10^{-3}	2.5×10^{-3}	2.5×10^7
pathogenic <i>E. coli</i>					2.9×10^{-5}	2.9×10^{-5}	2.9×10^5
<i>Campylobacter</i> spp.					1.4×10^{-5}	1.4×10^{-5}	1.4×10^5

6.4. Conclusions

In this study, the acceptable concentrations of pathogens in river water were calculated for the determination of the hygiene standard value in Chapter 7. In this calculation, we use the point estimation of QMRA approach. As a result, the acceptable concentration *Norovirus* GII and *Sapovirus* was 2.8×10^7 copies/L, and that of parhogenic *E. coli*, *Salmpnella* spp., and *Campylobacter* spp. were 2.9×10^5 copies/L, 2.5×10^7 copies/L, and 1.4×10^5 copies/L, respectively.

However, this approach is not enough to obtain the accurate acceptable concentration of pathogens, because the individual variability was not concerned in this calculation. If you want to obtain the more accurate information, you should perform the distribution estimation in QMRA. Thus, additional experiments and reports will be needed.

6.5. References

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Chapter 7

Establishment of the hygiene standard value of conventional and alternative indicators based on the acceptable risk of pathogen infection

7.1. Introduction

Fecal contamination of water with pathogenic microorganisms has been evaluated and managed by fecal indicator microorganisms, including coliforms, fecal coliforms, the concentration of *Escherichia coli* and the concentration of *Enterococcus* spp.. Although these indicator microorganisms have been used extensively for many years as indicators for determining the sanitary quality of surface, recreational, and shellfish growing waters (Scott et al. 2002), fundamental shortcomings of these conventional indicators are recently emphasized. For example, the concentrations of bacterial indicators and pathogens are independently fluctuated (Harwood et al. 2005; Savichtcheva et al. 2007), owing to the difference in biological and physicochemical properties of these microbiological entities, which causes a poor correlation between the amount of bacterial indicator microorganisms and that of pathogens. This makes it difficult occasionally to predict the presence of pathogens in water by detecting these conventional indicators.

Furthermore, the current water hygiene standards using these conventional indicators were not determined based on the possible adverse effects on human health. In other words, it is not clear that the density of indicator microorganisms below the current value of water hygiene standards must mean the low risk of infection by pathogenic microorganisms. WHO recommended the use of the epidemiological information and the quantitative microbial risk analysis (QMRA) (WHO, Guidelines for Drinking Water Quality 4th edition). Therefore, it is very critical to understand the occurrence characteristics of pathogens in water and to establish evidence-based water hygiene standards based on the acceptable risk of pathogen infection.

In this study, conventional indicators such as total coliforms and the concentration of *E. coli*, and alternative indicators such as *Bacteroides-Prevotella* 16S rRNA genetic markers and pharmaceutical compounds are focused on. Although many alternative indicators have been developed to identify sources of fecal contamination in water environments (Meays et al. 2004; Savichtcheva and Okabe 2006; Scott et al. 2002; Simpson et al. 2002), the genetic markers of 16S rRNA of *Bacteroides-Prevotella* have been regarded as the most promising alternative (Field and Samadpour 2007). Also, some pharmaceutical compounds such as carbamazepine have been expected as the alternative indicators (Cimenti et al. 2007; Gassera et al. 2011; Haack et al. 2009; Katz et al. 2009).

Based on these backgrounds, the objective of this study is to construct a framework to determine the evidence-based standard values of fecal pollution indicators including the conventional indicators (total coliforms and the concentration of *E. coli*) and alternative indicators (*Bacteroides-Prevotella* 16S rRNA genetic markers and pharmaceutical compounds), which is derived from an

acceptable risk of pathogen infection and occurrence characteristics of pathogens in water for microbiologically safe water management.

7.2. Framework for determining the acceptable concentration of indicators as water quality standard value

The framework is composed of the following five steps. 1) Concentrations of pathogens and indicators in environmental water are quantified in multiple sites for a period of time. 2) Bayesian model is used to estimate the concentration distribution of pathogens, indicators and the distribution of the ratio of a pathogen to an indicator. 3) The quantitative relationship between indicator and pathogen is calculated by using the distribution of the ratio of a pathogen to an indicator. 4) The acceptable concentration of pathogen in river water is calculated by the point estimation of quantitative microbial risk assessment (QMRA). 5) The quantity of indicator in water ensuring that the pathogen concentration is significantly below the acceptable concentration of pathogen is determined as the water quality standard value.

7.3. Materials and methods

7.3.1 Water samples and measurement of water quality parameter

River water samples were collected from Toyohira River (Station 1), Kamogamo River (Station 2), Nopporo River (Station 3), Atsubetsu River (Station 4), and Motsukisamu River (Station 5) in Sapporo, and Atsubetsu River (Station 6) in Ebetsu, Hokkaido, Japan. River water samples were collected twice a month from January 2012 to April 2013. No major contamination source of feces is located near Station 1. On the other hand, effluents from wastewater treatment plants are discharged nearby Stations 3 and 5. There are wild waterfowls such as wild ducks in Station 2 and 5. Domestic stock farms are located nearby Stations 4 and 6, so contamination with animal feces is expected in these stations. All stations are classified into “Class B” in environmental quality standards for conservation of the living environment in Japan.

Water quality parameters, including water temperature, pH, electric conductivity (EC), biochemical oxygen demand (BOD), suspended solids (SS), total coliforms, *E. coli*, total nitrogen (T-N) and total phosphorous (T-P) were measured for each water sample according the standard method (APHA et al., 2005).

7.3.2 Recovery of bacterial cells from water samples

To monitoring the DNA loss during the bacterial cell recovery and DNA extraction processes, MG1655 Δ lac::kan (Kobayashi et al. 2013b) was used as the sample process control (SPC) for genetic markers and pathogenic bacteria. 100 μ L of MG1655 Δ lac::kan were added in 5L of river water before the recovery of bacterial cells.

Bacterial cells suspended in 5L of river water were collected by pressure filtration with a 0.22- μ m-pore-size polyethersulfone membrane filter. Bacterial cells on the membrane filter were eluted by soaking in 30 ml of sterile PBS with gelatin buffer (NaH_2PO_4 : 0.58g, Na_2HPO_4 : 2.5g NaCl: 8.5g and Gelatin: 0.1g / L) and rigorously shaken by a vortex mixer (Cho and Kim 2000; Okabe et al. 2007). Suspended cells in the PBS with gelatin buffer were collected by centrifugation at 10,000 $\times g$ for 15 min at 4°C, and the pellet was resuspended in distilled MilliQ water. The harvested cells were resuspended in 0.8 ml of distilled MilliQ water. Resulting cell suspensions were subjected to DNA extraction and propidium monoazide (PMA) treatment as described below.

7.3.3 Recovery of virus particles from water samples

To monitoring the RNA loss during the virus particle recovery, RNA extraction and reverse transcription reaction processes, Murine *norovirus* (Kitajima et al. 2010) was used as the sample process control (SPC) for pathogenic viruses. 10 μ L of Murine *norovirus* were added in 1L of river water before the recovery of virus particles.

Recovery of virus particles from water samples with 2.5 M MgCl_2 were performed according to Katayama et al. 2002. Virus particles suspended in 1L of river water were collected by suction filtration with 0.45- μ m pore size of A type HA negatively charged membrane (Nihon Millipore, Tokyo, Japan). This membrane was rinsed with 200 mL of 0.5 mM H_2SO_4 (pH 1.0), and performed the elution with 10 mL of 1 mM NaOH (pH 10.8). The concentrates were neutralized upon elution with 0.1mL of 50 mM H_2SO_4 and 0.1mL of 1 $\times$ TE buffer (pH 8.0).

7.3.4 Treatment of bacterial cells with PMA

One half of the resuspended bacterial cells recovered from water samples were subjected to DNA extraction and the other half was subjected to PMA treatment according to Kobayashi et al. (2013b). PMA was dissolved in distilled MilliQ water to create 2 mM stock concentration and stored at -20°C in dark. The PMA solution was added to 400 μ L of resuspended bacterial cells in 1.5 mL centrifuge tube at final concentrations of 100 μ M, and incubated for 5 min in dark. Then, the tube was horizontally placed on ice and exposed to 500-W halogen light, which was placed 20 cm from the tube, for 10 min. The PMA-treated cells were then subjected to DNA extraction as described below.

extracted from virus particle suspensions (1 mL) obtained from water samples by using NucliSENS miniMAG (bioMérieux, Marcy l'Etoile, Lyon, France). Reverse transcription reaction for extracted RNA was performed by using PrimeScript RT reagent Kit (Perfect Real Time) (Takara Bio, Otsu, Japan). From November 2012, transcription reaction for extracted RNA was performed by using RNA UltraSense™ One-Step Quantitative RT-PCR System (Applied Biosystems, Forster City, CA, USA).

7.3.6 Quantitative PCR Assays

The concentrations of Total (Total Bac) and human-specific genetic markers (Human Bac) (Okabe et al. 2007), pathogenic bacteria (*Campylobacter* spp., *Salmonella* spp. and pathogenic *E. coli*) (Ishii et al. 2013), MG1655 Δ lac::kan (Kobayashi et al. 2013b) were quantified using a real-time PCR method and a real-time PCR with PMA method. The concentrations of pathogenic viruses (*Norovirus* GII (Allegra et al. 2005) and *Sapovirus* (Oka et al. 2006)) and Murine *norovirus* (Kitajima et al. 2010) were quantified using a real-time PCR method. Levels of PCR inhibition were evaluated by the addition of internal amplification control (IAC), (Chicken-Bac plasmid, Kobayashi et al. 2013b) to the sample DNA prior to qPCR. Amplification efficiencies of IAC were calculated as quantitative values of IAC in environmental DNA samples divided by the quantitative value of IAC in pure plasmid solution.

The qPCR assays were performed using SYBR Green chemistry to quantify the Total Bac, MG1655 Δ lac::kan and Chicken-Bac (IAC). TaqMan qPCR assay was also performed to quantify Human-Bac, *Campylobacter* spp., *Salmonella* spp., pathogenic *E. coli*, *Norovirus* GII, *Sapovirus* and Murine *norovirus*. In SYBR Green assays, each PCR mixture (25 μ l) composed of 1x SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan), 1x ROX Reference Dye (Takara Bio, Otsu, Japan), 400 nM each of forward and reverse primers, and 2 μ l of template DNA. In the TaqMan qPCR assay for Human-Bac, *Norovirus* GII, *Sapovirus* and Murine *norovirus*, each PCR mixture (25 μ l) composed of 1x Premix Ex Taq (Takara Bio, Otsu, Japan), 200 nM each of forward and reverse primers, 200 nM of fluorogenic probe, and 2.0 μ l of template DNA. In the TaqMan qPCR assay using RNA UltraSense™ One-Step Quantitative RT-PCR System (Applied Biosystems, Forster City, CA, USA), each PCR mixture (25 μ l) composed of 1x reaction Mix buffer, 1x ROX Reference Dye, 1x EnzymeMix, 500 nM each of forward primers, 900 nM each of reverse primers, 252 nM of fluorogenic probe, and 5.0 μ l of template DNA. In the TaqMan qPCR assay for *Campylobacter* spp., *Salmonella* spp. and pathogenic *E. coli*, each PCR mixture (20 μ l) composed of 1x FastStart TaqMan Probe Master (Roche, Deutschland), 900 nM each of forward and reverse primers, 250 nM of fluorogenic probe, and 2.0 μ l of template DNA. PCR reactions using SYBR Premix Ex Taq II

(Takara Bio, Otsu, Japan) and Premix Ex Taq (Takara Bio, Otsu, Japan) were performed in MicroAmp Optical 96-well reaction plates with Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Forster City, CA, USA). The reaction was carried out by heating at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing and extension at 60°C for 34 sec. Following to the amplification step, melting curve analysis was performed for SYBR Green assays to confirm no unexpected PCR products were obtained. PCR reactions using RNA UltraSense™ One-Step Quantitative RT-PCR System (Applied Biosystems, Forster City, CA, USA) were performed in MicroAmp Optical 96-well reaction plates with Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Forster City, CA, USA). The reaction was carried out by heating at 55°C for 15 min and 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 1 min and extension at 65°C for 1 min. PCR reactions using FastStart TaqMan Probe Master (Roche, Deutschland) was performed in MicroAmp Optical 96-well reaction plates with ABI PRISM 7000sequence detection system (Applied Biosystems, Forster City, CA, USA). The reaction was carried out by heating at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 1 min.

Standard sequences of the real-time PCR assays for genetic markers, viruses and pathogenic bacteria were prepared using recombinant pCR 2.1-TOPO vector plasmids inserted with target sequences. The standard plasmids for the quantification of these targets were prepared by TA cloning system. For the standard plasmid for the quantification of strain MG1655Δlac::kan, pUC19 vector carrying the PCR amplicon generated from strain MG1655Δlac::kan with the primer set Kan-res-F and DS-Kan-R was used. The ligated products were transformed into *E. coli* TOP10 competent cells (Invitrogen, Carlsbad, CA, USA). Plasmids were extracted and purified from *E. coli* cells by using QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). The concentrations of plasmid DNA was adjusted from 10^{-1} to 10^{-8} ng per μL and used to generate standard curves. Standard curves were generated by linear regression analysis between threshold cycles (C_T) and the concentration of the plasmid DNA by using Applied Biosystems 7500 Real-Time PCR System software ver. 2.0.4 (Applied Biosystems, Forster City, CA, USA). The quantification limit was defined as the lowest concentration of plasmid DNA that was amplified within the linear range of the standard curve.

7.3.5 DNA and RNA extraction and reverse transcription reaction

Total DNA was extracted from bacteria cell suspensions (200 μL) obtained from water samples by using PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Total RNA was

7.3.7 Recovery and quantification of pharmaceutical compounds

1L of river water samples were pre-filtered by using Advantec Grade GB140 Glass Fiber Filters (Advantec, CA, USA), and pH of filtrates were controlled up to pH 2.0 by using 12N of hydrochloric acid. To monitoring the pharmaceutical compound recovery, 1ng of fenoprop (Sigma-Aldrich, MO, USA) and 10,11-dehydrocarbamazepine (Sigma-Aldrich, MO, USA) were added in filtrates as internal control. The solid-phase extractions were performed by using Oasis MCX 3 cc Vac Cartridge, 60 mg Sorbent per Cartridge (Waters, Tokyo, Japan). The conditioning process by using 1mL of methanol and MilliQ water (pH 2.0) had been performed to Oasis MCX 3 cc Vac Cartridge, 60 mg Sorbent per Cartridge (Waters, Tokyo, Japan), beforehand. These cartridges were washed four times with 1mL of acetone and eluted materials were collected. For detection of pharmaceutical compounds by GC/MS (QP-5050, Shimadzu, Kyoto, Japan), the derivatization reactions by using N-(t-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTF) (Advantec, CA, USA) were performed. The targets of pharmaceutical compounds are Ibuprofen (IBU), Ketoprofen (KPF), Mefenamic acid (MFA), Carbamazepine (CAB) and Diclofenac (DCF).

7.3.8 Estimation of the predictive distribution of indicators and pathogens concentrations and the distribution of the ratio of indicator to pathogen

Estimation of the predictive distribution of genetic markers and pathogens concentrations were performed according to Kato et al. (2013). In this estimation, Bayesian model was employed to obtain the model parameter. This approach allows us to describe the positive sample concentrations without the substitution of the non-detect data. The predictive distributions of genetic markers and pathogens concentrations depend on the number of quantified values, and eight or more quantified values from river water are required for its accurate estimation. The distributions of the ratio of genetic marker to pathogen were also estimated according to Kato et al. (2013).

7.3.9 Calculation of the quantitative relationship between indicator and pathogen concentration

The quantitative ratio of a pathogen to a genetic marker was simulated 10,000 times from the estimated distribution of the ratio, and consequently 10,000 values of the quantitative ratio of a pathogen to a genetic marker were obtained. On the other hand, a value of the genetic marker concentration was tentatively assigned such as 10 copies/mL. Then, 10,000 values of the pathogen concentrations were subsequently obtained by subtracting each the simulation value of the quantitative ratio from the tentative value of the genetic marker concentration. The generation of

10,000 values of the pathogen concentration was repeatedly performed using several tentatively assigned values of the genetic marker concentration.

7.4. Results and discussion

7.4.1 Water quality parameter in river water and recovery ratio of SPCs

Water quality parameters such as water temperature, pH, electrical conductivity (EC), dissolved oxygen (DO), biological oxygen demand (BOD), suspended solids (SS), total nitrogen (T-N), and total phosphorous (T-P) were basically adhered to the environmental quality standards for conservation of the living environment. The single correlations between the recovery ratio of SPCs and the water quality parameters including water temperature, pH, EC, DO, BOD, SS, T-N, and T-P were assessed (previously shown in Figure 4.7). As a result, no particular parameter was found to show a statistically significant correlation with the recovery ratio of SPCs.

If the recovery ratio of SPCs was abnormally low or high, the analysis of this sample was performed again from DNA or RNA extraction. Then if reanalysis data was still abnormal, this data was removed in next estimation by using test of rejection of Smirnov-Grubbs' outlier test ($\alpha=0.05$).

7.4.2 Posterior predictive distributions of indicators and pathogens concentrations

a) Conventional indicators

Figure 7.1 shows the posterior predictive distributions of total coliforms and *E. coli*. All samples of total coliforms and concentration of *E. coli* were positive results. Total coliforms are approximately ten times larger than the concentration of *E. coli*, because total coliforms include *E. coli*, *Citrobacter* spp., *Klebsiella* spp., and *Enterobacter* spp.. And, total coliforms and the concentrations of *E. coli* increased in the summer season. These trends have already reported by many researchers (An et al. 2002; Baudisová 1997; Kobayashi et al. 2013a; Okabe and Shimazu 2007).

b) Total and Human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers

Figure 7.2 shows the posterior predictive distributions of total and human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers. All samples of the concentration of total and human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers were positive results. Figure 7.3 shows the posterior predictive distributions of total and human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers based on the data by using qPCR with PMA. 99% (142/144) of samples of total and 95% (137/144) of samples of human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers were positive results. Ideal indicators need the nonpathogenicity, the rapid detection method,

the easy enumeration method, similar survival characteristics to pathogens (Scott et al. 2002). The concentration of total and human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers were higher than that of conventional indicators. And the detection method and enumeration method based on qPCR technique are more rapid than the culture-based methods. Thus, these genetic markers are expected as alternative indicators.

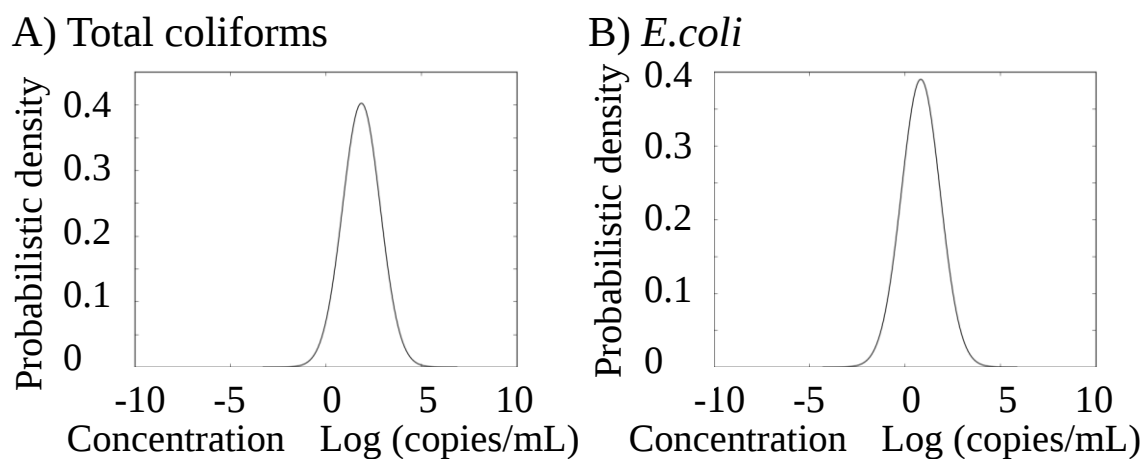


Figure 7.1 Posterior predictive distributions of total coliforms and *E. coli*.

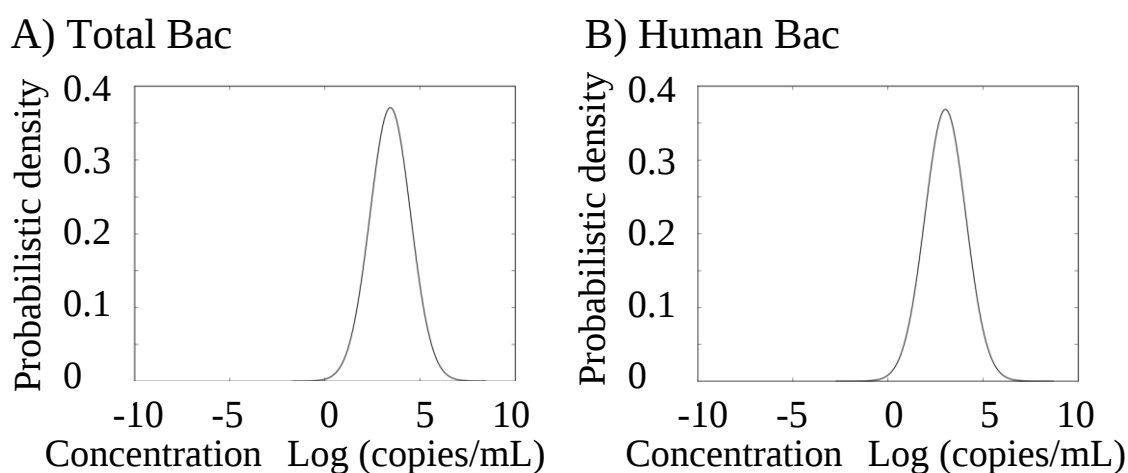


Figure 7.2 Posterior predictive distributions of total and human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers.

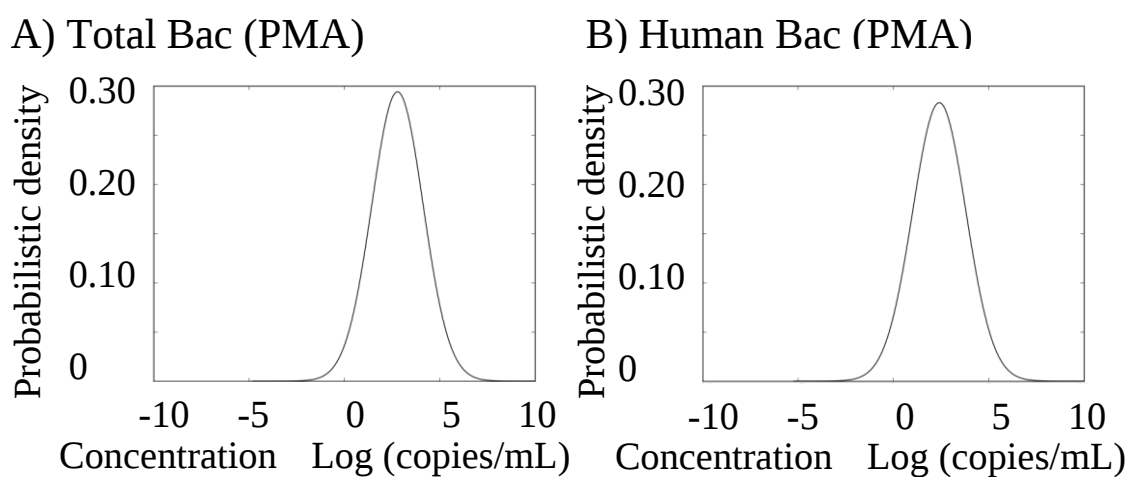


Figure 7.3 Posterior predictive distributions of total and human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers based on the data by using qPCR with PMA.

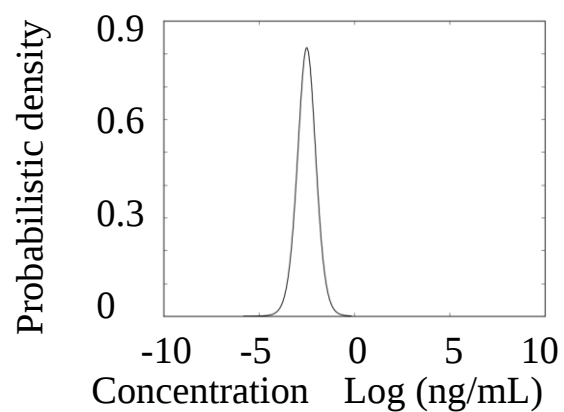
c) Pharmaceutical compounds (IBU, KPF, MFA, DCF and CAB)

Figure 7.4 shows the posterior predictive distributions of IBU, KPF, MFA and CAB. The posterior predictive distributions of pharmaceutical compounds were estimated by using data from Station 3. All samples of the concentration of KPF and CAB were positive results. 88% (14/16) of samples of IBU and 63% (10/16) of samples of MFA were positive results. The posterior predictive distributions of DCF were not able to estimate, because all samples of the concentration of DCF were negative results. The detection frequency of pharmaceutical compounds was lower than that of conventional indicators and the other alternative indicators. The range of posterior predictive distribution derived from data of pharmaceutical compounds was smaller than that of the others. This means that the dispersion of pharmaceutical compounds was small. The difference of concentration between pharmaceutical compounds and conventional indicators or the other chemical markers has sometimes been reported (Sauvé et al. 2012).

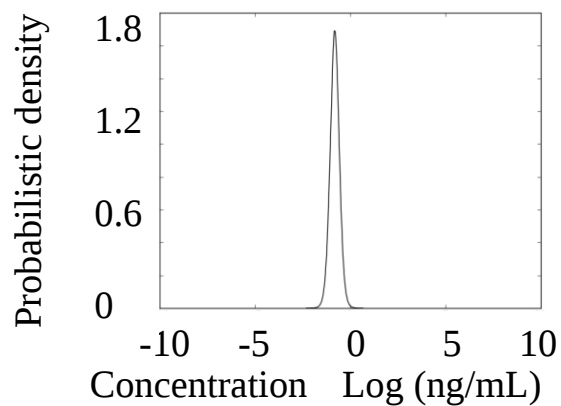
d) Pathogens

Figure 7.5 shows the posterior predictive distributions of *Norovirus* GII, *Sapovirus*, pathogenic *E. coli*, *Salmonella* spp. and *Campylobacter* spp.. 24% (34/144) of samples of *Norovirus*, 4.2% (6/144) of samples of *Sapovirus*, 24% (39/144) of samples of pathogenic *E. coli*, 17% (25/144) of samples of *Salmonella* spp. and 19% (28/144) of samples of *Campylobacter* spp. were positive results. Figure 7.6 shows the posterior predictive distributions of pathogenic *E. coli*, *Salmonella* spp. and *Campylobacter* spp. based on the data by using qPCR with PMA. 19% (27/144) of samples of pathogenic *E. coli*, 18% (26/144) of samples of *Salmonella* spp. and 21% (30/144) of samples of *Campylobacter* spp. were positive results. The detection frequency of *Sapovirus* was lower than that of the other pathogens. Kato et al. (2013) has reported that 8 or larger numbers of sample must be required for accurately estimating the posterior predictive distribution. The posterior predictive distributions and the distribution of the ratio of indicator to *Sapovirus* concentrations can be estimated numerically. However, *Sapovirus* data was removed from the determination of the hygiene standard value, because the sample size was not enough to obtain the accurate results.

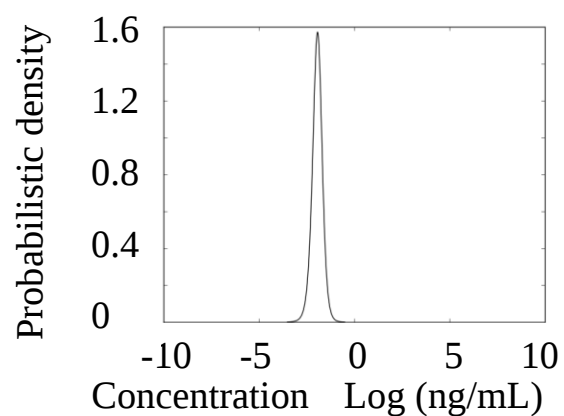
A) IBU



B) KPF



C) MFA



D) CAB

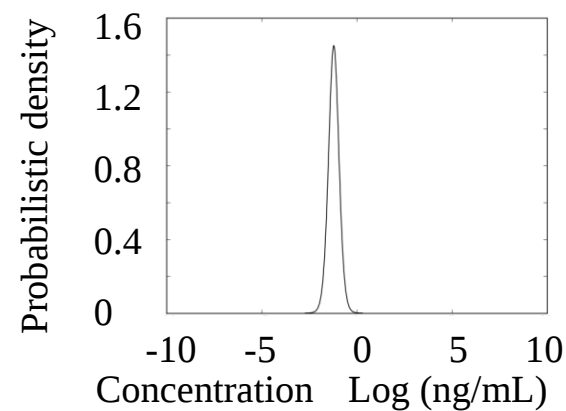


Figure 7.4 Posterior predictive distributions of IBU, KPF, MFA and CAB.

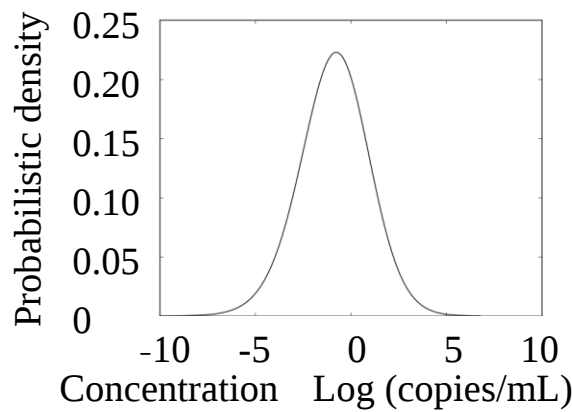
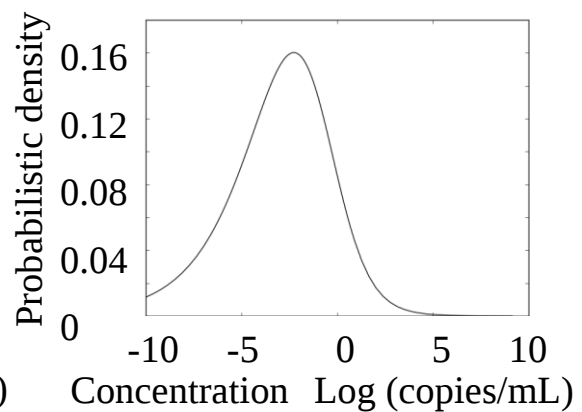
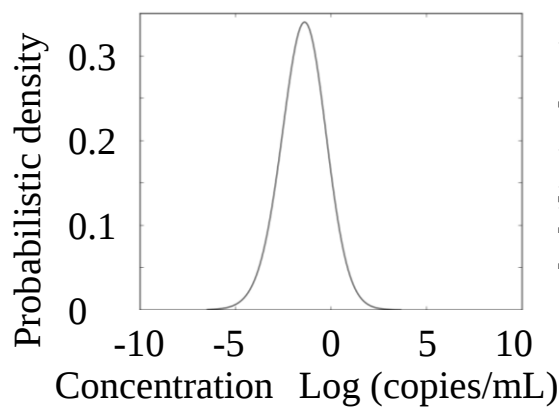
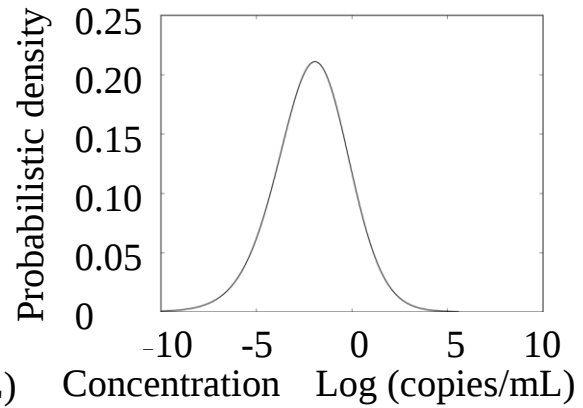
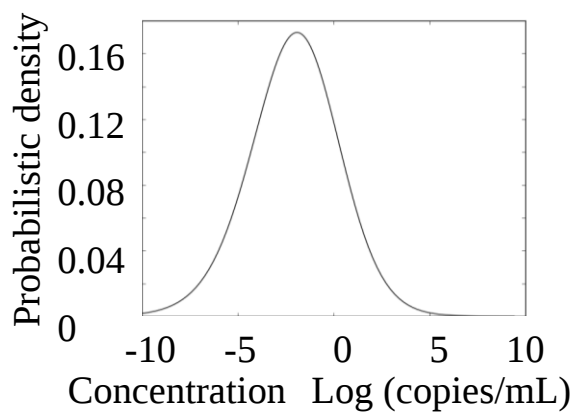
C) *Norovirus* GIID) *Sapovirus*E) pathogenic *E. coli*F) *Salmonella* spp.G) *Campylobacter* spp.

Figure 7.5 Posterior predictive distributions of *Norovirus* GII, *Sapovirus*, pathogenic *E. coli*, *Salmonella* spp. and *Campylobacter* spp..

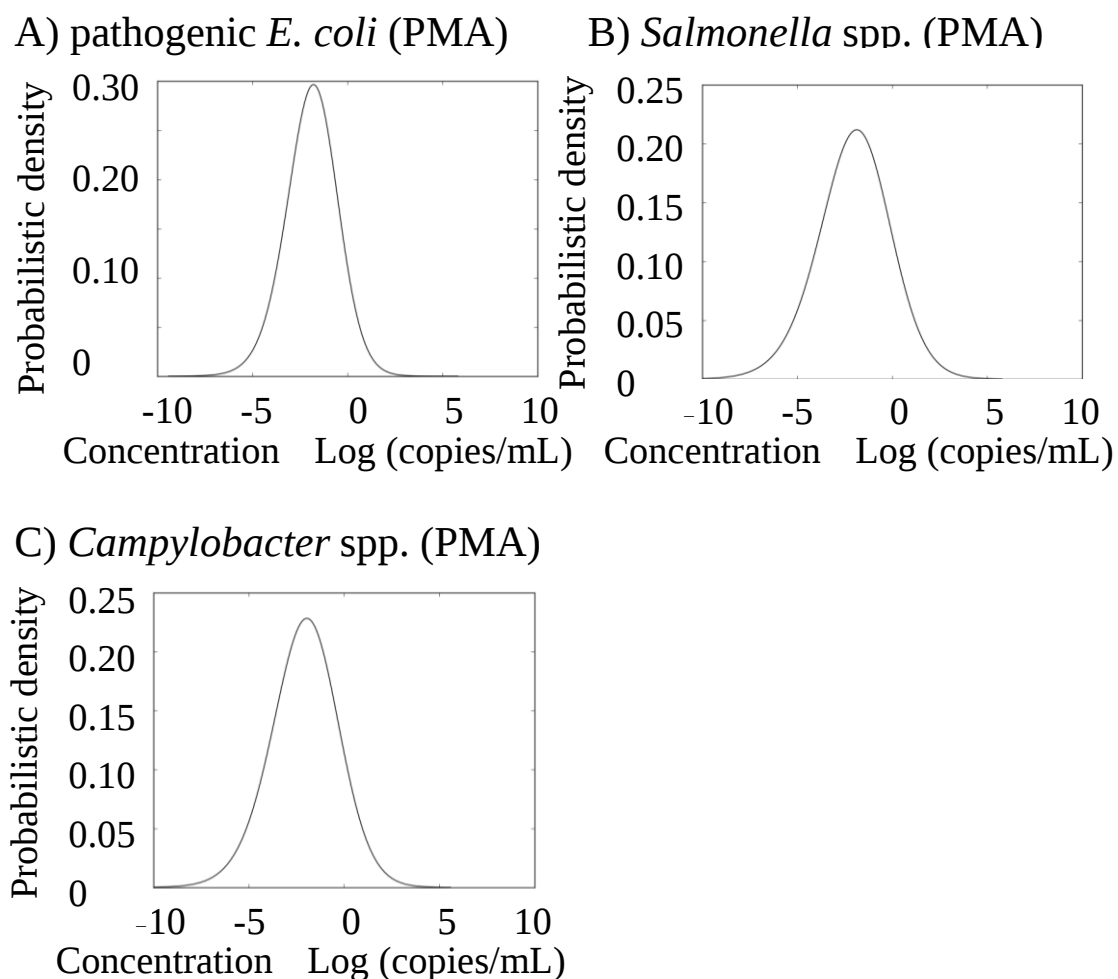


Figure 7.6 Posterior predictive distributions of pathogenic *E. coli*, *Salmonella* spp. and *Campylobacter* spp. based on the data by using qPCR with PMA.

7.4.3 Distribution of the ratio of indicator to pathogen and quantitative relationship between indicator and pathogen concentration

a) Total coliforms and pathogens (W/O PMA)

Figure 7.7 shows the distribution of the ratio of total coliforms to pathogen concentrations that are based on the normal qPCR. Figure 7.8 shows the 1 and 99 percentile of total coliforms versus pathogen concentrations that are based on the normal qPCR.

b) Total coliforms and pathogens (W PMA)

Figure 7.9 shows the distribution of the ratio of total coliforms to pathogen concentrations that are based on the qPCR with PMA. Figure 7.10 shows the 1 and 99 percentile of total coliforms versus pathogen concentrations that are based on the qPCR with PMA.

c) *E. coli* and pathogens (W/O PMA)

Figure 7.11 shows the distribution of the ratio of *E. coli* concentrations to pathogen concentrations that are based on the normal qPCR. Figure 7.12 shows the 1 and 99 percentile of *E. coli* concentrations versus pathogen concentrations that are based on the normal qPCR.

d) *E. coli* and pathogens (W PMA)

Figure 7.13 shows the distribution of the ratio of *E. coli* concentrations to pathogen concentrations that are based on the qPCR with PMA. Figure 7.14 shows the 1 and 99 percentile of *E. coli* concentrations versus pathogen concentrations that are based on the qPCR with PMA.

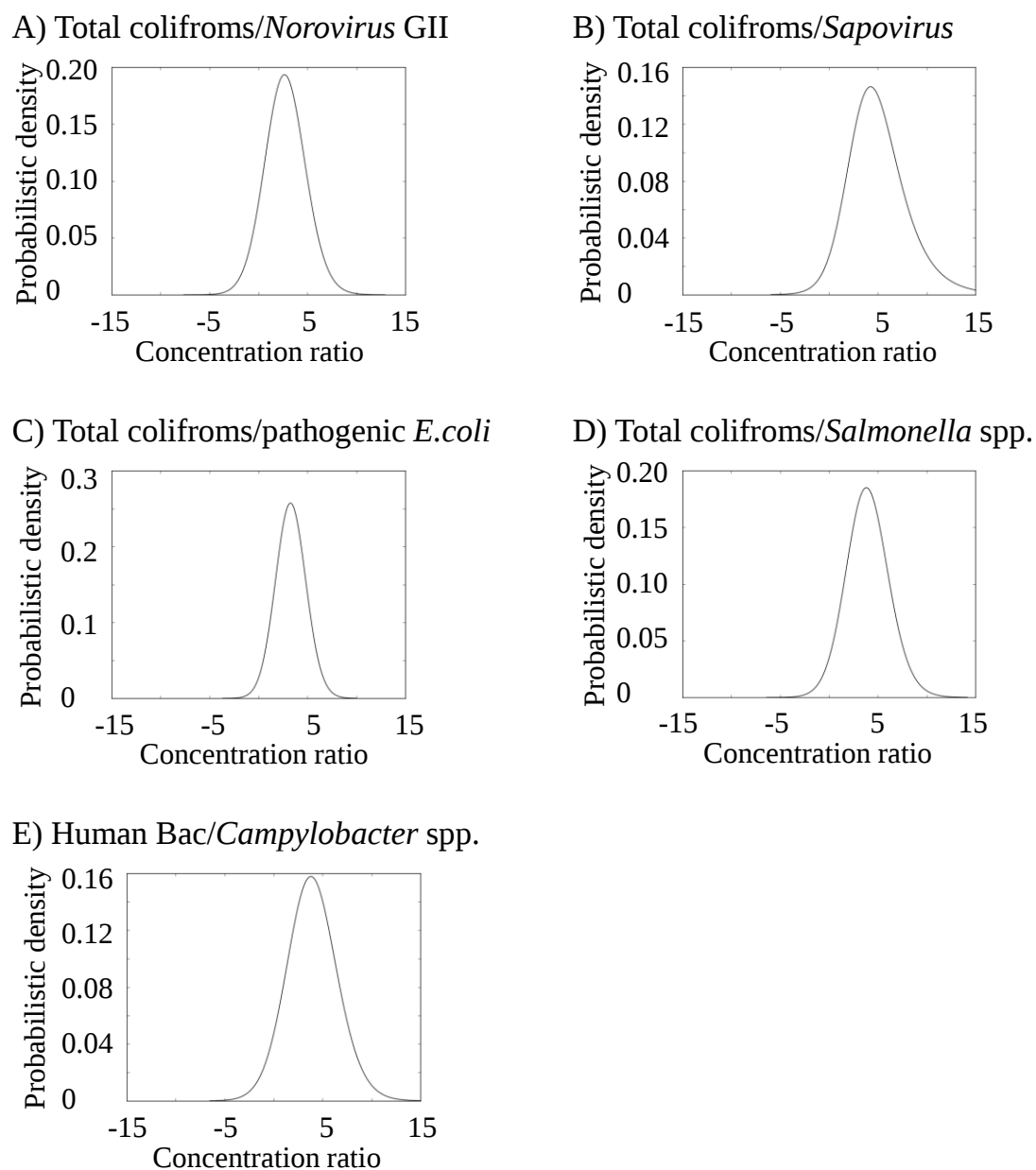


Figure 7.7 Distribution of the ratio of total coliforms to pathogen concentrations that are based on the normal qPCR.

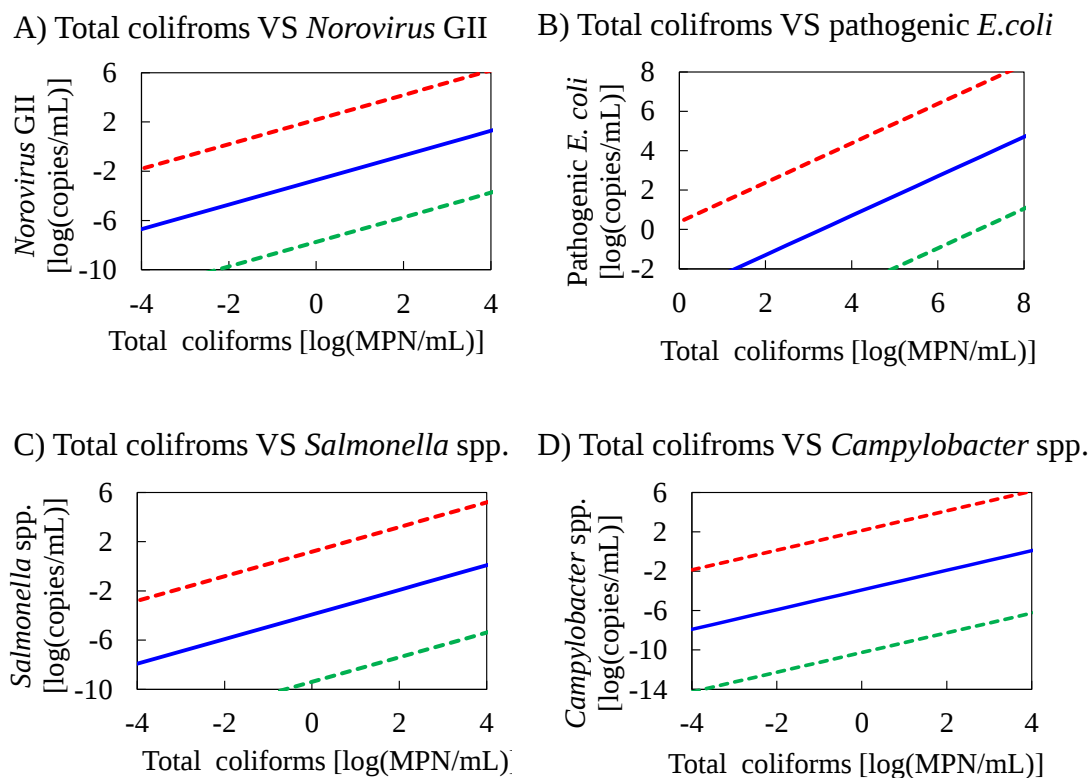


Figure 7.8 1 and 99 percentile of total coliforms versus pathogen concentrations that are based on the normal qPCR (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

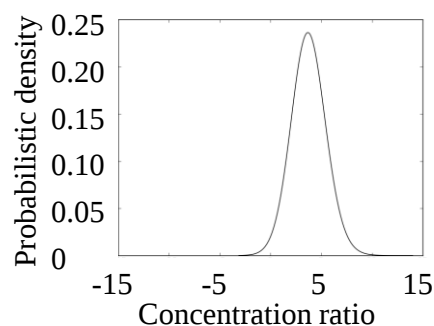
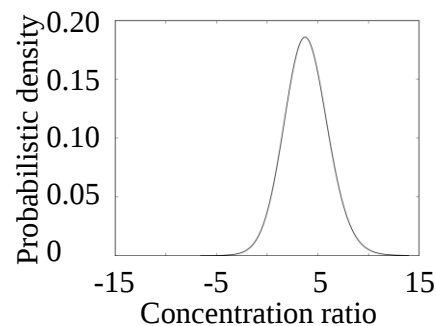
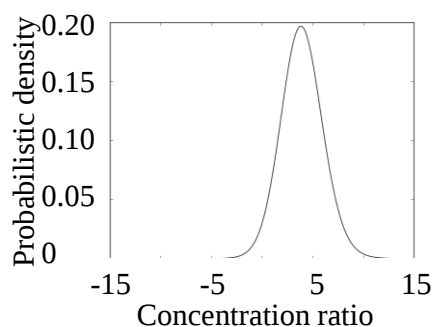
A) Total coliforms/pathogenic *E.coli* (PMA)B) Total coliforms/*Salmonella* spp. (PMA)C) Total coliforms/*Campylobacter* spp. (PMA)

Figure 7.9 Distribution of the ratio of total coliforms to pathogen concentrations that are based on the qPCR with PMA.

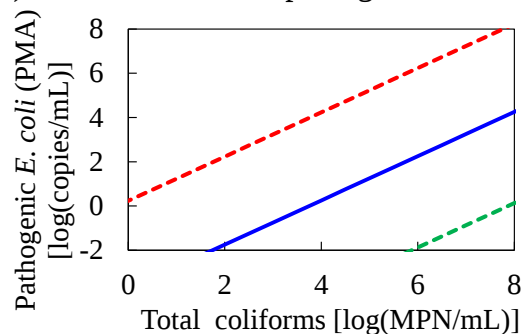
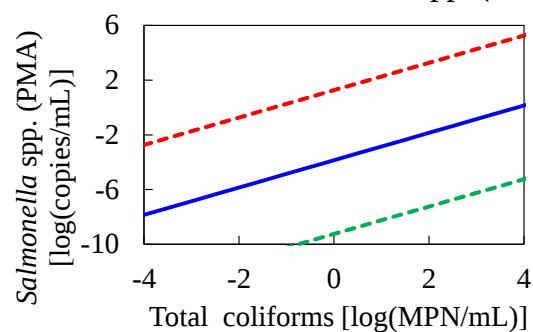
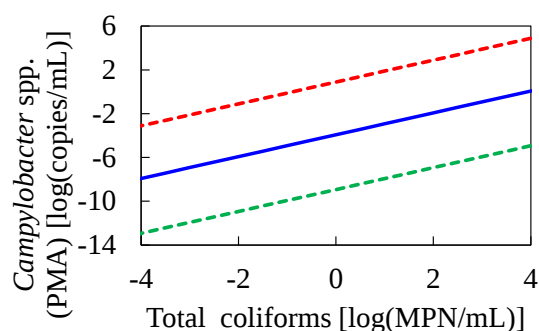
A) Total coliforms VS pathogenic *E.coli* (PMA)B) Total coliforms VS *Salmonella* spp. (PMA)C) Total coliforms VS *Campylobacter* spp. (PMA)

Figure 7.10 1 and 99 percentile of total coliforms versus pathogen concentrations that are based on the qPCR with PMA (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

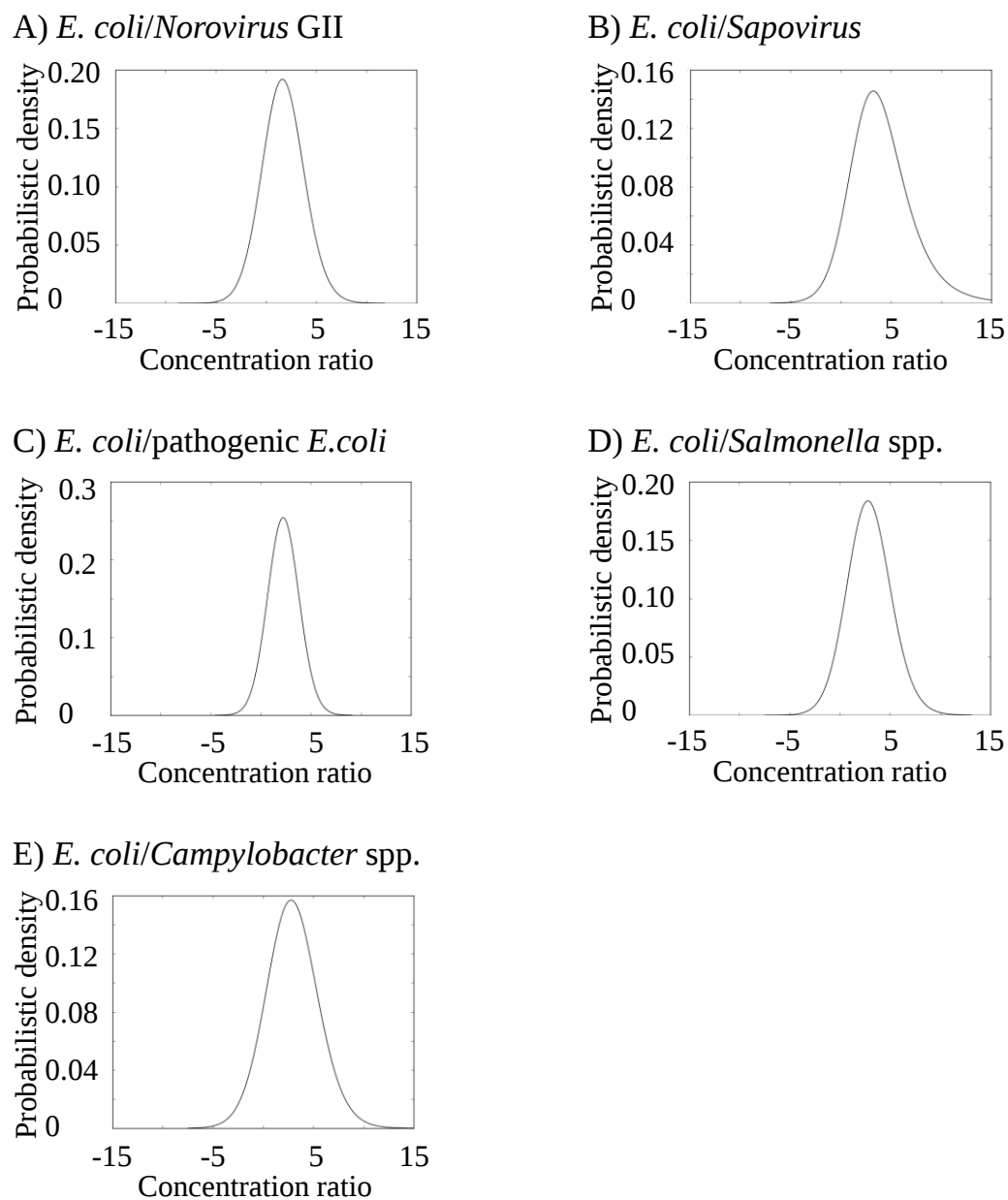


Figure 7.11 Distribution of the ratio of *E. coli* concentrations to pathogen concentrations that are based on the normal qPCR.

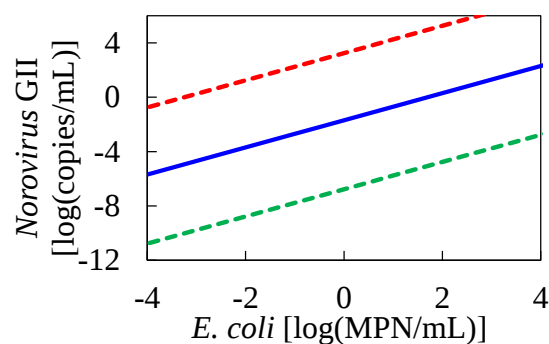
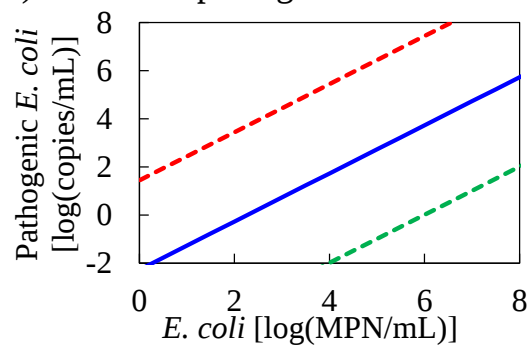
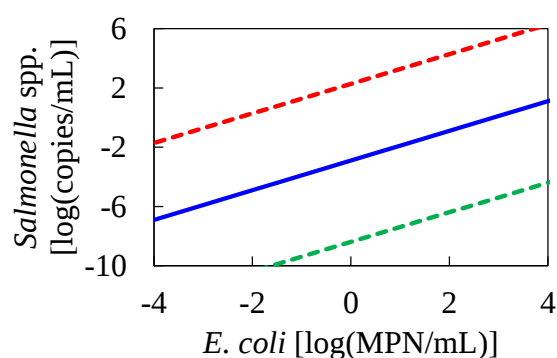
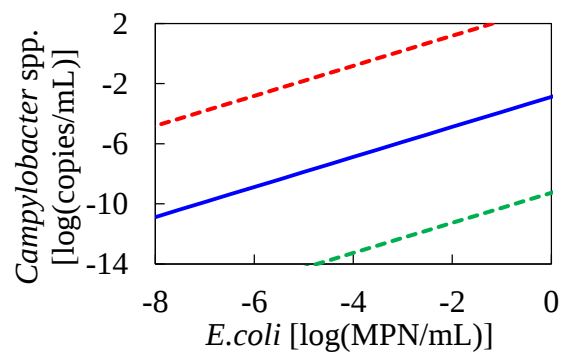
A) *E. coli* VS Norovirus GIIB) *E. coli* VS pathogenic *E. coli*C) *E. coli* VS *Salmonella* spp.D) *E. coli* VS *Campylobacter* spp.

Figure 7.12 1 and 99 percentile of *E. coli* concentrations versus pathogen concentrations that are based on the normal qPCR (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

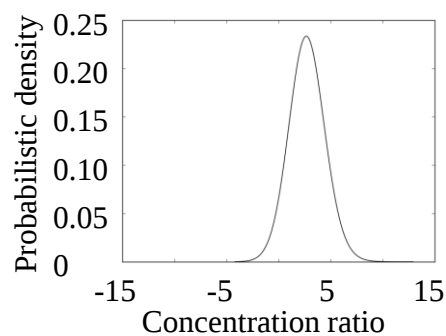
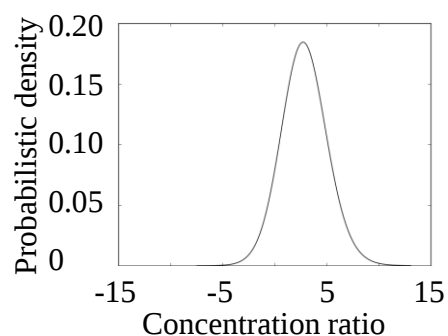
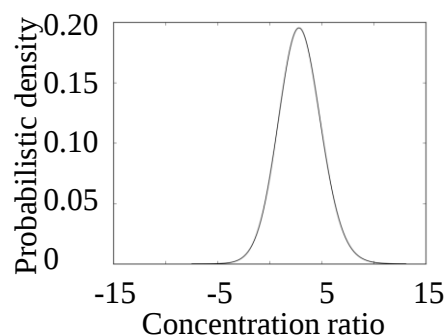
A) *E. coli*/pathogenic *E.coli* (PMA)B) *E. coli*/*Salmonella* spp. (PMA)C) *E. coli*/*Campylobacter* spp. (PMA)

Figure 7.13 Distribution of the ratio of *E. coli* concentrations to pathogen concentrations that are based on the qPCR with PMA.

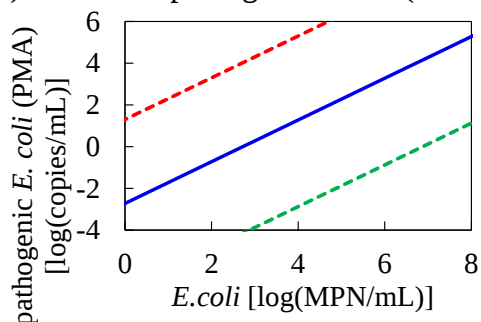
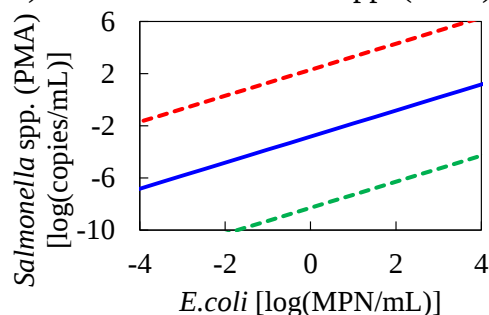
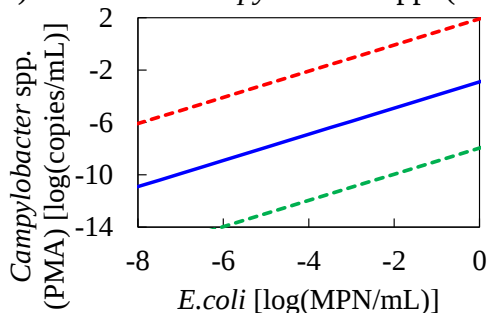
A) *E. coli* VS pathogenic *E.coli* (PMA)B) *E. coli* VS *Salmonella* spp. (PMA)C) *E. coli* VS *Campylobacter* spp. (PMA)

Figure 7.14 1 and 99 percentile of *E. coli* concentrations versus pathogen concentrations that are based on the qPCR with PMA (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

e) Total *Bacteroides-Prevotella* 16S rRNA genetic markers (W/O PMA) and pathogens (W/O PMA)

Figure 7.15 shows the distribution of the ratio of total *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations to pathogen concentrations that are based on the normal qPCR. Figure 7.16 shows the 1 and 99 percentile of total *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the normal qPCR, versus pathogen concentrations that are based on the normal qPCR.

f) Total *Bacteroides-Prevotella* 16S rRNA genetic markers (W/O PMA) and pathogens (W PMA)

Figure 7.17 shows the distribution of the ratio of total *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations that are based on the normal qPCR to pathogen concentrations that are based on the qPCR with PMA. Figure 7.18 shows the 1 and 99 percentile of total *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the normal qPCR, versus pathogen concentrations that are based on the qPCR with PMA.

g) Total *Bacteroides-Prevotella* 16S rRNA genetic markers (W PMA) and pathogens (W/O PMA)

Figure 7.19 shows the distribution of the ratio of total *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations that are based on the qPCR with PMA to pathogen concentrations that are based on the normal qPCR. Figure 7.20 shows the 1 and 99 percentile of total *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the qPCR with PMA, versus pathogen concentrations that are based on the normal qPCR.

h) Total *Bacteroides-Prevotella* 16S rRNA genetic markers (W PMA) and pathogens (W PMA)

Figure 7.21 shows the distribution of the ratio of total *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations to pathogen concentrations that are based on the qPCR with PMA. Figure 7.22 shows the 1 and 99 percentile of total *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the qPCR with PMA, versus pathogen concentrations that are based on the qPCR with PMA.

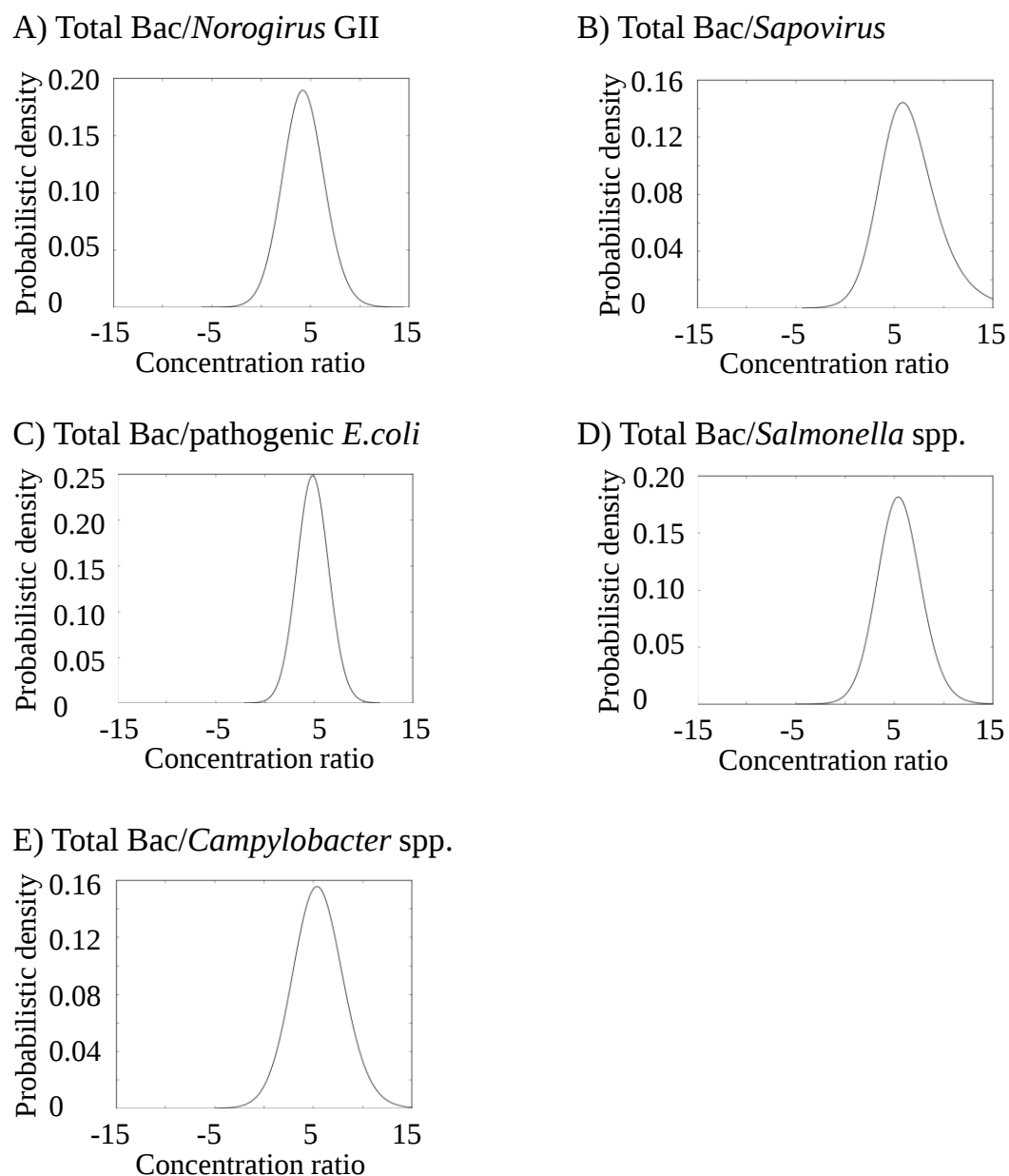


Figure 7.15 Distribution of the ratio of total *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations to pathogen concentrations that are based on the normal qPCR.

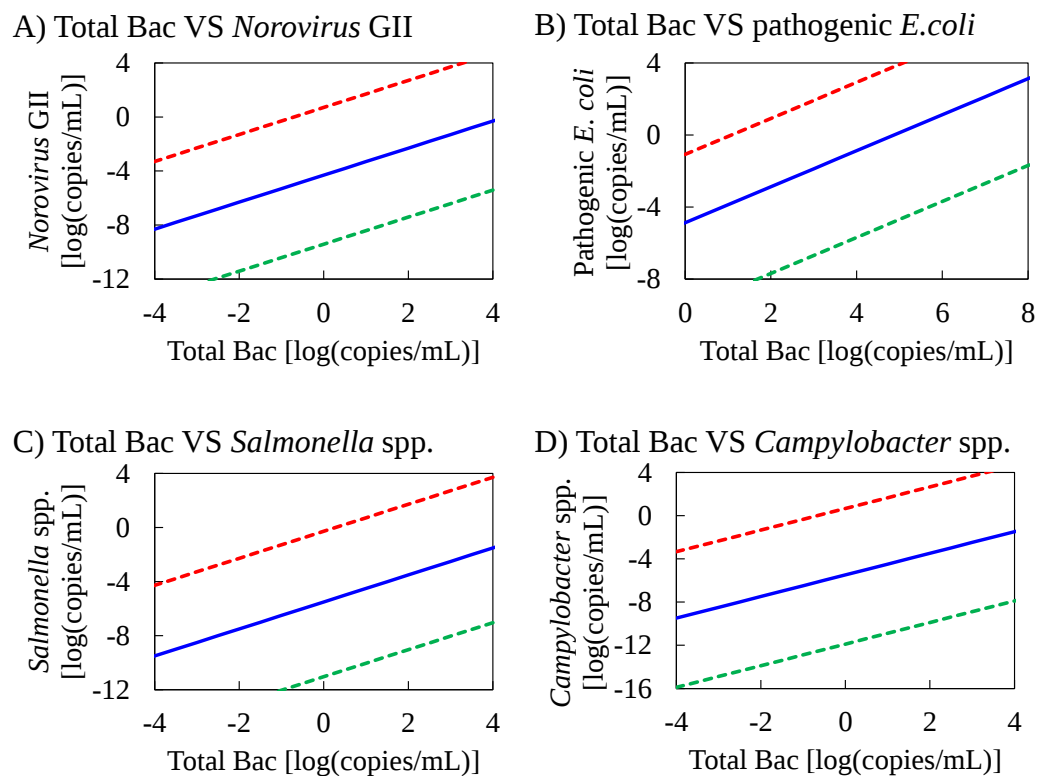


Figure 7.16 1 and 99 percentile of total *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the normal qPCR, versus pathogen concentrations that are based on the normal qPCR (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

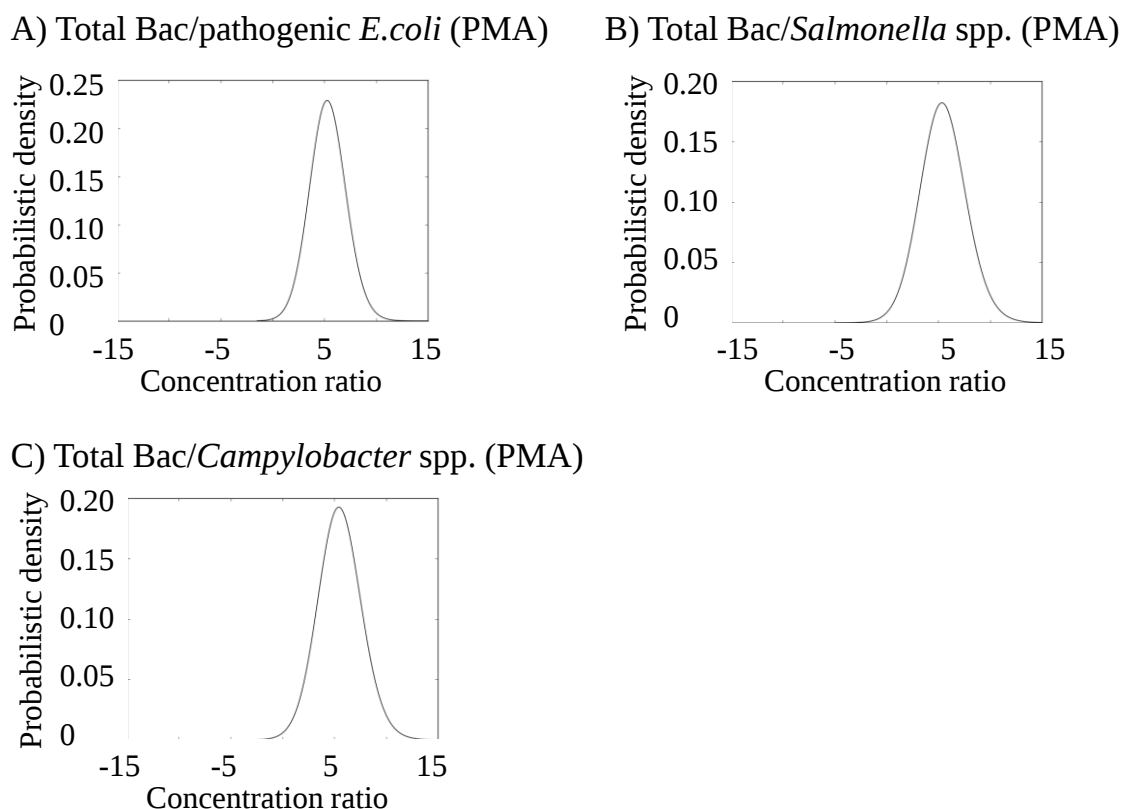


Figure 7.17 Distribution of the ratio of total *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations that are based on the normal qPCR to pathogen concentrations that are based on the qPCR with PMA.

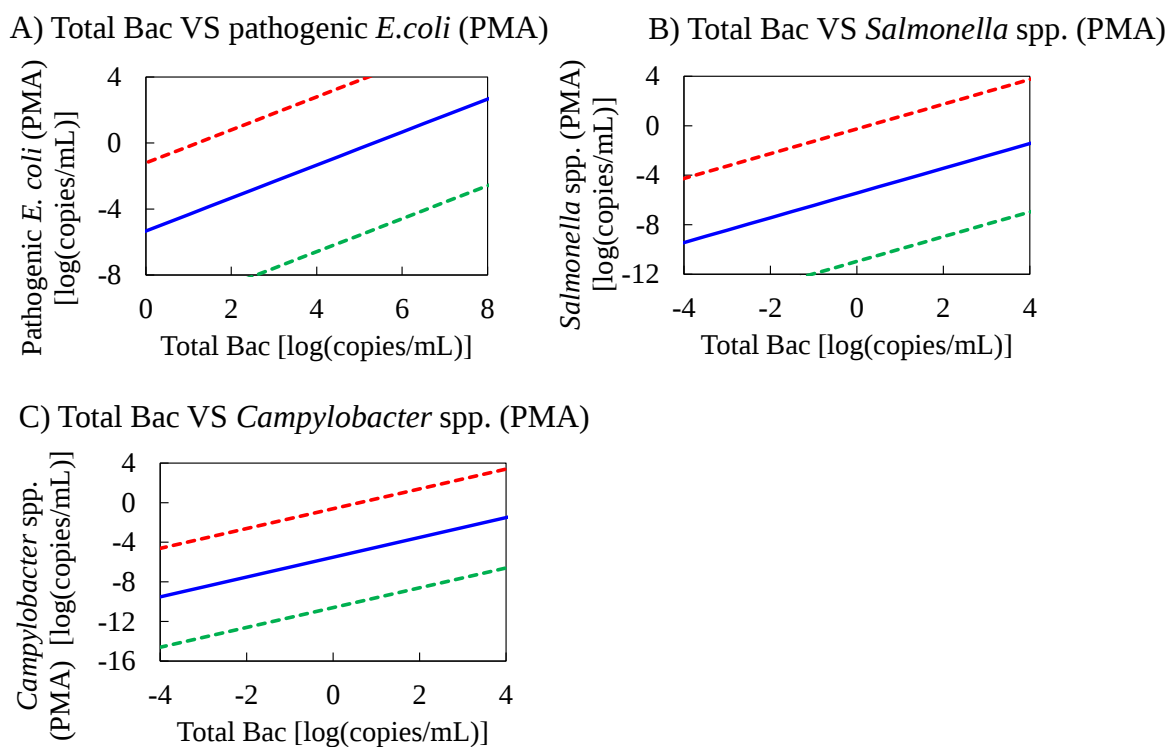


Figure 7.18 1 and 99 percentile of total *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the normal qPCR, versus pathogen concentrations that are based on the qPCR with PMA (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

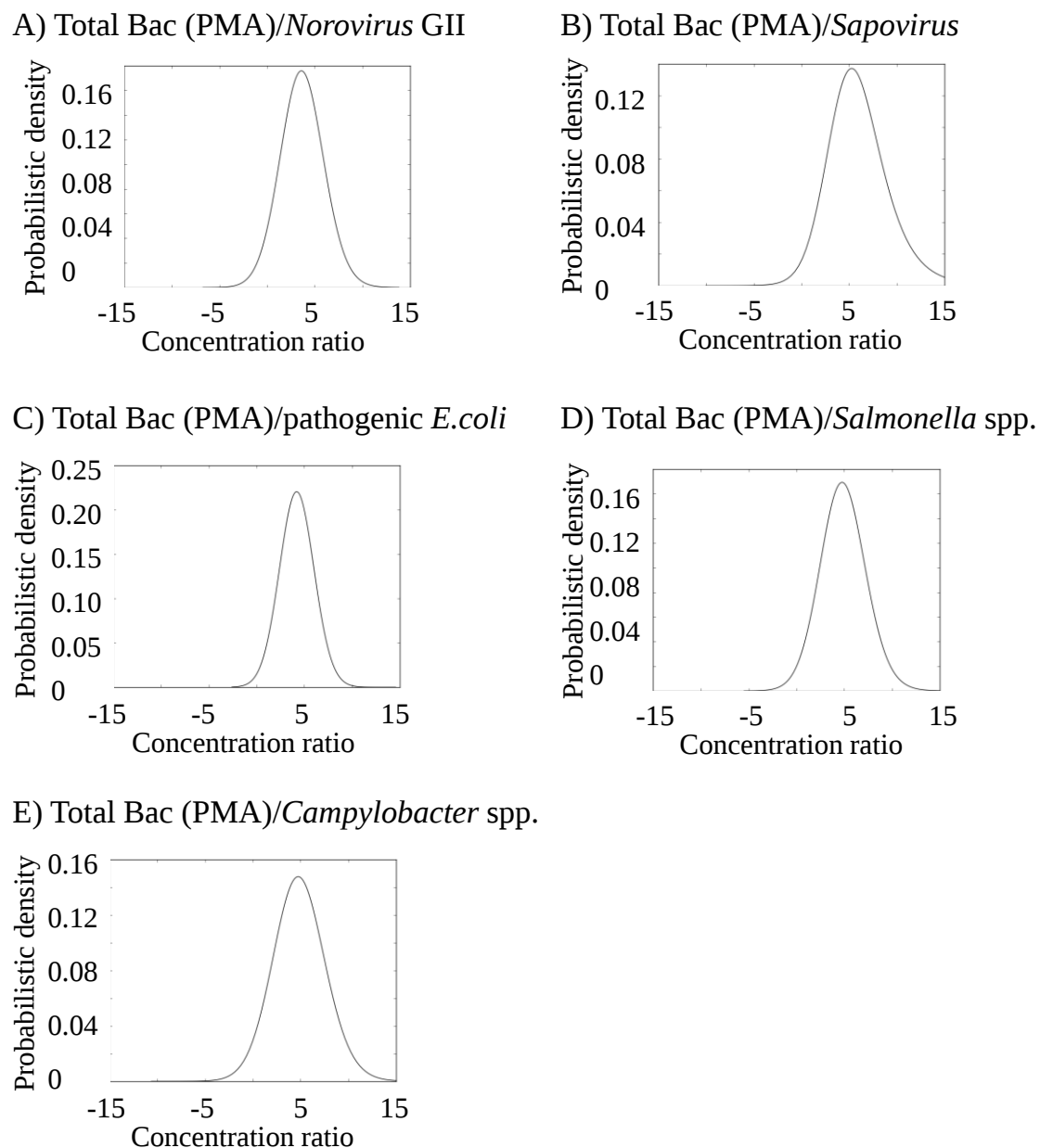


Figure 7.19 Distribution of the ratio of total *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations that are based on the qPCR with PMA to pathogen concentrations that are based on the normal qPCR.

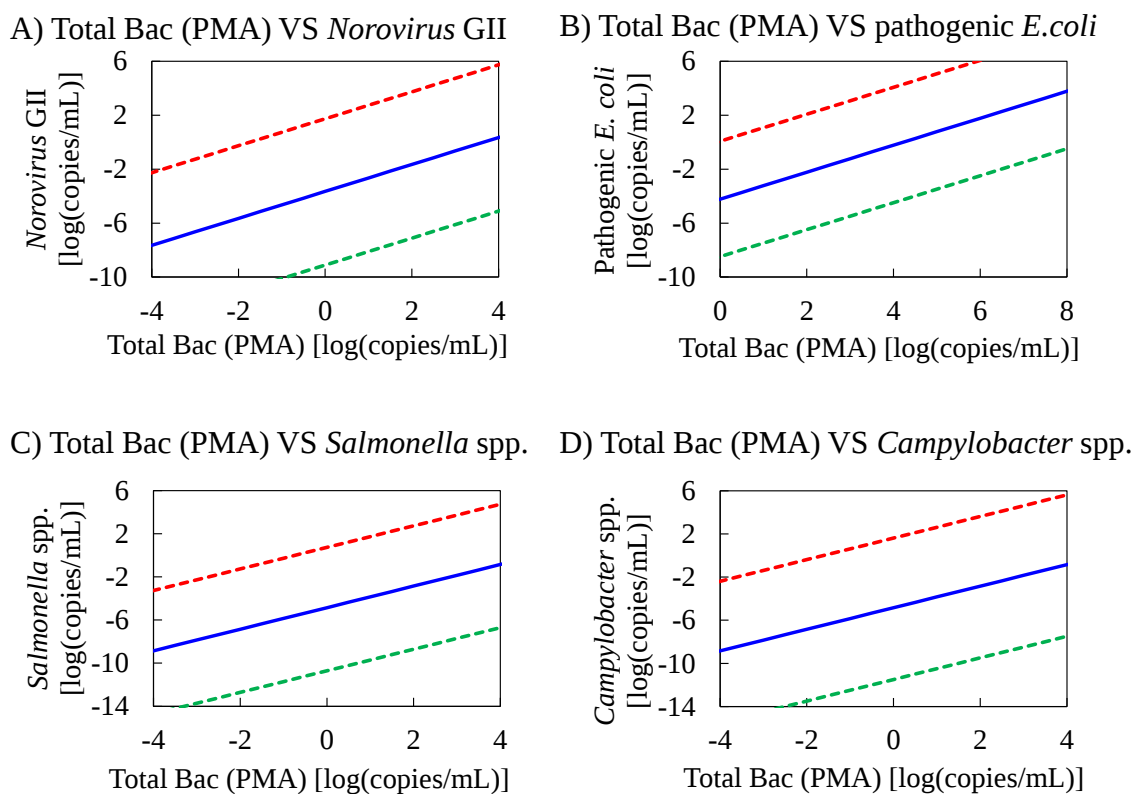
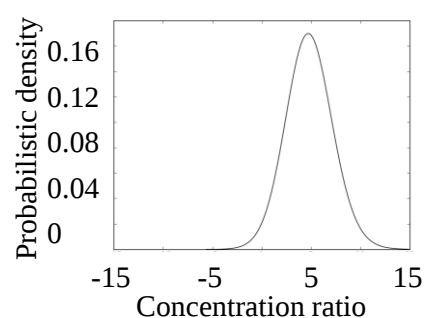
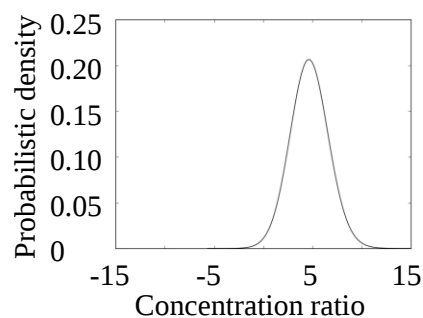


Figure 7.20 1 and 99 percentile of total *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the qPCR with PMA, versus pathogen concentrations that are based on the normal qPCR (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

A) Total Bac (PMA)/pathogenic *E.coli* (PMA) B) Total Bac (PMA)/*Salmonella* spp. (PMA)



C) Total Bac (PMA)/*Campylobacter* spp. (PMA)

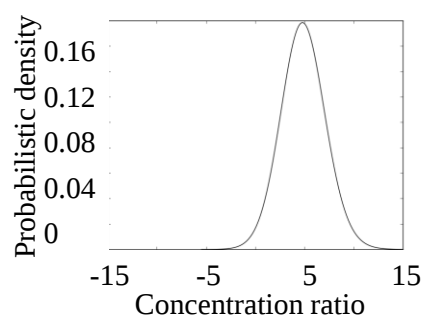
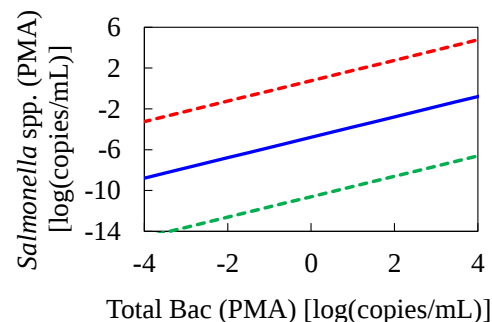
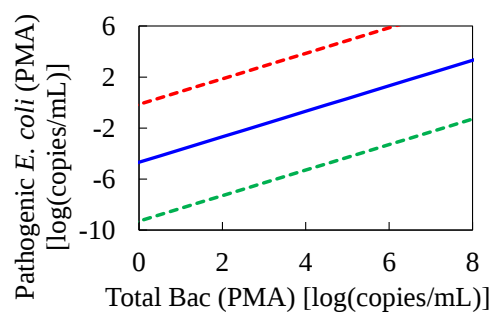


Figure 7.21 Distribution of the ratio of total *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations to pathogen concentrations that are based on the qPCR with PMA.

A) Total Bac (PMA) VS pathogenic *E.coli* (PMA) B) Total Bac (PMA) VS *Salmonella* spp. (PMA)



C) Total Bac (PMA) VS *Campylobacter* spp. (PMA)

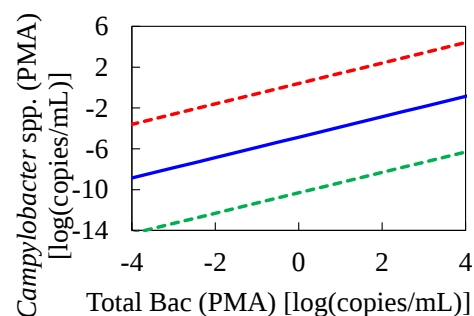


Figure 7.22 1 and 99 percentile of total *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the qPCR with PMA, versus pathogen concentrations that are based on the qPCR with PMA (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

i) Human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers (W/O PMA) and pathogens (W/O PMA)

Figure 7.23 shows the distribution of the ratio of human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations to pathogen concentrations that are based on the normal qPCR. Figure 7.24 shows the 1 and 99 percentile of human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the normal qPCR, versus pathogen concentrations that are based on the normal qPCR.

j) Human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers (W/O PMA) and pathogens (W PMA)

Figure 7.25 shows the distribution of the ratio of human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations that are based on the normal qPCR to pathogen concentrations that are based on the qPCR with PMA. Figure 7.26 shows the 1 and 99 percentile of human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the normal qPCR, versus pathogen concentrations that are based on the qPCR with PMA.

k) Human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers (W PMA) and pathogens (W/O PMA)

Figure 7.27 shows the distribution of the ratio of human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations that are based on the qPCR with PMA to pathogen concentrations that are based on the normal qPCR. Figure 7.28 shows the 1 and 99 percentile of human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the qPCR with PMA, versus pathogen concentrations that are based on the normal qPCR.

l) Human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers (W PMA) and pathogens (W PMA)

Figure 7.29 shows the distribution of the ratio of human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations to pathogen concentrations that are based on the qPCR with PMA. Figure 7.30 shows the 1 and 99 percentile of human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the qPCR with PMA, versus pathogen concentrations that are based on the qPCR with PMA.

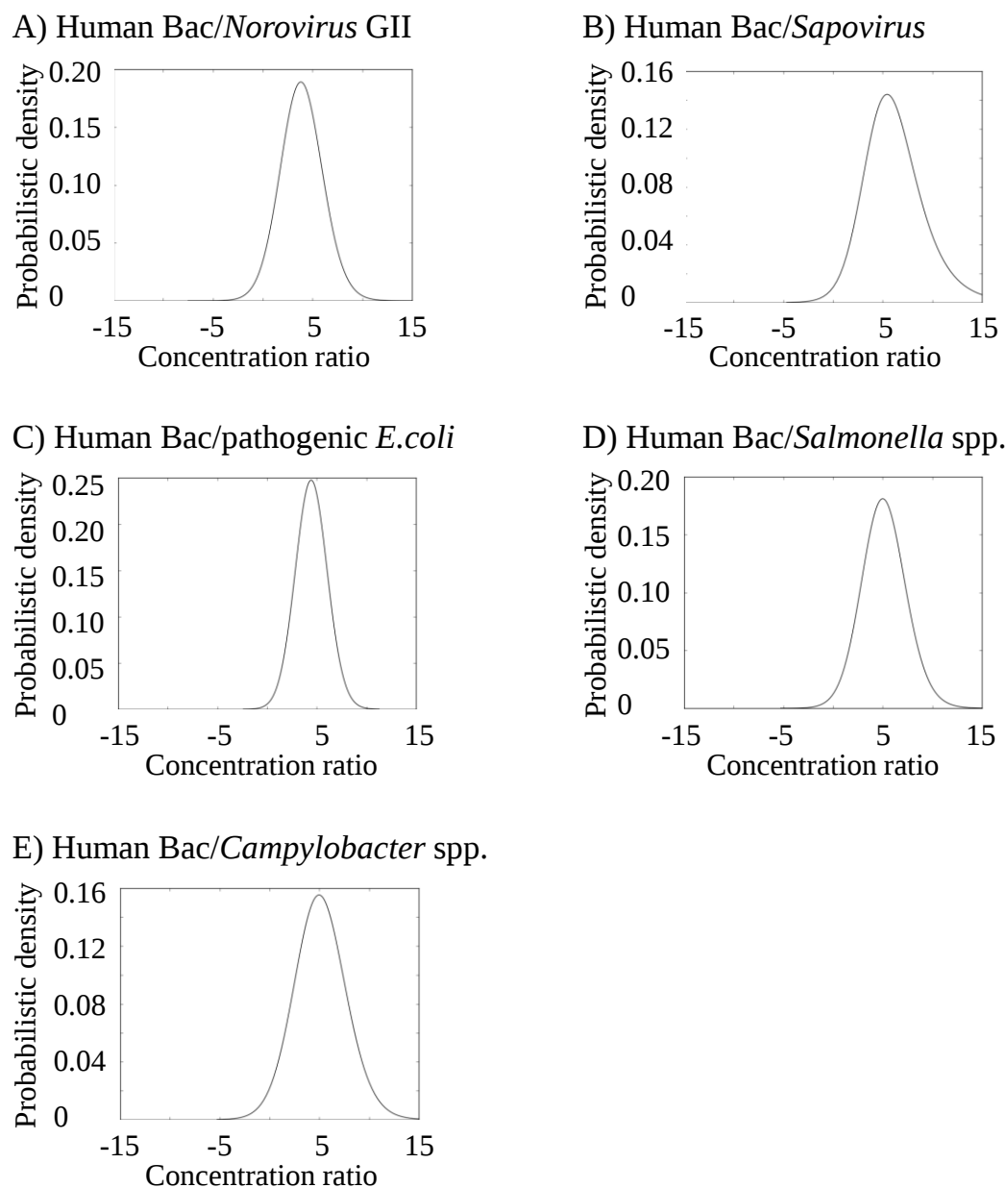


Figure 7.23 Distribution of the ratio of human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations to pathogen concentrations that are based on the normal qPCR.

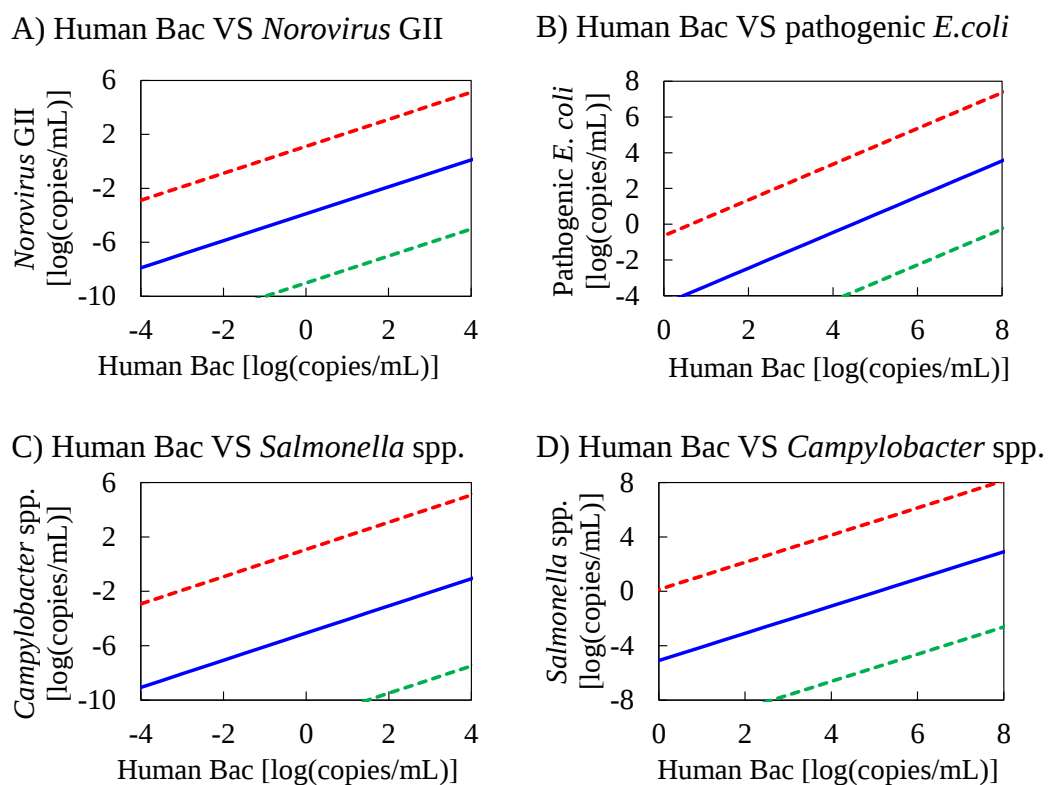


Figure 7.24 1 and 99 percentile of human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the normal qPCR, versus pathogen concentrations that are based on the normal qPCR (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

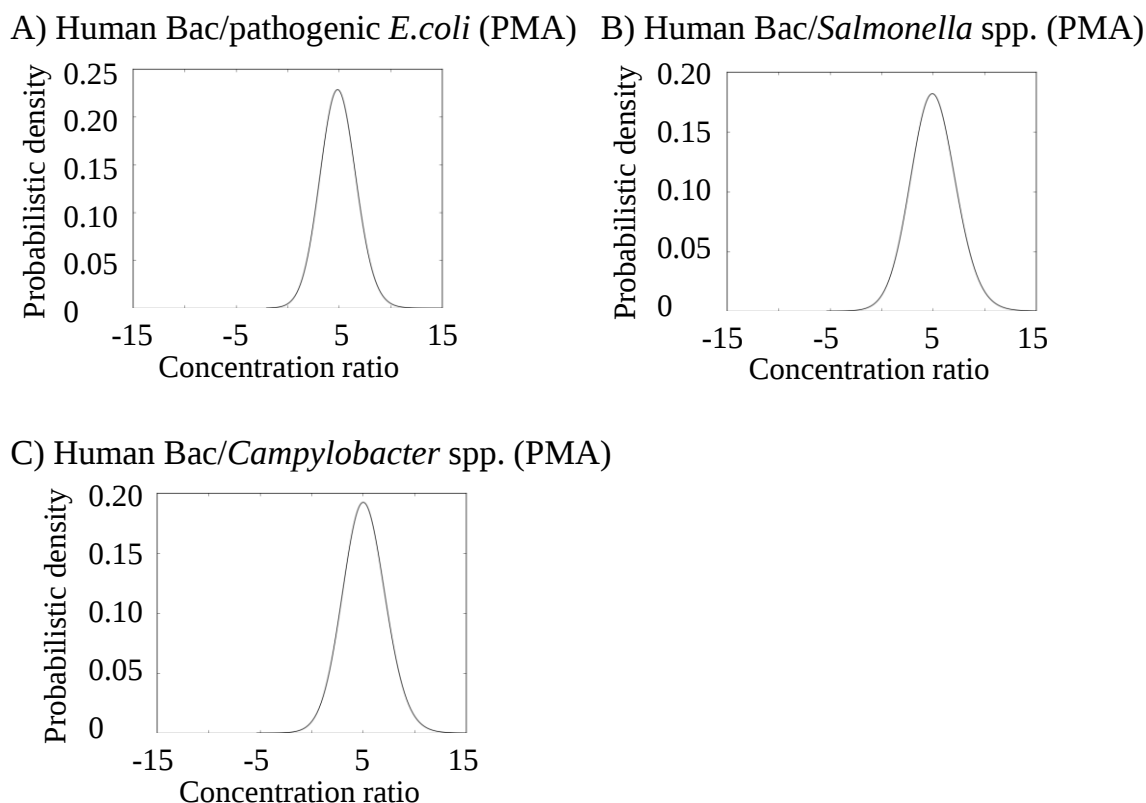


Figure 7.25 Distribution of the ratio of human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations that are based on the normal qPCR to pathogen concentrations that are based on the qPCR with PMA.

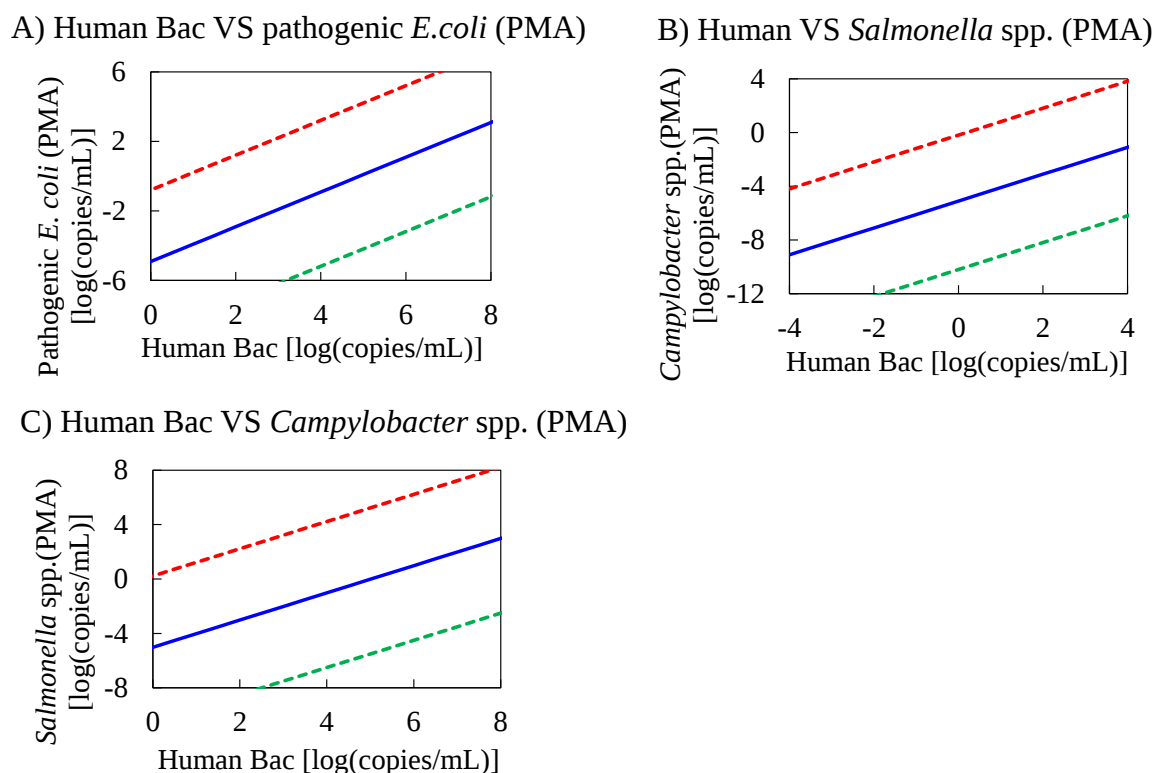


Figure 7.26 1 and 99 percentile of human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the normal qPCR, versus pathogen concentrations that are based on the qPCR with PMA (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

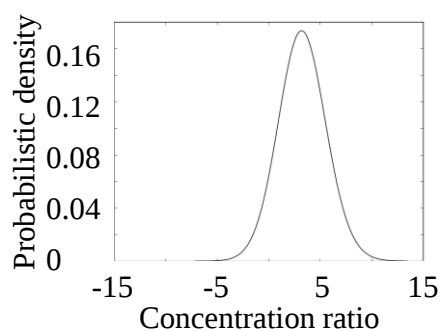
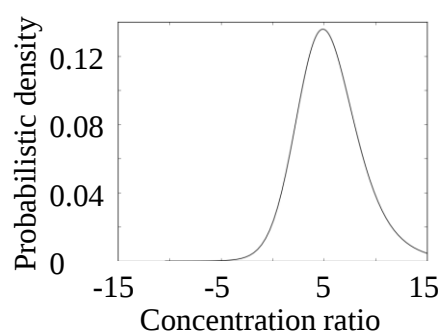
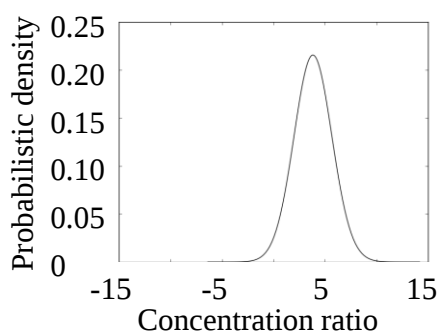
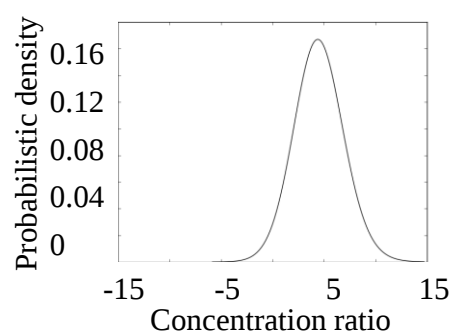
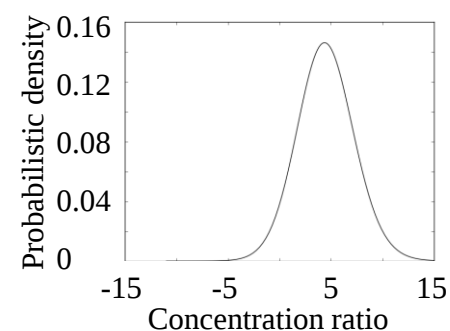
A) Human Bac (PMA)/*Norovirus* GIIB) Human Bac (PMA)/*Sapovirus*C) Human Bac (PMA)/pathogenic *E.coli*D) Human Bac (PMA)/*Salmonella* spp.E) Human Bac (PMA)/*Campylobacter* spp.

Figure 7.27 Distribution of the ratio of human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations that are based on the qPCR with PMA to pathogen concentrations that are based on the normal qPCR.

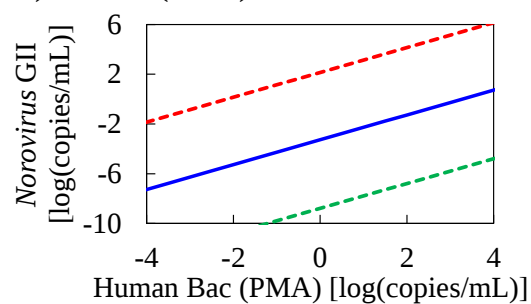
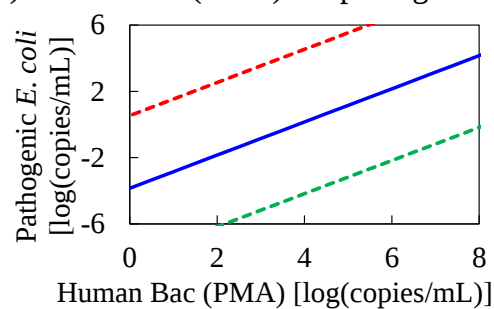
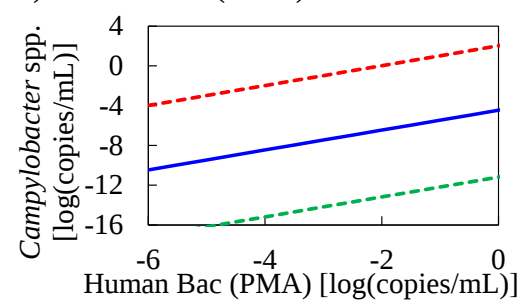
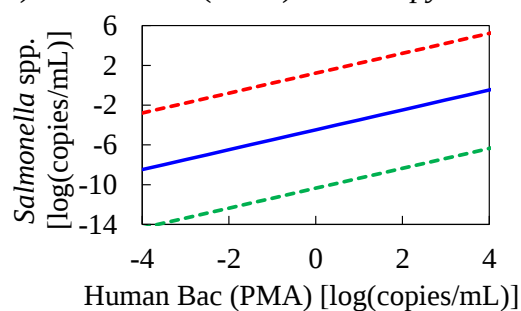
A) Human (PMA) VS *Norovirus* GIIB) Human Bac (PMA) VS pathogenic *E.coli*C) Human Bac (PMA) VS *Salmonella* spp.D) Human Bac (PMA) VS *Campylobacter* spp.

Figure 7.28 1 and 99 percentile of human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the qPCR with PMA, versus pathogen concentrations that are based on the normal qPCR (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

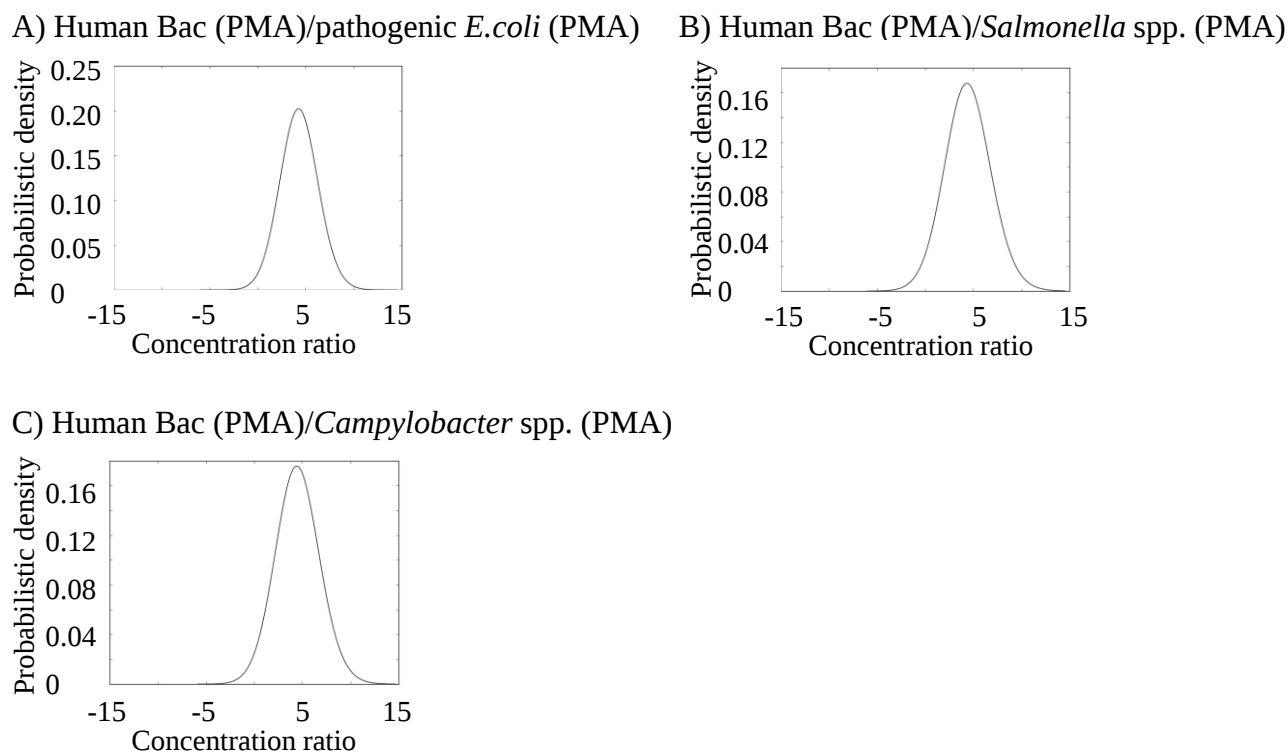


Figure 7.29 Distribution of the ratio of human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker concentrations to pathogen concentrations that are based on the qPCR with PMA

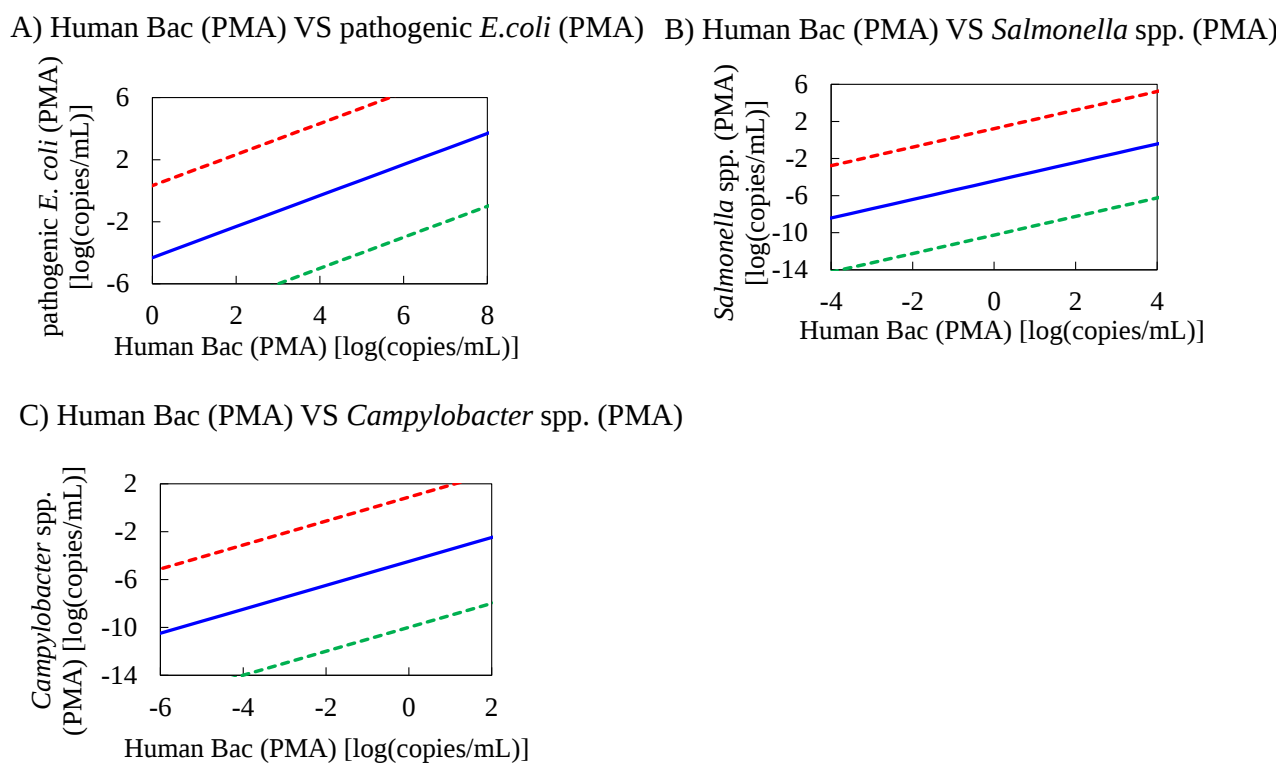


Figure 7.30 1 and 99 percentile of human-specific *Bacteroides-Prevotella* 16S rRNA genetic markers that are based on the qPCR with PMA, versus pathogen concentrations that are based on the qPCR with PMA (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

m) IBU and pathogens (W/O PMA)

Figure 7.31 shows the distribution of the ratio of IBU concentrations to pathogen concentrations that are based on the normal qPCR. Figure 7.32 shows the 1 and 99 percentile of IBU concentration versus pathogen concentrations that are based on the normal qPCR.

n) IBU and pathogens (W PMA)

Figure 7.33 shows the distribution of the ratio of IBU concentrations to pathogen concentrations that are based on the qPCR with PMA. Figure 7.34 shows the 1 and 99 percentile of IBU concentration versus pathogen concentrations that are based on the qPCR with PMA.

o) KPF and pathogens (W/O PMA)

Figure 7.35 shows the distribution of the ratio of KPF concentrations to pathogen concentrations that are based on the normal qPCR. Figure 7.36 shows the 1 and 99 percentile of KPF concentration versus pathogen concentrations that are based on the normal qPCR.

p) KPF and pathogens (W PMA)

Figure 7.37 shows the distribution of the ratio of KPF concentrations to pathogen concentrations that are based on the qPCR with PMA. Figure 7.38 shows the 1 and 99 percentile of KPF concentration versus pathogen concentrations that are based on the qPCR with PMA.

q) MFA and pathogens (W/O PMA)

Figure 7.39 shows the distribution of the ratio of MFA concentrations to pathogen concentrations that are based on the normal qPCR. Figure 7.40 shows the 1 and 99 percentile of MFA concentration versus pathogen concentrations that are based on the normal qPCR.

r) MFA and pathogens (W PMA)

Figure 7.41 shows the distribution of the ratio of MFA concentrations to pathogen concentrations that are based on the qPCR with PMA. Figure 7.42 shows the 1 and 99 percentile of MFA concentration versus pathogen concentrations that are based on the qPCR with PMA.

s) CAB and pathogens (W/O PMA)

Figure 7.43 shows the distribution of the ratio of CAB concentrations to pathogen concentrations that are based on the normal qPCR. Figure 7.44 shows the 1 and 99 percentile of CAB concentration versus pathogen concentrations that are based on the normal qPCR.

t) CAB and pathogens (W PMA)

Figure 7.45 shows the distribution of the ratio of CAB concentrations to pathogen concentrations that are based on the qPCR with PMA. Figure 7.46 shows the 1 and 99 percentile of CAB concentration versus pathogen concentrations that are based on the qPCR with PMA.

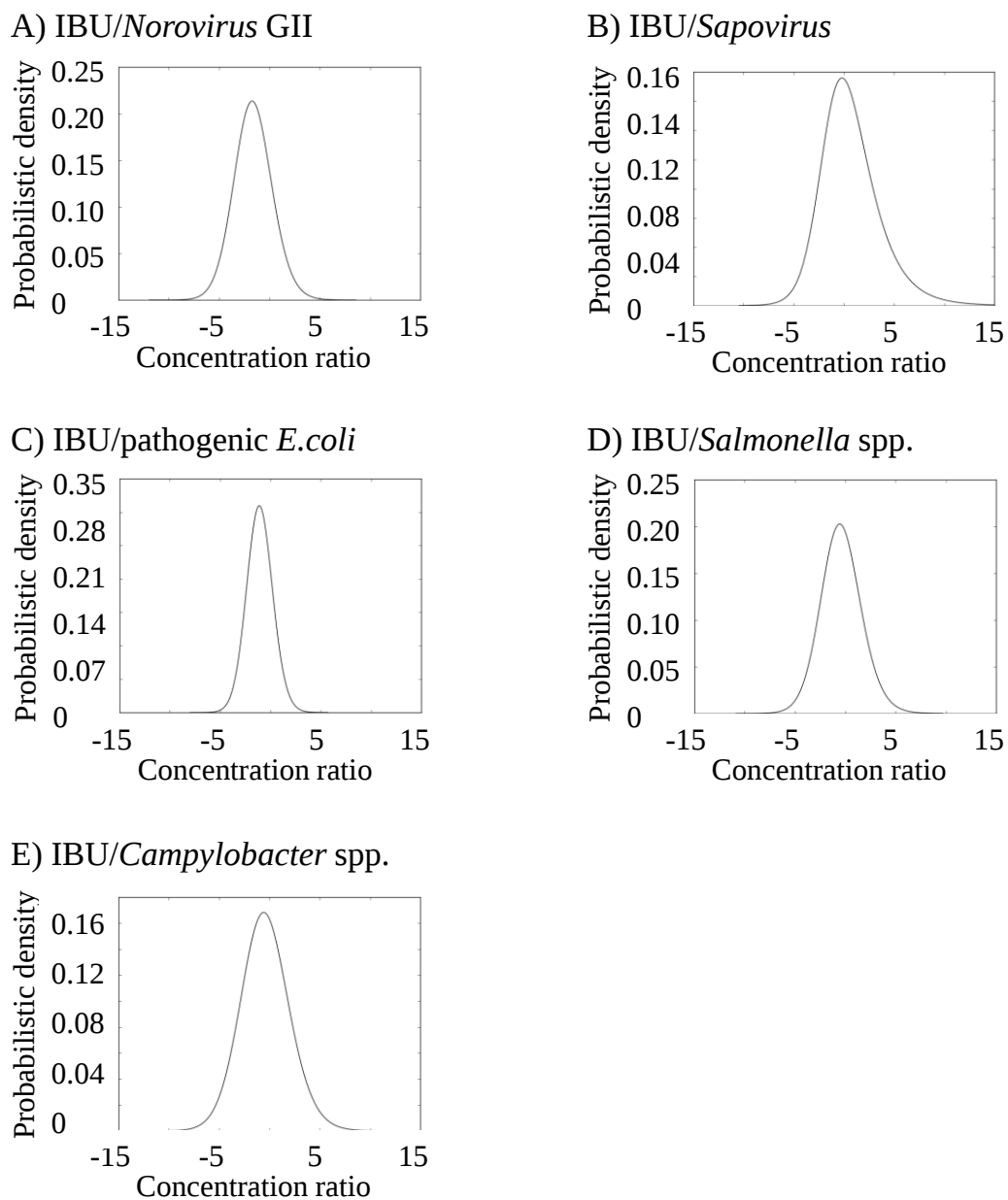


Figure 7.31 Distribution of the ratio of the ratio of IBU concentrations to pathogen concentrations that are based on the normal qPCR.

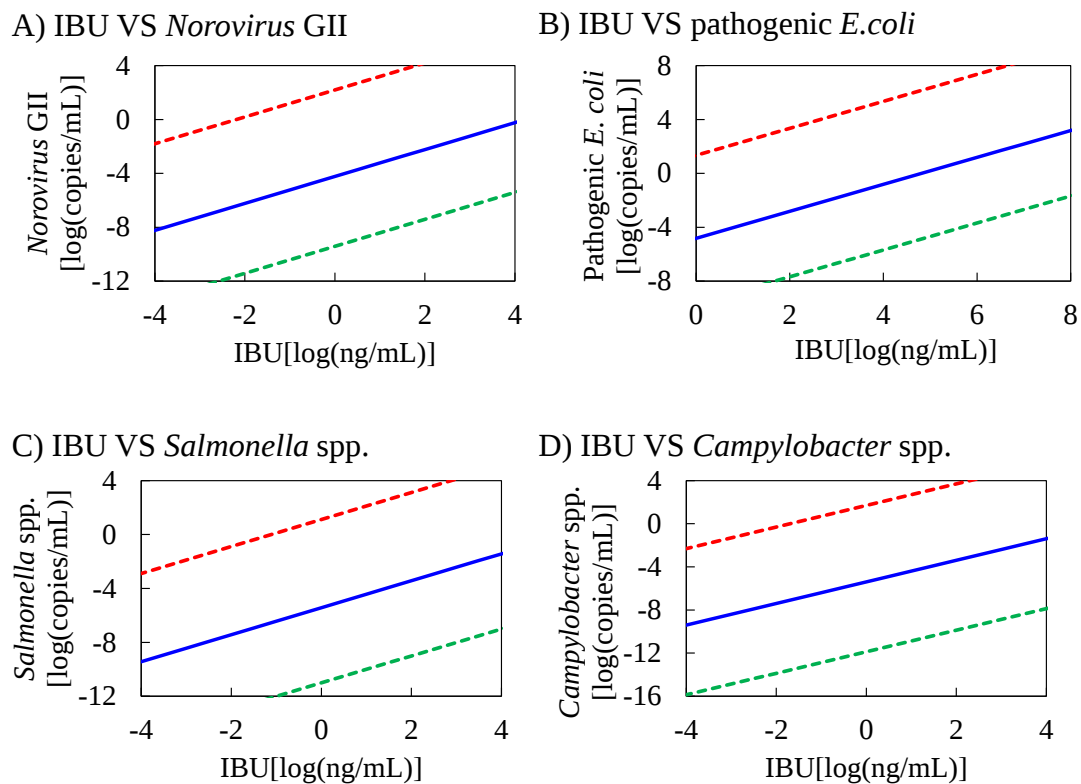


Figure 7.32 1 and 99 percentile of IBU concentration versus pathogen concentrations that are based on the normal qPCR (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

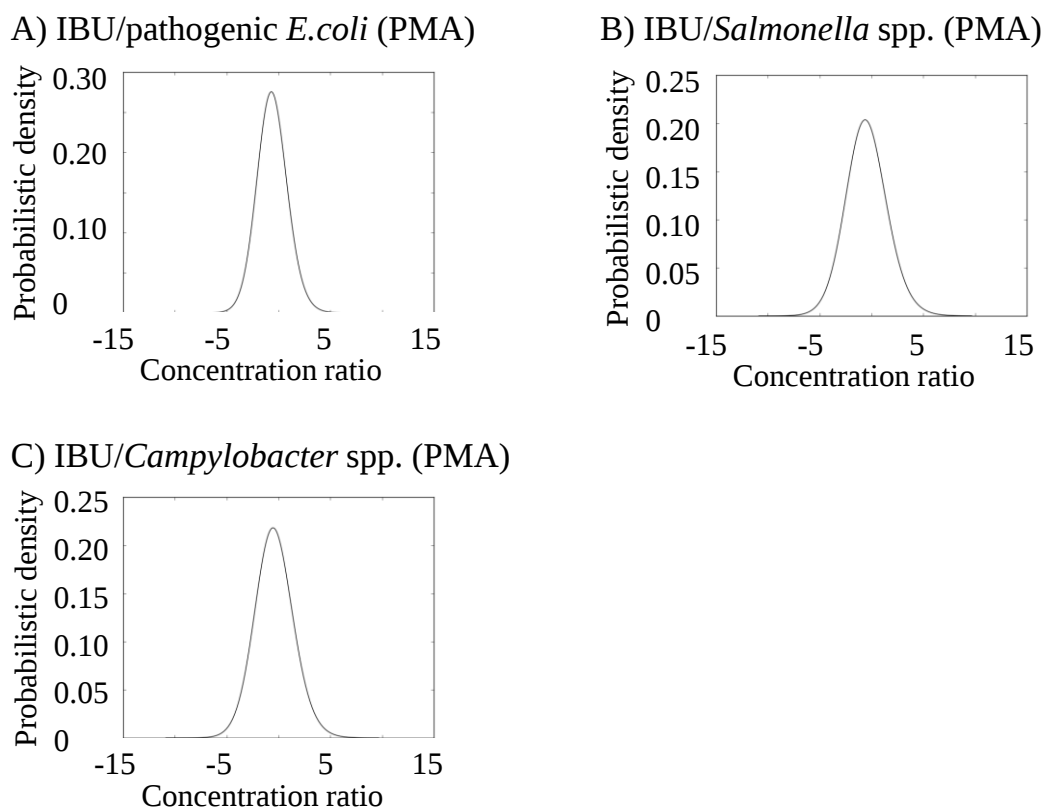


Figure 7.33 Distribution of the ratio of IBU concentrations to pathogen concentrations that are based on the qPCR with PMA.

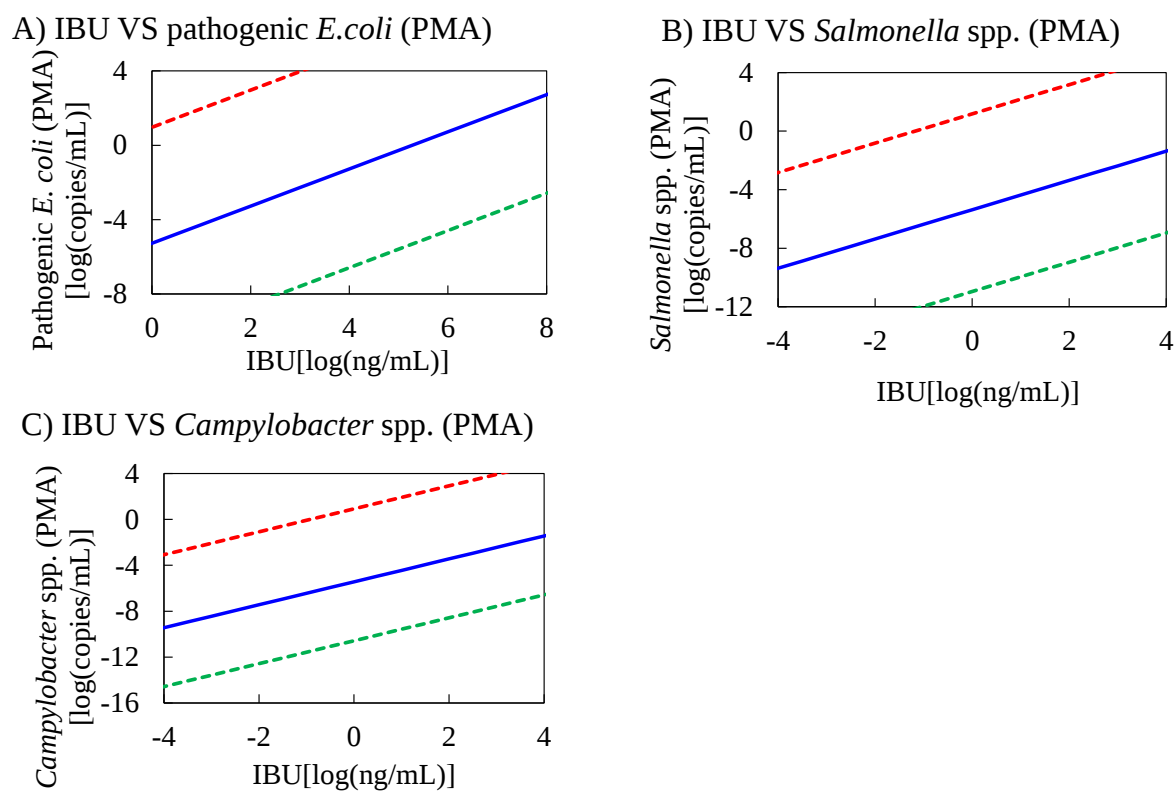


Figure 7.34 1 and 99 percentile of IBU concentration versus pathogen concentrations that are based on the qPCR with PMA (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

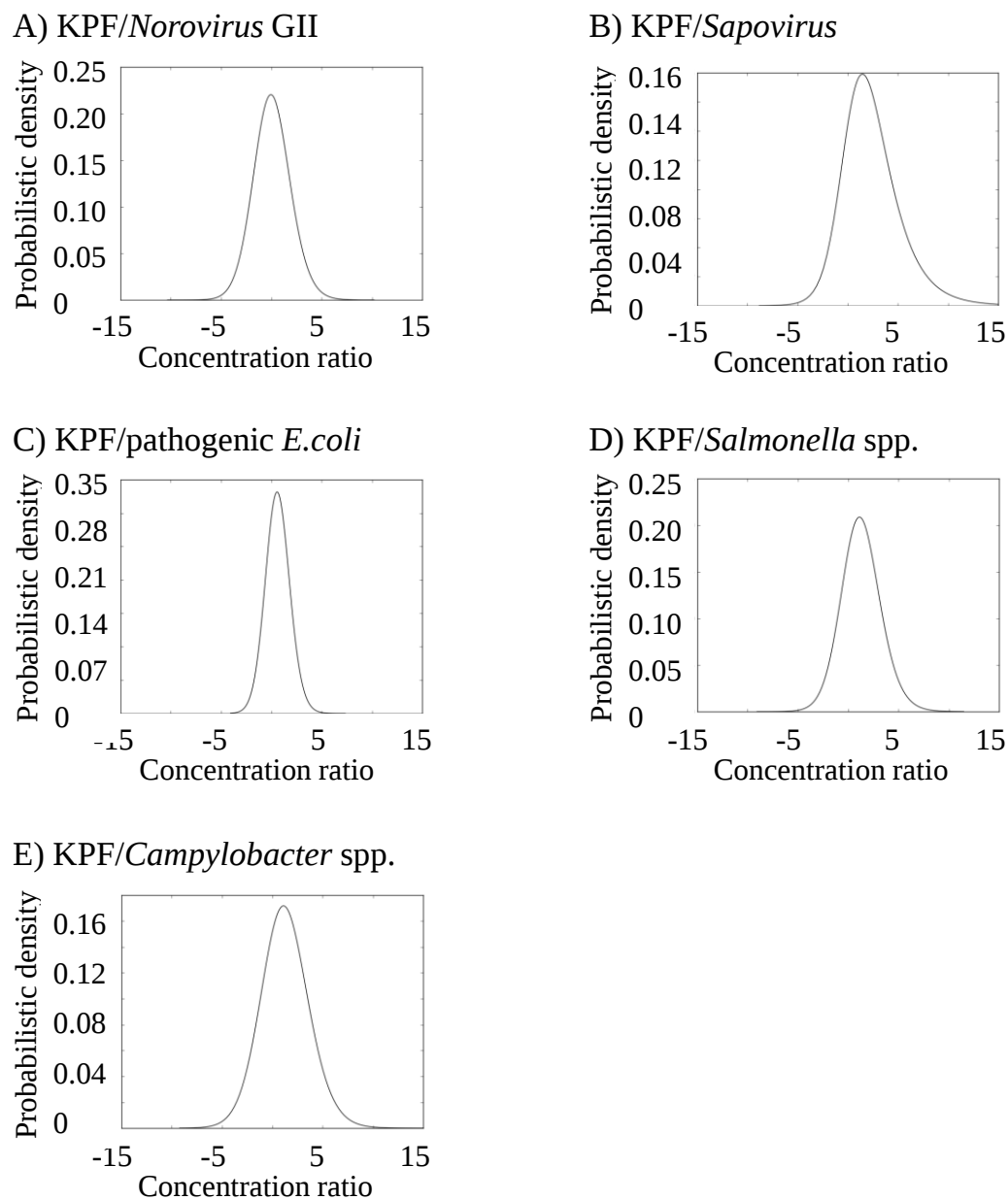


Figure 7.35 Distribution of the ratio of KPF concentrations to pathogen concentrations that are based on the normal qPCR.

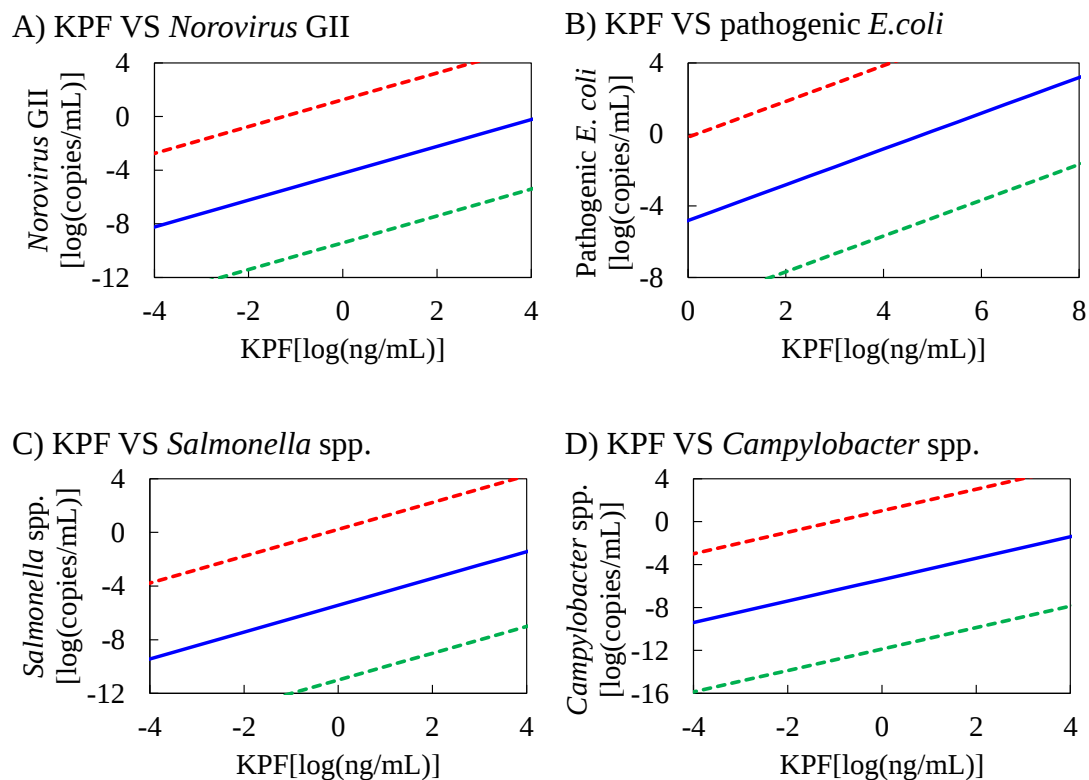


Figure 7.36 1 and 99 percentile of KPF concentration versus pathogen concentrations that are based on the normal qPCR (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

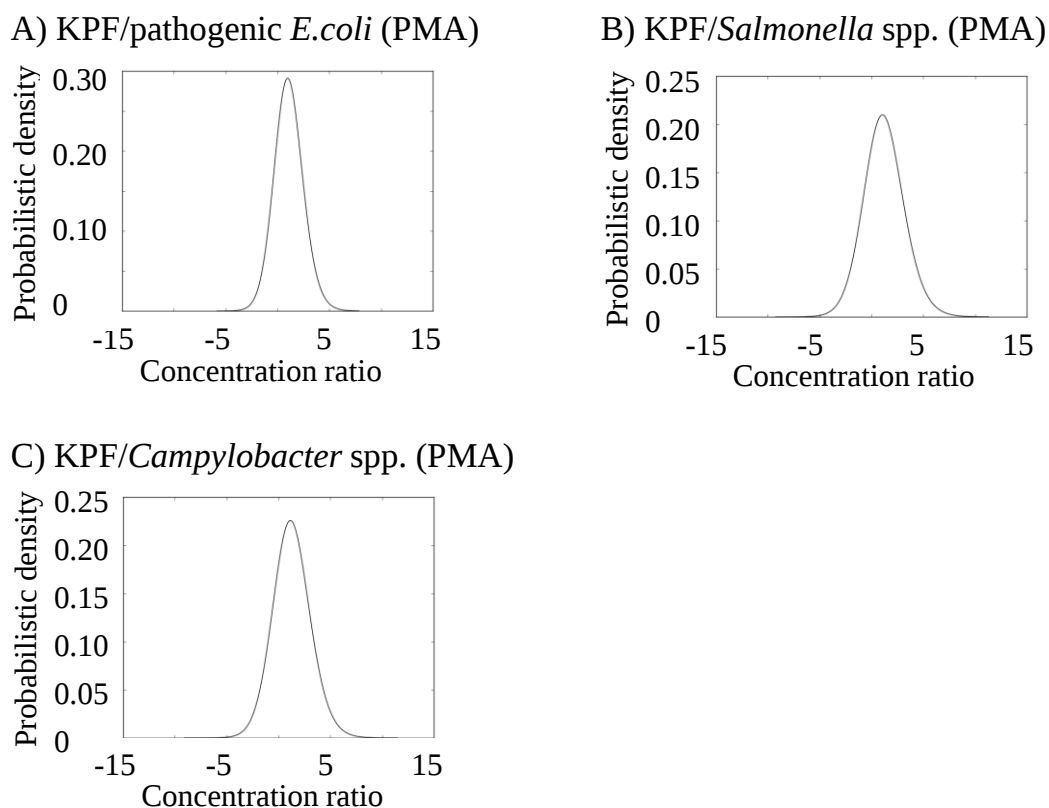


Figure 7.37 Distribution of the ratio of KPF concentrations to pathogen concentrations that are based on the qPCR with PMA.

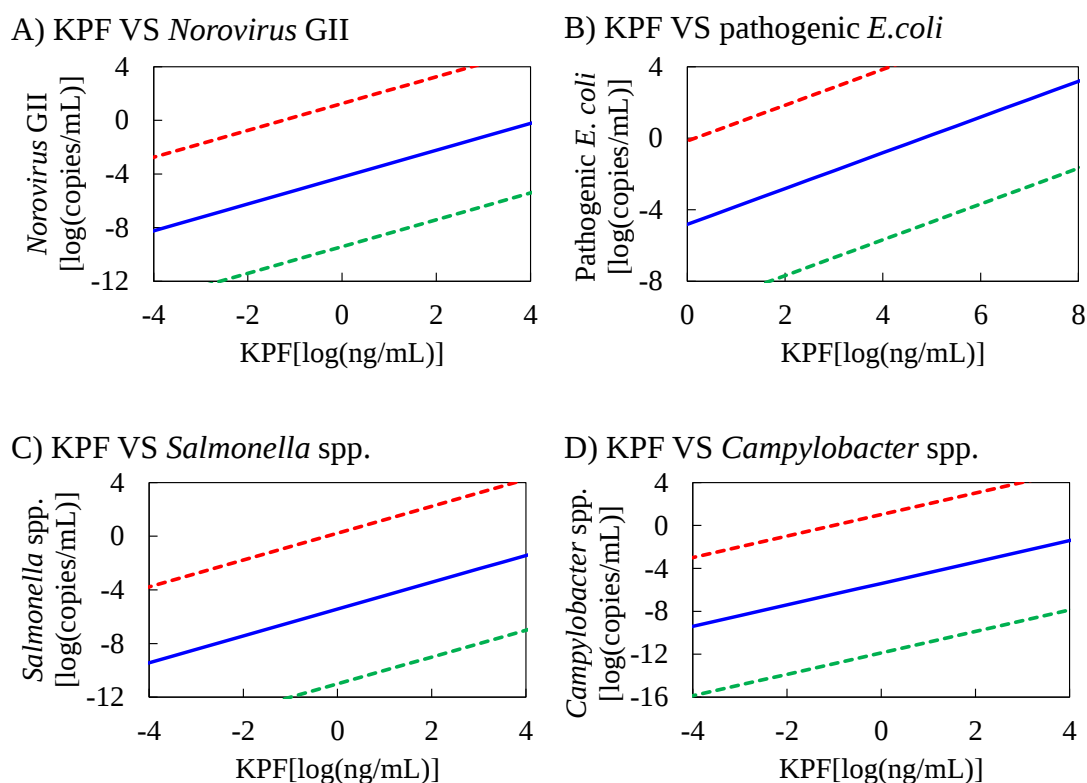


Figure 7.38 1 and 99 percentile of KPF concentration versus pathogen concentrations that are based on the qPCR with PMA (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

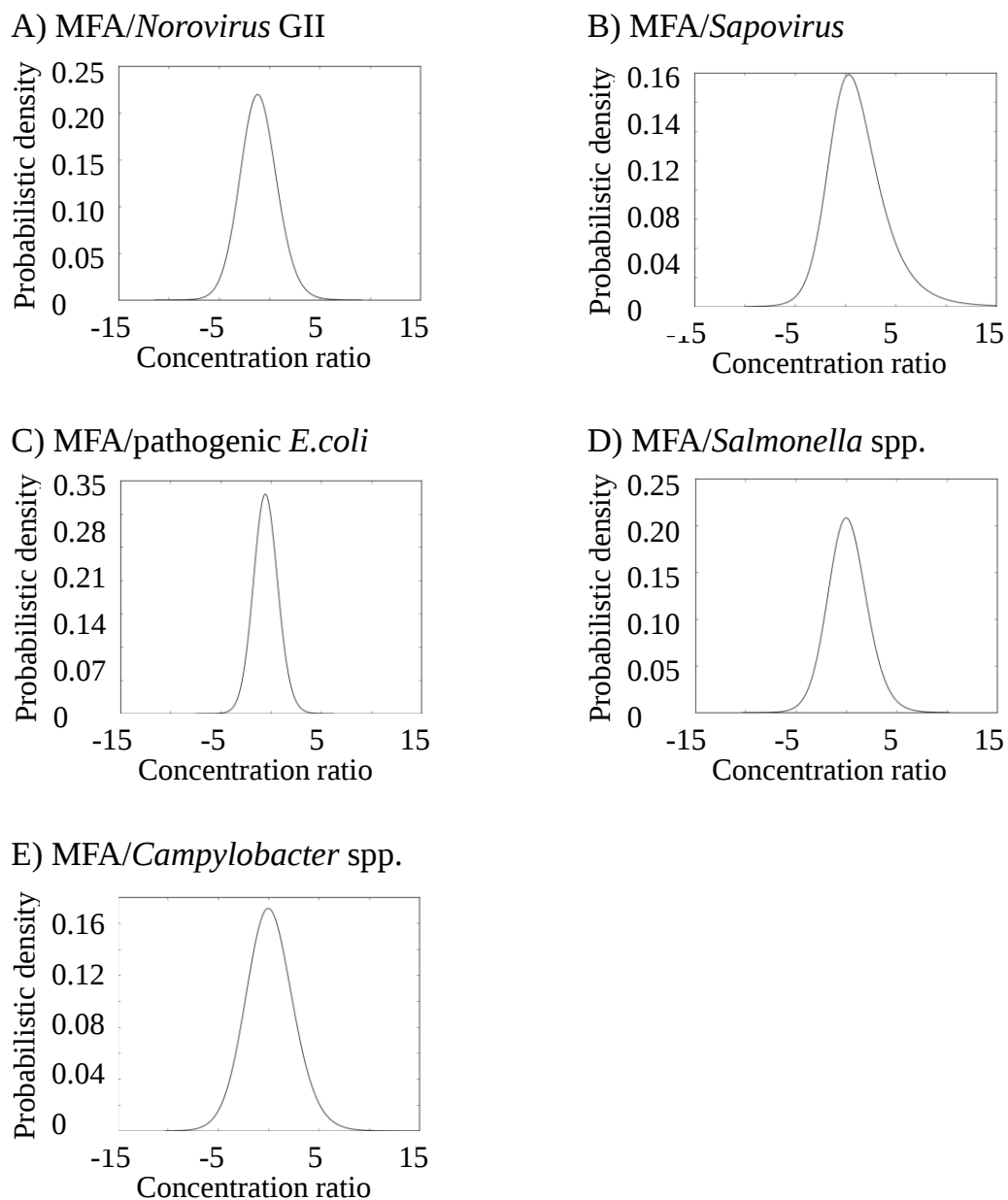


Figure 7.39 Distribution of the ratio of MFA concentrations to pathogen concentrations that are based on the normal qPCR

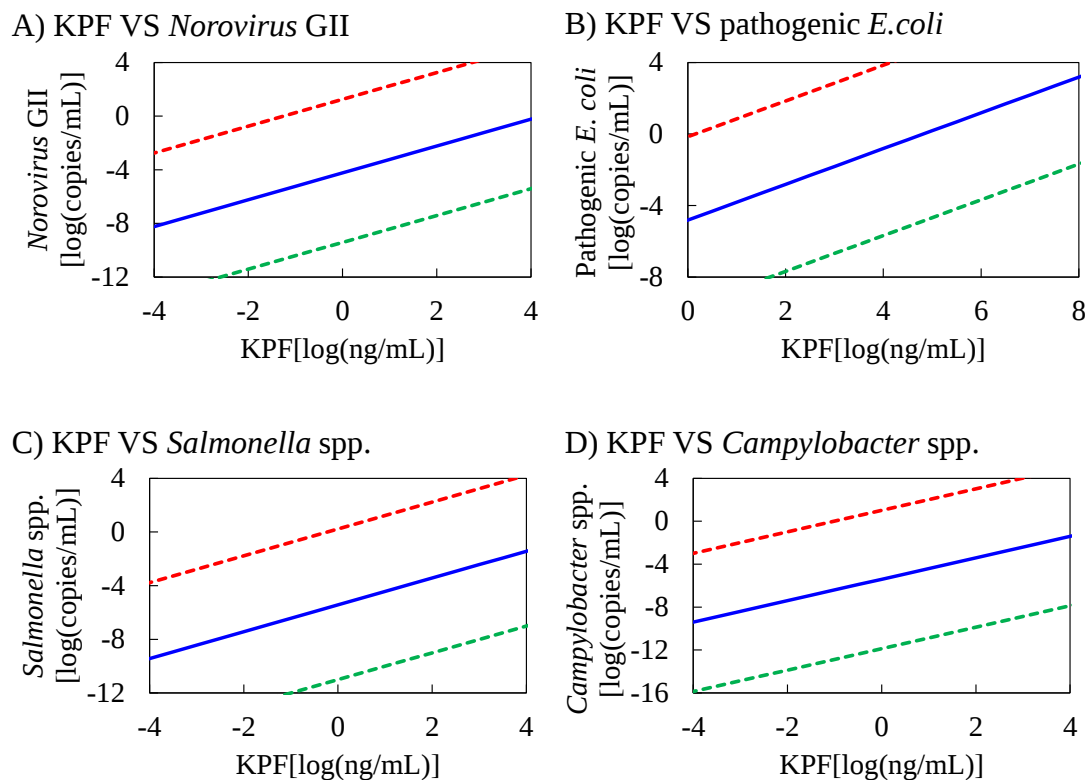


Figure 7.40 1 and 99 percentile of MFA concentration versus pathogen concentrations that are based on the normal qPCR (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

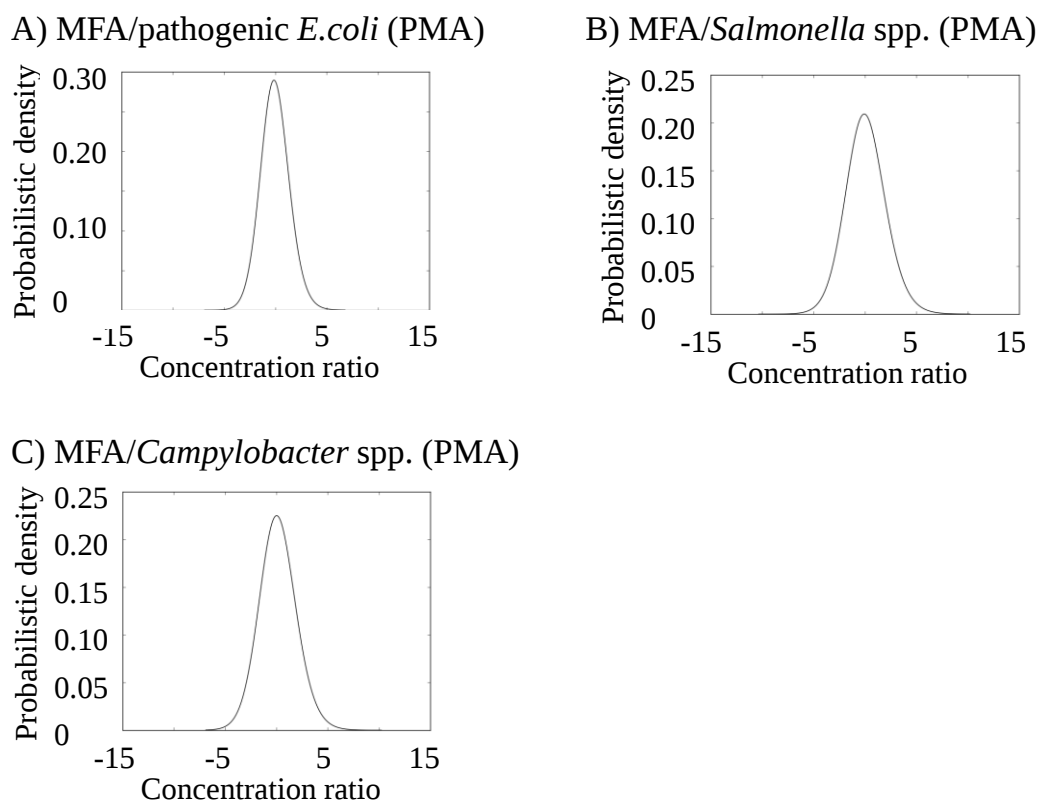


Figure 7.41 Distribution of the ratio of MFA concentrations to pathogen concentrations that are based on the qPCR with PMA.

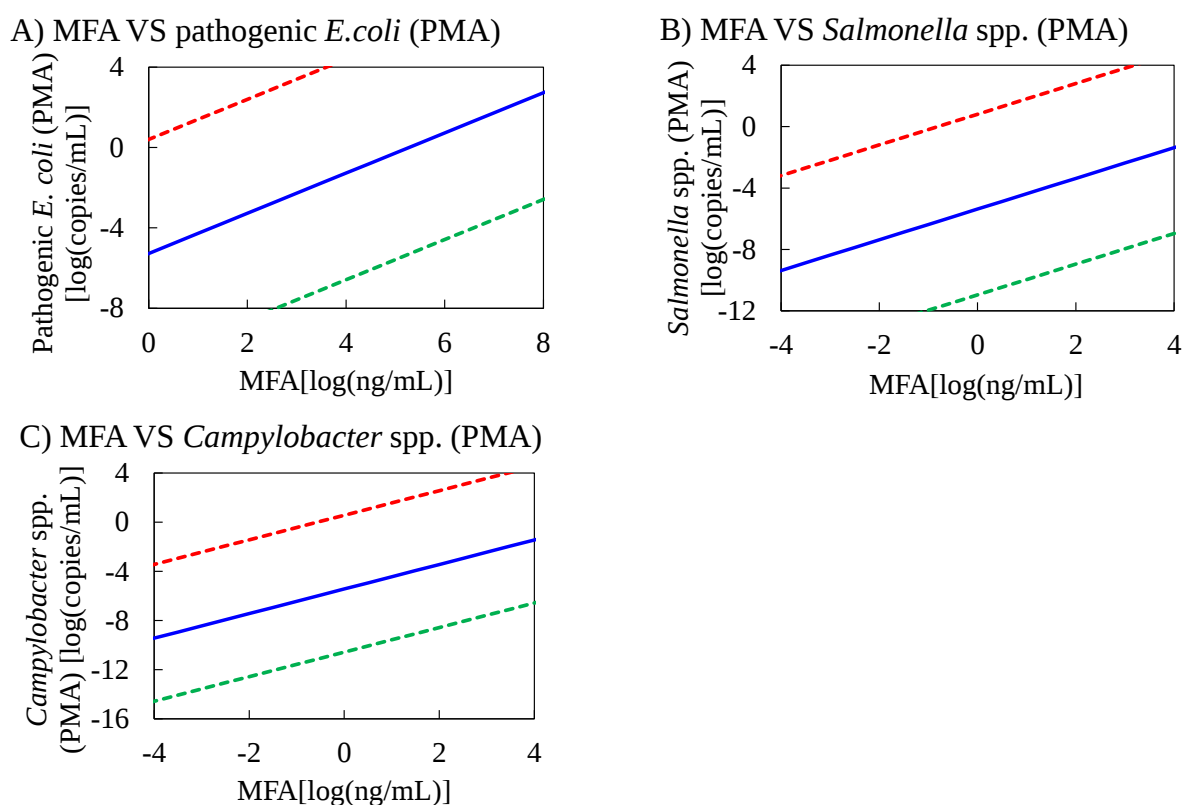


Figure 7.42 1 and 99 percentile of MFA concentration versus pathogen concentrations that are based on the qPCR with PMA (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

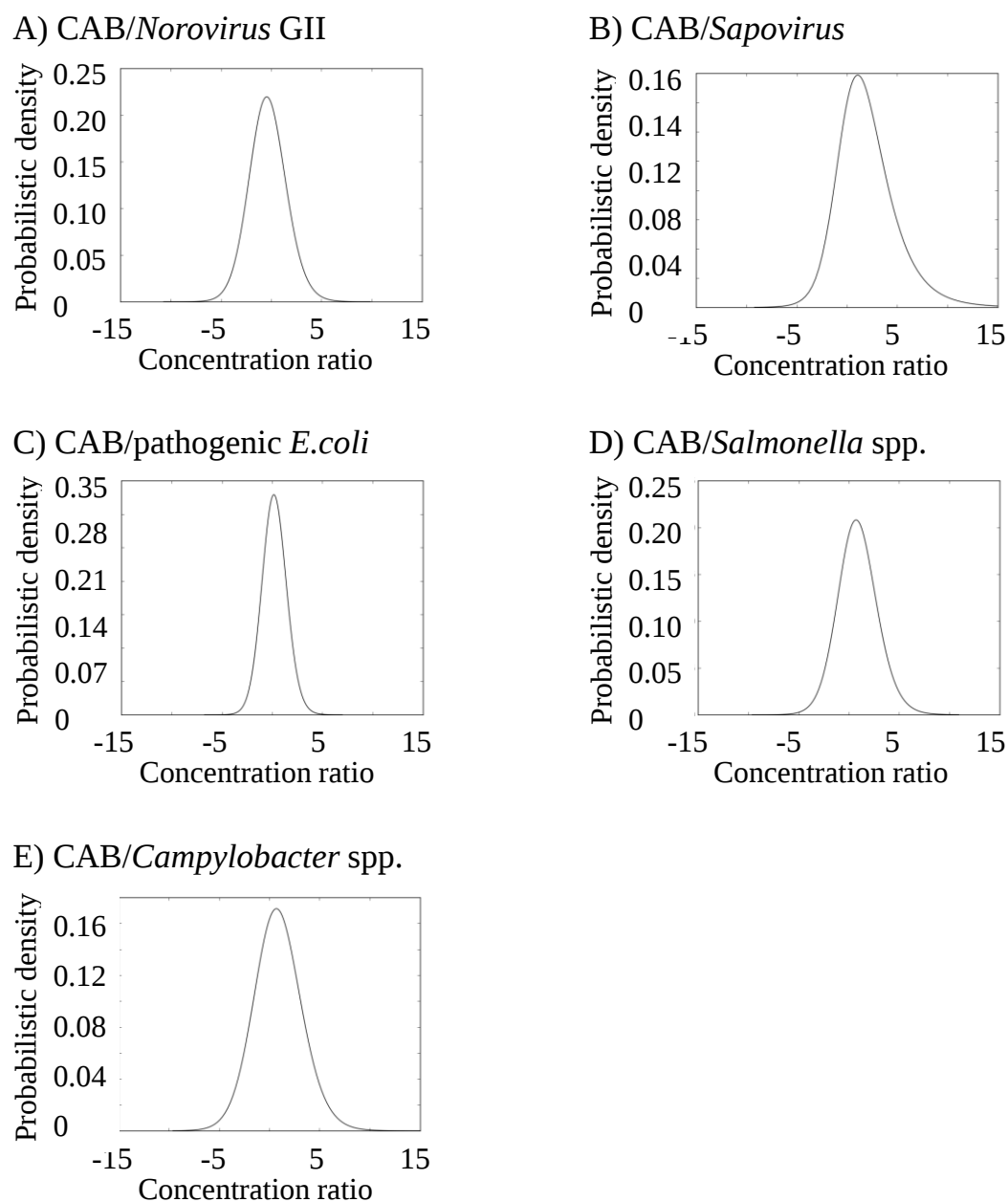


Figure 7.43 Distribution of the ratio of CAB concentrations to pathogen concentrations that are based on the normal qPCR.

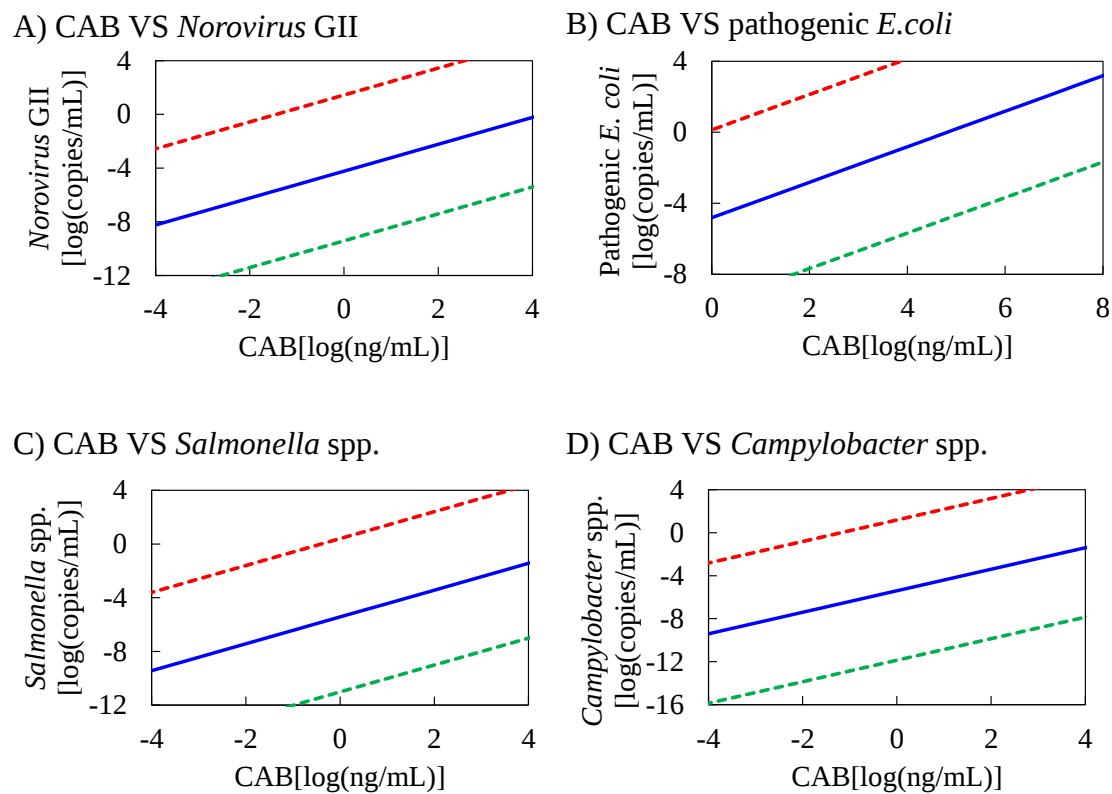


Figure 7.44 1 and 99 percentile of CAB concentration versus pathogen concentrations that are based on the normal qPCR (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

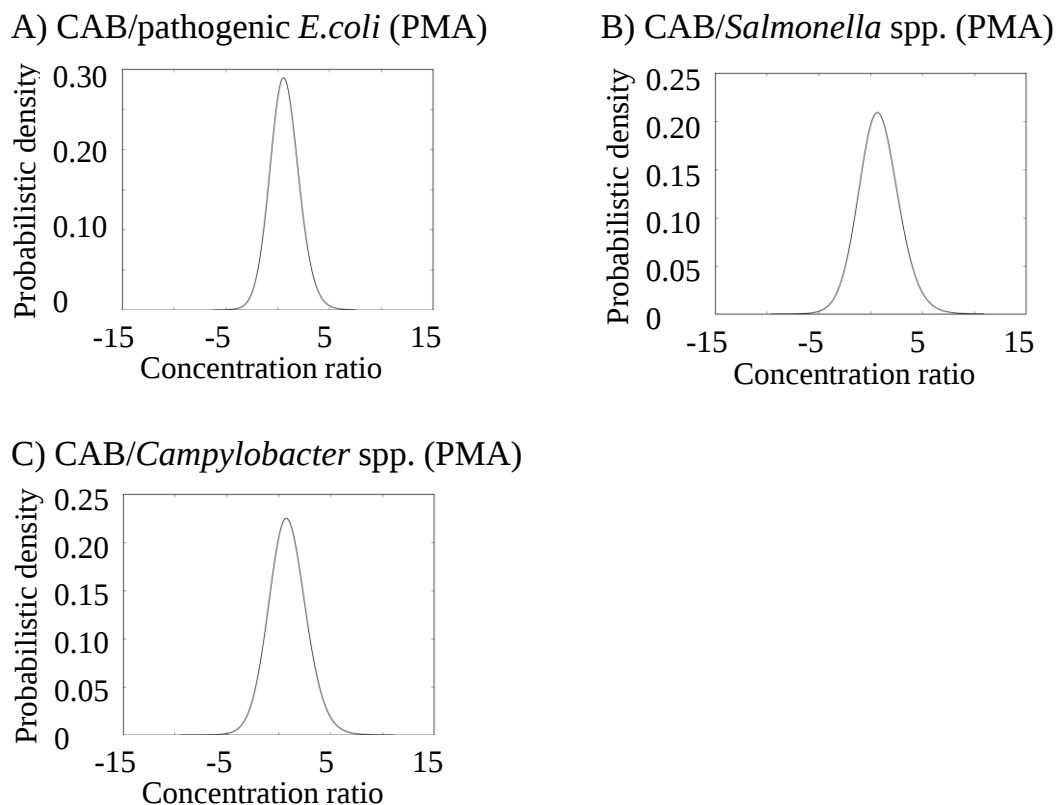


Figure 7.45 Distribution of the ratio of CAB concentrations to pathogen concentrations that are based on the qPCR with PMA.

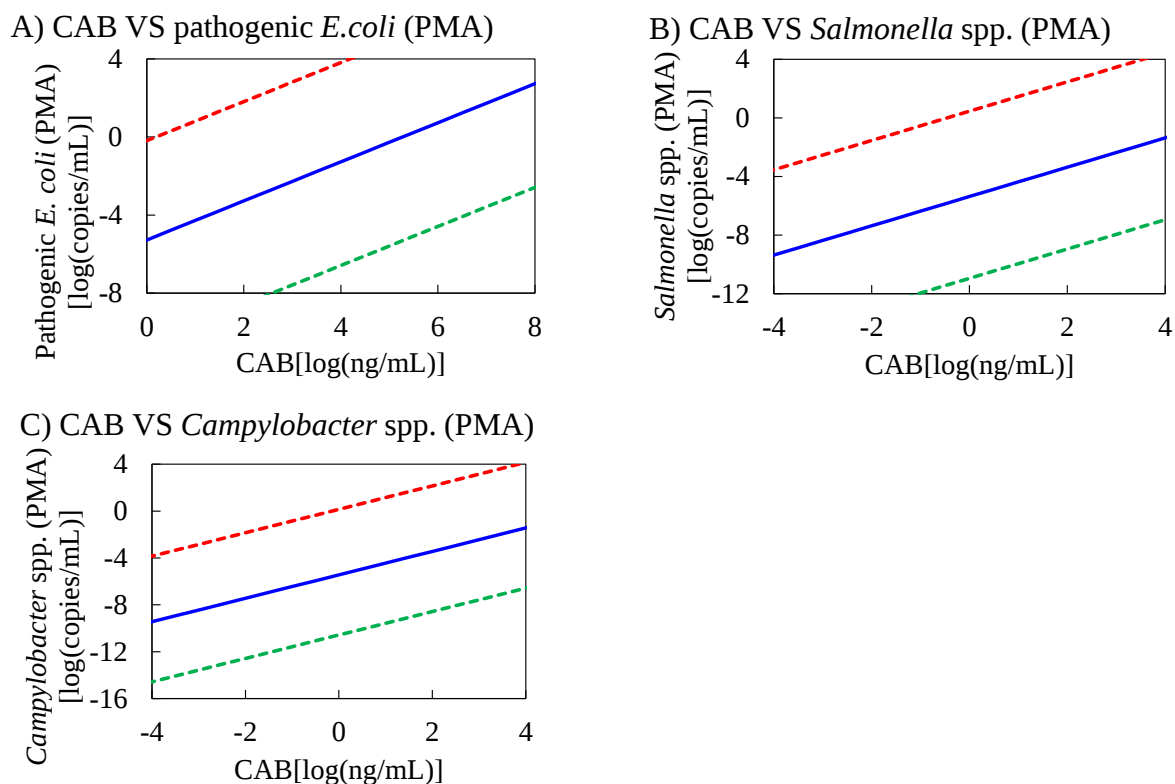


Figure 7.46 1 and 99 percentile of CAB concentration versus pathogen concentrations that are based on the qPCR with PMA (Red line: 99 %tile, Blue line: Median, Green line; 1 %tile).

7.4.3 Determination of the hygiene standard value

The indicator concentration as a hygiene standard value was determined by using the 99 percentile of indicator concentration versus pathogen concentrations and the acceptable concentrations of pathogens (previously shown in Table 6.2). In this study, the safety factor of 10 was used. Calculation of the acceptable concentration of pathogens was performed by using point estimation of QMRA in Chapter 6. This safety factor means the individual variability against a pathogen infection. For example, the acceptable concentration of *Norovirus* GII was calculated to be 2.8×10^4 copies/mL as shown in Table 6.2. If the safety factor of 10 was used, the final acceptable concentration of *Norovirus* GII becomes 2.8×10^3 copies/mL. The indicator concentration as a hygiene standard value is a concentration at intersection point of the 99 percentile of indicator concentration versus pathogen concentrations and the final acceptable concentration (Figure 7.47). Thus, if 2.2×10^2 copies/mL of human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker is used as a water quality standard value, *Norovirus* GII concentration exceeds the acceptable level once in 100 times investigation. In similar way, each indicator concentrations as a hygiene standard value against each pathogens were calculated, and these values were shown in Table 7.1 to 7.8.

The failure rate of pathogen control (1% in this case; 99 percentile= 1% of failure rate) is a tentative one, and can be changed from 1% to the other values such as 0.1% and 0.01%. If you would like to control all these pathogens under the failure rate of 1%, the lowest concentration of indicator should be set as a hygiene standard value. Thus, if you want to use total coliforms as hygiene indicator, total coliforms as a hygiene standard value should be set at 1.1×10^2 MPN/L (based on the normal qPCR) or 1.8×10^3 MPN/L (based on the qPCR with PMA). If you want to use *E. coli* as hygiene indicator, *E. coli* concentration as a hygiene standard value should be set at 1.0×10^1 MPN/L (based on the normal qPCR) or 1.7×10^2 MPN/L (based on the qPCR with PMA). These results were lower than the water quality standard value of fecal pollution indicators in Japan. For example, total coliforms and the concentration of *E. coli* have been defined at 5.0×10^4 MPN/L and 5.0×10^3 MPN/L respectively in environmental quality standards for conservation of the living environment. Thus, if you would like to control the water quality based on the acceptable risk of pathogen infection, you should change the water quality standard value into approximately 1/100 of present value.

On the other hand, if you want to use total *Bacteroides-Prevotella* 16S rRNA genetic marker (based on the normal qPCR) as hygiene indicator, total *Bacteroides-Prevotella* 16S rRNA genetic marker concentration as a hygiene standard value should be set at 3.2×10^3 copies/L (based on the normal qPCR) or 5.9×10^4 copies/L (based on the qPCR with PMA). If you want to use viable total *Bacteroides-Prevotella* 16S rRNA genetic marker (based on the qPCR with PMA) as hygiene

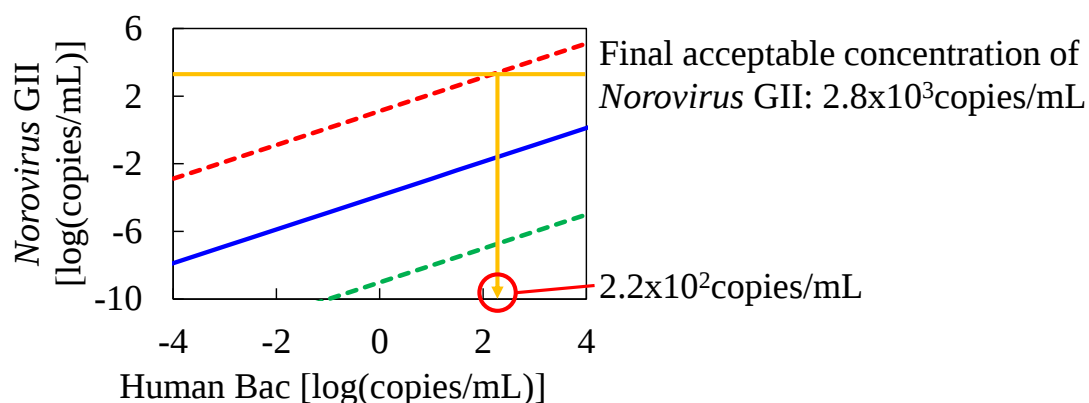


Figure 7.47 The indicator concentration as a hygiene standard value; the intersection point of the 99 percentile of indicator concentration versus pathogen concentrations and the final acceptable concentration

Table 7.1 Total coliforms as a hygiene standard value

Pathogens	Data set (MPN-Pathogen)	Acceptable concentration (copies/L)	Failure rate	Water quality standard value (MPN/L)
<i>Norovirus</i> GII	MPN-Normal	2.8x10 ⁷	1%	1.8x10 ⁴
<i>Sapovirus</i>	MPN-Normal			-
<i>Salmonella</i> spp.	MPN-Normal	2.5x10 ⁷		1.6x10 ⁵
	MPN-PMA			1.4x10 ⁵
pathogenic <i>E.coli</i>	MPN-Normal	2.9x10 ⁵		1.2x10 ⁴
	MPN-PMA			1.7x10 ⁴
<i>Campylobacter</i> spp.	MPN-Normal	1.4x10 ⁵		1.1x10 ²
	MPN-PMA			1.8x10 ³

Table 7.2 The concentration of *E. coli* as a hygiene standard value

Pathogens	Data set (MPN-Pathogen)	Acceptable concentration (copies/L)	Failure rate	Water quality standard value (MPN/L)
<i>Norovirus</i> GII	MPN-Normal	2.8x10 ⁷	1%	1.6x10 ³
<i>Sapovirus</i>	MPN-Normal			-
<i>Salmonella</i> spp.	MPN-Normal	2.5x10 ⁷		1.3x10 ⁴
	MPN-PMA			1.3x10 ⁴
pathogenic <i>E.coli</i>	MPN-Normal	2.9x10 ⁵		1.1x10 ³
	MPN-PMA			1.5x10 ³
<i>Campylobacter</i> spp.	MPN-Normal	1.4x10 ⁵		1.0x10 ¹
	MPN-PMA			1.7x10 ²

Table 7.3 The concentration of total *Bacteroides-Prevotella* 16S rRNA genetic marker as a hygiene standard value

Pathogens	Data set (Bac-Pathogen)	Acceptable concentration (copies/L)	Failure rate	Water quality standard value (copies/L)
<i>Norovirus</i> GII	Normal-Normal	2.8×10^7	1%	5.6×10^5
	PMA-Normal			5.1×10^4
<i>Sapovirus</i>	Normal-Normal	2.8×10^7		5.6×10^5
	PMA-Normal			5.2×10^4
<i>Salmonella</i> spp.	Normal-Normal	2.5×10^7		4.8×10^6
	Normal-PMA			4.6×10^6
	PMA-Normal			4.7×10^5
	PMA-PMA			4.4×10^5
pathogenic <i>E.coli</i>	Normal-Normal	2.9×10^5		3.5×10^5
	Normal-PMA			4.6×10^5
	PMA-Normal			2.4×10^4
	PMA-PMA			4.0×10^4
<i>Campylobacter</i> spp.	Normal-Normal	1.4×10^5		3.2×10^3
	Normal-PMA			5.9×10^4
	PMA-Normal			3.5×10^2
	PMA-PMA			5.6×10^3

Table 7.4 The concentration of human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker as a hygiene standard value

Pathogens	Data set (Bac-Pathogen)	Acceptable concentration (copies/L)	Failure rate	Water quality standard value (copies/L)
<i>Norovirus</i> GII	Normal-Normal	2.8×10^7	1%	2.2×10^5
	PMA-Normal			2.0×10^4
<i>Sapovirus</i>	Normal-Normal	2.8×10^7		1.7×10^6
	PMA-Normal			2.0×10^4
<i>Salmonella</i> spp.	Normal-Normal	2.5×10^7		1.9×10^6
	Normal-PMA			1.5×10^6
	PMA-Normal			1.6×10^5
	PMA-PMA			1.5×10^5
pathogenic <i>E.coli</i>	Normal-Normal	2.9×10^5		1.3×10^5
	Normal-PMA			1.8×10^5
	PMA-Normal			8.5×10^3
	PMA-PMA			1.4×10^4
<i>Campylobacter</i> spp.	Normal-Normal	1.4×10^5		1.2×10^3
	Normal-PMA			2.2×10^4
	PMA-Normal			1.4×10^2
	PMA-PMA			1.9×10^3

Table 7.5 The concentration of IBU as a hygiene standard value

Pathogens	Data set (GC/MS -Pathogen)	Acceptable concentration (copies/L)	Failure rate	Water quality standard value (ng/L)
<i>Norovirus</i> GII	GC/MS-Normal	2.8x10 ⁷	1%	1.8x10 ⁴
<i>Sapovirus</i>	GC/MS-Normal			-
<i>Salmonella</i> spp.	GC/MS-Normal	2.5x10 ⁷		2.0x10 ⁵
	GC/MS-PMA			1.7x10 ⁵
pathogenic <i>E.coli</i>	GC/MS-Normal	2.9x10 ⁵		1.3x10 ³
	GC/MS-PMA			3.1x10 ³
<i>Campylobacter</i> spp.	GC/MS-Normal	1.4x10 ⁵		2.9x10 ²
	GC/MS-PMA			1.7x10 ³

Table 7.6 The concentration of KPF as a hygiene standard value

Pathogens	Data set (GC/MS -Pathogen)	Acceptable concentration (copies/L)	Failure rate	Water quality standard value (ng/L)
<i>Norovirus</i> GII	GC/MS-Normal	2.8x10 ⁷	1%	1.6x10 ⁵
<i>Sapovirus</i>	GC/MS-Normal			-
<i>Salmonella</i> spp.	GC/MS-Normal	2.5x10 ⁷		1.5x10 ⁶
	GC/MS-PMA			1.3x10 ⁶
pathogenic <i>E.coli</i>	GC/MS-Normal	2.9x10 ⁵		4.2x10 ⁴
	GC/MS-PMA			7.6x10 ⁴
<i>Campylobacter</i> spp.	GC/MS-Normal	1.4x10 ⁵		1.4x10 ³
	GC/MS-PMA			1.5x10 ⁴

Table 7.7 The concentration of MFA as a hygiene standard value

Pathogens	Data set (GC/MS -Pathogen)	Acceptable concentration (copies/L)	Failure rate	Water quality standard value (ng/L)
<i>Norovirus</i> GII	GC/MS-Normal	2.8x10 ⁷	1%	4.0x10 ⁴
<i>Sapovirus</i>	GC/MS-Normal			-
<i>Salmonella</i> spp.	GC/MS-Normal	2.5x10 ⁷		4.2x10 ⁵
	GC/MS-PMA			3.9x10 ⁵
pathogenic <i>E.coli</i>	GC/MS-Normal	2.9x10 ⁵		4.6x10 ³
	GC/MS-PMA			1.1x10 ⁴
<i>Campylobacter</i> spp.	GC/MS-Normal	1.4x10 ⁵		4.7x10 ²
	GC/MS-PMA			3.9x10 ³

Table 7.8 The concentration of CAB as a hygiene standard value

Pathogens	Data set (GC/MS -Pathogen)	Acceptable concentration (copies/L)	Failure rate	Water quality standard value (ng/L)
<i>Norovirus</i> GII	GC/MS-Normal	2.8x10 ⁷	1%	1.0x10 ⁵
<i>Sapovirus</i>	GC/MS-Normal			-
<i>Salmonella</i> spp.	GC/MS-Normal	2.5x10 ⁷		1.0x10 ⁶
	GC/MS-PMA			8.9x10 ⁵
pathogenic <i>E.coli</i>	GC/MS-Normal	2.9x10 ⁵		2.1x10 ⁴
	GC/MS-PMA			4.5x10 ⁴
<i>Campylobacter</i> spp.	GC/MS-Normal	1.4x10 ⁵		9.4x10 ²
	GC/MS-PMA			1.0x10 ⁴

indicator, viable total *Bacteroides-Prevotella* 16S rRNA genetic marker concentration as a hygiene standard value should be set at 3.5×10^2 copies/L (based on the normal qPCR) or 5.6×10^3 copies/L (based on the qPCR with PMA). If you want to use human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker (based on the normal qPCR) as hygiene indicator, human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker concentration as a hygiene standard value should be set at 1.2×10^3 copies/L (based on the normal qPCR) or 2.2×10^4 copies/L (based on the qPCR with PMA). If you want to use viable human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker (based on the qPCR with PMA) as hygiene indicator, viable human-specific *Bacteroides-Prevotella* 16S rRNA genetic marker concentration as a hygiene standard value should be set at 4.1×10^2 copies/L (based on the normal qPCR) or 1.9×10^3 copies/L (based on the qPCR with PMA). The concentrations of conventional indicators and genetic markers as the hygiene standard value are approximately same level with these quantification limit values. Thus, the use of these hygiene standard values requires a re-thinking of concentration method.

And, if you want to use IBU as hygiene indicator, IBU concentration as a hygiene standard value should be set at 2.9×10^2 ng/L (based on the normal qPCR) or 1.7×10^3 ng/L (based on the qPCR with PMA). If you want to use KPF as hygiene indicator, KPF concentration as a hygiene standard value should be set at 1.4×10^3 ng/L (based on the normal qPCR) or 1.5×10^4 ng/L (based on the qPCR with PMA). If you want to use MFA as hygiene indicator, MFA concentration as a hygiene standard value should be set at 4.7×10^2 ng/L (based on the normal qPCR) or 3.9×10^3 ng/L (based on the qPCR with PMA). If you want to use CAB as hygiene indicator, CAB concentration as a hygiene standard value should be set at 9.4×10^2 ng/L (based on the normal qPCR) or 1.0×10^4 ng/L (based on the qPCR with PMA). The concentrations of pharmaceutical compounds as the hygiene standard value are higher than these quantification limit values.

These indicator concentration as the hygiene standard value will be changed, if the other pathogens were concerned in this framework. Depending on the considerable waterborne diseases in the target water body, you should change the target pathogens. For example, the increased number of cryptosporidiosis infections in three EU countries (Netherlands, the United Kingdom and Germany) in the same period of time is unusual and is a great public health concern (Fournet et al. 2013). In these countries, the concentration of *Cryptosporidium* spp. should be concerned in the determination of hygiene standard value.

In addition to it, this framework can be used for the determination of the other water quality value such as drinking water quality standard, recreational water quality standard, reclaimed water quality standard value. If you want to use this framework for determination of the other water quality

value, you should survey the indicator and pathogen concentration in water system, and change the scenario of QMRA.

7.5. Conclusions

The establishment of water hygiene standards based on the possible adverse effects on human health has been critically needed. In this study, we constructed a framework to determine the evidence-based standard values of fecal pollution indicators including the conventional indicators (total coliforms and the concentration of *E. coli*) and alternative indicators (*Bacteroides-Prevotella* 16S rRNA genetic markers and pharmaceutical compounds), which is derived from an acceptable risk of pathogen infection and occurrence characteristics of pathogens in water for microbiologically safe water management. For example, if you want to use total coliforms as hygiene indicator, total coliforms as a hygiene standard value should be set at 1.1×10^2 MPN/L (based on the normal qPCR) or 1.8×10^3 MPN/L (based on the qPCR with PMA). These results were lower than the water quality standard value of fecal pollution indicators in Japan. Thus, if you would like to control the water quality based on the acceptable risk of pathogen infection, you should change the water quality standard value into approximately 1/100 of present value.

7.6. References

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Chapter 8

Conclusions

8.1. Conclusions

The final goal of this study is to construct the framework to determine the hygiene standard values based on the acceptable risk of pathogen infection. To achieve this final goal, individual tasks were accomplished. Firstly, *Bacteroides-Prevotella* 16S rRNA genetic markers were constructed for identifying each fecal contamination source. Secondly, for the use of these genetic markers in the detection and the monitoring of fecal contamination in environmental water, quantification method of them was standardized. Thirdly, the acceptable risks of pathogen infection were calculated by the point estimation of quantitative microbial risk analysis. Fourthly, the quantitative relationships of indicators and pathogens were calculated by using surveillance data of pathogen and indicator concentrations. Finally, the hygiene standard values were determined based on the acceptable risk of pathogen infection.

The followings are the conclusions derived in this study.

- We identified chicken and duck-associated genetic markers (Chicken/Duck-Bac, Chicken-Bac and Duck-Bac) in the 16S rRNA genes of the *Bacteroides* and *Prevotella* genera, and developed real-time PCR assays to quantify these markers in environment water.
- We established a genetically-engineered *Escherichia coli* K12 strain (designated as strain MG1655 Δ lac::kan) as a sample process control (SPC) for the qPCR assays to more accurately quantify the host specific *Bacteroides-Prevotella* 16S rRNA genetic markers in environmental water samples.
- We investigated the fate of some human-specific genetic markers in river water and compared with that of conventional indicator microorganisms at various water temperature conditions. In addition to that, the possible effect of predators on the persistency of bacterial genetic markers in natural water environment was also investigated.
- We calculated the acceptable concentrations of pathogens in river water for the determination of the hygiene standard values. In this calculation, we use the point estimation of QMRA approach.
- We constructed a framework to determine the evidence-based standard values of fecal pollution indicators including the conventional indicators (total coliforms and the concentration of *E. coli*) and alternative indicators (*Bacteroides-Prevotella* 16S rRNA genetic markers and pharmaceutical compounds), which were derived from an acceptable risk of pathogen infection and occurrence characteristics of pathogens in water for microbiologically safe water management.

8.2. Future perspectives

In this study, I constructed the framework to determine the hygiene standard values based on the acceptable risk of pathogen infection. However, some issues to be managed in this framework still remain. For example, QMRA approach employed in chapter 6 is not enough to obtain the accurate acceptable concentration of pathogens, because the individual variability was not taken into account. If you want to obtain more accurate information, you should perform the distribution estimation in QMRA. In addition to it, many information such as removal capacity of pathogens in advanced water treatment and the persistence of pathogens in environmental water is still scarce. Further experiments and reports will be needed. Moreover, the needs of more appropriate alternative indicators for pathogenic viruses are suggested, because the correlations between pathogenic viruses and fecal pollution indicators were very low in some settings. Finally, nation-wide investigation of the occurrence of pathogens and indicator microorganisms in environmental water is necessary to derive the hygiene standard value that was applicable as national standard.

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