Investigation of bacterial community composition and abundance in a lowland arable catchment

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Abstract

This study aimed to characterise the bacterial community composition and abundance in the River Wensum in Norfolk using epifluorescence microscopy (EFM), automated ribosomal intergenic analysis (ARISA) and 454 pyrosequencing. It also aimed to determine the effects of spatial and temporal variations and environmental factors on bacterial community composition and abundance in this intensively farmed lowland catchment. The three techniques provided the same trends in bacterial community composition and abundance across the Wensum catchment. Total bacterial numbers ranged from 0.21×10^6 cells/mL to 5.34×10^6 cells/mL (mean = 1.1×10^6 cells/mL). The bacterial community composition and abundance showed significant differences between sites and times and were related to environmental parameters, with temperature and flow rate explaining most of the variation in bacterial community composition and abundance. Bacterial abundance increases as water moves downstream, while bacterial diversity decreases as water moves downstream. Some operational taxonomic units (OTUs) become commoner as the water moves downstream (3^{rd} and 4^{th} order streams). This presumably reflects the fact that these bacteria are actively growing in the river, and reducing the abundance of other taxa. Consequently, the community becomes less diverse moving downstream, although a small number of sites do not fit this pattern. The River Wensum is dominated by the phyla Proteobacteria, Bacteroidetes, Cyanobacteria and Actinobacteria. Members of these phyla are well known to be responsible for biogeochemical processes, such as nitrogen cycling. The commonest bacteria at upstream sites were Proteobacteria (OTUs 2 and 4), Deltaproteobacteria (OTU29), Gammaproteobacteria (OTU32), Sphingobacteria (OUT9) and Flavobacteria (OTUs 12 and 23). Most OTUs (2, 9, 17, 29 and 32) are considered to be soil bacteria, suggesting that these bacteria are terrestrial in origin and are flushed into the lower order streams. Most of the upstream bacteria showed positive relationships with total nitrogen (TN) and total carbon (TC) and the presence of arable areas. On the other hand, the commonest bacteria at downstream sites were Cyanobacteria (OTU1), Flavobacteria (OTUs 3, 10 and 19), Cytophagia (OTU14), Actinobacteria (OTUs 20, 21 and 25) and Alphaproteobacteria (OTU26). Most of the downstream bacterial OTUs showed a positive relationship with TP and the presence of urban areas. The results of this research, however, do not provide strong evidence that competition is an important process structuring these bacterial communities. In addition, the correlations between environmental parameters and bacterial composition and abundance are not strong and do not clearly distinguish the most impacted sites from others. This suggests that bacterial community composition cannot be used as an indicator of the ecological status to assess compliance with Water Framework Directive (WFD) in a moderately impacted lowland catchment like the Wensum.

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List of Abbreviations

AO	Acridine orange
ANOISM	Analysis of similarities
ANOVA	Analysis of variance
ARDRA	Amplified ribosomal DNA restriction analysis
ARISA	Automated ribosomal intergenic spacer analysis
Bb	Base pair
BC	Blackwater catchment
BLAST	Basic local alignment search tool
BSC1	Blackwater sub-catchment 1
BSC2	Blackwater sub-catchment 2
CFU	Colony forming unit
CTC	5-cyno-2, 3-ditolyl tetrazolium chloride
DAPI	4',6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
EB	Ethidium bromide
EDTA	Ethylene-diamine-tetraacetic acid
EFM	Epifluorescence microscopy

6-FAM	Phosphoramidite fluorescence 5-carboxy-fluorescein
FCM	Flow cytometry
FISH	Fluorescent in situ hybridization
HEX	Hexachlorofluorescein
HPCs	Heterotrophic plate counts
IGS	Intergenic spacer
ITS	Intergenic transcribed spacer
INT	2-(p- iodophenyl)-3-(p-nitrophenyl)-5phenyl tetrazolium chloride
LCT	Lower catchment tributaries
MCT	Mid catchment tributaries
MDS	Multidimentional scaling
MPN	Most probable number
NCBI	National Centre for Biotechnology Information
OUT	Operational taxonomic unit
PCA	Plate count agar
PCR	Polymerase chain reaction
Rep-PCR	Repetitive extragenic palindromic-Polymerase chain reaction
RISA	Ribosomal intergenic spacer analysis
SEM	Scanning electron microscopy
SIMPER	Similarity percentages
SSCP	Single strand confirmation polymorphism
STWs	Sewage treatment works
TC	Total carbon

TGGE	Temperature gradient gel electrophoresis
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorus
T-RFLP	Terminal restriction fragment length polymorphism
TSS	Total suspended solids
UW	Upper Wensum
WB	Wensum Beck
WCM	Wensum Costessey Mill
WSM	Wensum Swanton Morley

Chapter One

General introduction

The purpose of this chapter is to discuss bacterial community composition, structure and abundance and the roles that they play in river ecosystems and the way these are affected by land use, spatial and temporal factors and physicochemical and biological parameters.

1.1 The quality of river water and the study of microbes

Water represents a common and essential chemical compound on our planet (Szewzyk *et al.* 2000). Freshwater is a necessary element for life and also is an important source for several activities, such as agriculture, human consumption and industrial processes (Hahn 2006). However, it remains an important factor in transmitting many diseases, especially in less developed countries (Ozler and Aydin 2008). Approximately, 2.5% of the total volume of water in the world represents freshwaters but much of this is in the form of ice, making it inaccessible. Despite the widespread uses of freshwater, it receives less research effort in microbiology compared with marine environments (Debroas *et al.* 2009). Freshwater consists of various microbes that can be affected by the quality of this type of water. The physical and chemical condition of freshwater can influence the microbial species composition and abundance. Microbes exhibit different responses to these conditions, for example, some are found in a wide range of condition, others are either sensitive or intolerant to contamination (Raibole and Singh 2011).

Rivers in urban areas are normally used to provide people with freshwater (Zhang *et al.* 2012). They are also the main source of water for agricultural and industrial purposes (Kenzaka *et al.* 2001). However, rivers are an open system and linked to their adjacent areas. This may cause changes in the ecological structure and function of rivers (Velimirov *et al.* 2011). Also, effluents after or before treatment are normally discharged into rivers (Zhang *et al.* 2012). In an urban area, river ecosystem can be threatened by overloading of wastes, affecting suitability for consumption and causing public health risks (Olayemi 1994). Hence, rivers as an aquatic ecosystem need to be in a healthy state and one of the most important factors to restore river health is to study and understand the microbial community composition and activity (Zhang *et al.* 2012).

Rivers represent active ecosystems. For example, the important concentrations of organic matter come from different sources and can be carried by rivers (Skorczewski and Mudryk 2009). Due to the variation of dissolved organic matter (DOM) content and concentrations in

rivers, they are considered to be an ideal environment to discover the link between bacterial community structure and DOM components (Kirchman *et al.* 2004). Organic matter can be highly utilized and decomposed by bacterioplankton communities (Yamakanamardi and Goulder 1995).

All types of water bodies can be inhibited by various groups of microorganisms. For each of microorganism to be active and grow needs specific physical and chemical conditions that can be provided by water bodies (Małecka and Donderski 2006). Although these bodies differ in terms of the availability of these parameters, an important feature shared by all water bodies is that they select species or groups of species in the determined clusters of bacteria (Zwart *et al.* 2002). Microorganisms in various types of freshwater should be essentially assessed and those bacteria considered to be important microbes should be monitored (Velimirov *et al.* 2011). Also, to prevent changes in aesthetic parameters of water quality, such as water odour, bacterial growth should be controlled (Hammes *et al.* 2008).

Surface waters in the United Kingdom are exposed to high concentrations of nutrients, particularly as a result of agricultural practices (Heathwaite *et al.* 1996), although waste water treatment works also discharge nutrients (European-Commission 2006). Nitrogen and phosphorus are the nutrients that show the greatest enrichment of concentrations (White and Hammond 2007) and have exceeded the permissible limits of the concentrations of nitrogen and phosphorous, respectively, leading to extensive eutrophication and poor water quality (Cherry *et al.* 2008). The introduction of the Water Framework Directive (WFD, 2000/60/EC) has helped to protect the water bodies in the European Union. By 2015, the quality of water bodies must achieve good and non-degrading conditions in all member states of the European Union. Good ecological and chemical conditions of surface and ground waters should therefore be achieved (Cherry *et al.* 2008). In the WFD the ecological status of rivers can be classified on the basis of shifts of biological community structure and changes in the overall ecosystem functioning, particularly in response to anthropogenic nutrient loadings. To evaluate the impacts of anthropogenic pressures, five ecological elements are usually examined by the WFD with the most important one being phytoplankton. Diatoms are widely used as indicators of the quality of river water for WFD assessments (European-Commission 2006; Solimini *et al.* 2006). Kelly *et al.* (2008) used diatoms to describe the ecological status of different rivers in the UK.

The bacterial community can also be used to estimate the ecological status of river systems. For example, Velimirov *et al.* (2011) assessed bacterial community composition in the River Danube in Europe and concluded that once sufficient data are available at various spatiotemporal scales, bacterial community could be used as an indicator for evaluating the ecological status of rivers. Lear *et al.* (2009) investigated the bacterial community in some streams exposed to anthropogenic pressures, and found that the bacterial community is able to discriminate the most impacted sites from others. However, Lear *et al.* (2009) concluded that to use the bacterial community as an indicator for determining ecological status in freshwater, high-throughput sequencing and statistical tools would be needed to improve sensitivity of the analyses of the bacterial community.

1.2 The study of bacteria in aquatic environments - why it is so important?

Prokaryotes have been proven to be responsible for biological, geological and chemical processes in aquatic environments. They have a huge diversity and numbers compared with other creatures (Matcher *et al.* 2011). All organisms in ecosystems rely on the activity of microorganisms (Kirk *et al.* 2004). In many aquatic systems, increased phytoplankton numbers, such as algae are followed by increases in heterotrophic bacterial activities and production (Albright 1977). An important proportion of planktonic biomass is dominated by bacteria, and the function and metabolism of ecosystems are highly influenced by bacterial activities (Gasol *et al.* 1999). Active bacteria are involved in uptake of substrates, cell respiration and division (Freese *et al.* 2006). The term active also describes the live bacteria with high DNA content and Gasol et al. (1999) found that marine bacterioplankton with high DNA contents were the most active members of total bacterial populations. In aquatic ecosystems, Zimmermann *et al.* (1978) found that 36% of the total number of bacteria were involved in respiration in freshwater samples, while in sea water samples they represented only 12%. Also, in freshwater environments, the highest respiration rates were attributable to large size bacterial cells (about 2.4 µm diameter), while in sea water samples, small sizes (0.4 µm in diameter) of bacterial cells were responsible for respiring more than other sizes. Similarly, Fuhrman (1981) found that marine bacterioplankton cells smaller than 0.6 µm in diameter have had the highest activities compared to other bacterial sizes.

All bacteria that can grow on organic matter are referred to as heterotrophic bacteria (Allen *et al.* 2004). In the 1940s, the important role of bacteria in freshwater ecosystems was first recognised by Lindeman (Newton *et al.* 2011). Autotrophic and heterotrophic processes are mainly controlled by bacteria (Porter and Feig 1980), which play an important role in the food web in stream systems through biodegradation of organic matter and support higher trophic levels of the food web (Zimmermann *et al.* 1978; Beier *et al.* 2008). Also, fine benthic organic matter is highly dominated by bacteria where they play a major role in converting this matter into soluble nutrients, which are released into the water of streams to support other creatures (Fierer *et al.* 2007). Bacteria and archaea play an important role in freshwater ecosystems through converting nutrients to other forms (Findlay 2010), and they contribute to the cycling of nitrogen (Hahn 2006) and metals (Lisle *et al.* 2004). More than fifty phyla of bacteria and archaea are responsible for dissolved organic matter (DOM) decomposition in natural aquatic environments (Kirchman *et al.* 2004).

Two living forms of bacteria in streams are bacterioplankton and biofilm bacteria. The bacteria in biofilms can be found either free on suspended particular matter or growing on stones (Brummer *et al.* 2003; Dorigo *et al.* 2010). Bacteria and other microbes associated with biofilms play an important role in the decomposition of pollutants and organic matter in aquatic environments (Moss *et al.* 2006). Biofilms represent important habitats to bacterial activities. Bacterial biofilms are able to catch and concentrate nutrients from ambient water compared with bacterioplankton. Also, they are resistant to deleterious chemicals and harsh environmental conditions (Moss *et al.* 2006). Biofilms play an important role in protecting microbial communities from external hazards and providing energy sources (O'Sullivan *et al.*

2002). For example, bacterial growth is protected and supported by biofilms through reducing the effects resulting from disinfecting materials in water environment (Bai *et al.* 2010). The study conducted by Albright *et al.* (1980) revealed that these are about two orders of magnitude difference between total numbers of bacterioplankton and sessile bacteria in the Ogilvie and Swift rivers in US. However, cycling of nutrients and organic matter in aquatic ecosystems can be better understood by studying bacterioplankton compared to biofilm bacteria (Glavin *et al.* 2004).

It is important to determine the dominant bacterial groups of heterotrophic bacteria in freshwater ecosystems because of their importance in taking up and controlling dissolved organic matter and also their contribution to other processes in the environment, such as the carbon cycle (Kirchman *et al.* 2003). Also, to understand the responsible bacterial cells for most activities in the environment, for example, decomposition of organic matter, bacterial identity and viability should be determined (Kenzaka *et al.* 2001). Also, in the study of microbial production and growth rates, active bacteria should be enumerated (Schumann *et al.* 2003).

1.3 Bacterial diversity and structure

All cell forms in life can be linked to one of the three primary domains; bacteria, archaea and eukarya (plants and animals) (Woese and Fox 1977; Woese 1987; Woese *et al.* 1990). According to the uses of comparative analysis of rRNA sequences and cultivation methods, twelve bacterial phyla were well defined and delineated first by Woese (1987). As a result of these works that have done by Woese and his colleagues, the basic phylogenetic framework in microbial ecology has been gained. The second feature is that these works ultimately allowed the development of techniques to resolve the big issue in microbial ecology regarding the failure to cultivate 90% of bacteria (Head *et al.* 1998).

Traditional tools, such as microscopy and culture-dependent tools have limited our information about microbial diversity (Muyzer 1999), and dominant microbes are little known about as a result (Torsvik *et al.* 1990). Approximately nine thousand prokaryotic species have been detected in soil using traditional tools (Horner-Devine *et al.* 2004) and just about 5000 of bacterial species have been successfully characterized in natural habitats (Lee *et al.* 1996). However, microorganisms are known to have a large diversity compared to other creatures, such as animals and plants (Weisse 2006) and they are estimated to have more than 10^{30} members (Kopczynski *et al.* 1994). More than 90% of microorganisms in aquatic ecosystems are represented by the bacteria (Hahn 2009; Findlay 2010). Sequencing rRNA genes derived from environmental samples has expanded our information about the diversity of microbes (Rappe and Giovannoni 2003). The number of bacterial phyla has highly increased to about 85 as a result. The minority of these phyla have representatives of cultured bacteria (Rappe and Giovannoni 2003; Stewart 2012). In addition, the employment of culture-independent techniques using 16S rRNA has revealed higher uncultivable microbes than cultivable, for example, 54,655 genes of prokaryotes that are not cultivable were deposited in GenBank compared with 21,466 genes of prokaryotes that are cultivable (Riesenfeld *et al.* 2004). One

millilitre of marine water contains 100 to 200 bacterial species, while in soil, they are about 4000 species per g (Riesenfeld *et al.* 2004).

Biodiversity of freshwater microbes is still unclear due to the lack of techniques that are able to describe them (Weisse 2006). In addition, Brummer *et al.* (2003) stated that the exact estimation of bacterial composition in rivers is difficult because of the fact that river and stream waters are continuously shifted following flow conditions. The other reason is the difficult in determining interactions between microbial communities in rivers and bacteria in streams that can come from different sources, either exterior sources (allochthonous) or interior sources (autochthonous). The mixtures of aquatic and terrestrial bacteria in rivers may increase the diversity of them. From soils, for example, microbes can be flushed from terrestrial areas into freshwater systems through runoff and mixed with bacteria in streams (Cottrell *et al.* 2005).

Despite the increase in the number of studies to determine bacterial community composition in freshwaters, such as rivers and streams, they remain less than the number of studies in marine and soil environments (Brummer *et al.* 2003; Jezberova *et al.* 2010). Rappe and Giovannoni (2003) mentioned in their review that, in 2002, 3951 freshwater sequences of 16S rRNA genes have been published in GenBank compared with 6104 and 6037 sequences derived from seawater and soil environments, respectively. Also, riverine bacteria are little known about compared to other aquatic environments. For example, 1000 sequences of riverine bacteria and archaea have been deposited in GenBank in 2003 compared with 17 times more sequences that have been deposited from marine and lakes ecosystems (Cottrell *et al.* 2005).

The big challenge for microbial ecologists is the identification of the composition and activity of bacterial communities in ecosystems (Kenzaka *et al.* 2001). Understanding how to determine the species present in the sample community is important to facilitate the study of the diversity of a group of microbes (Kirk *et al.* 2004). Community structure of microorganisms can be fully described by determining their diversity (number of species within microbial community), evenness (number of cells within species) (Tiedje *et al.* 1999), total number and distribution of species in the natural environment (Kirk *et al.* 2004).

In water bodies, a large group of microorganisms are represented by heterotrophic bacteria. They are able to decompose autochthonous and allochthonous organic matter, supporting other microorganisms and purifying water environments (Małecka and Donderski 2006).The majority of heterotrophic bacteria in the Brda river in Germany, documented by Małecka and Donderski (2006), were gram-positive bacteria, such as Actinobacteria. Also, the common bacterial types were ammonifying bacteria, while the less common bacteria were pectinolytic bacteria. A high percentage of heterotrophic bacteria of bacterial isolates in the Asa River in Nigeria identified by Olayemi (1994) belonged to faecal coliforms, such as *Escherichia coli* and *Salmonella,* attributed to human wastes which highly impacted the quality of the river water. The predominant heterotrophic bacteria in the Meduxnekeag and Dunbar rivers in USA discovered by Bell *et al.* (1982) were psychrotrophic bacteria and *Pseudomonas spp.* with an apparent diversity within these groups reported between seasons. Some of the heterotrophic bacterial phyla seem to be abundant in freshwater ecosystems, such as rivers and lakes, for

example, the most dominant heterotrophic bacterial groups in freshwater ecosystems belong to Bacteroidetes and Proteobacteria (Kirchman *et al.* 2003). Also, Kirchman *et al.* (2004) stated that Cytophaga-Flavobacterium, alpha, beta and Gammaproteobacteria are the abundant heterotrophic bacterial phyla, using culture-dependent methods combined with molecular techniques. They are responsible for mineralizing dissolved organic matter.

In order to prove that freshwater ecosystems contain distinct bacteria, Zwart *et al.* (2002) investigated and compared available 16S RNA gene sequences (689 sequences) deposited in GenBank from different types of freshwater bacterioplankton around the world, and they included also 24 new sequences derived from Parker River samples in USA. They demonstrated that freshwater harboured a specific and typical community of bacterioplankton and this may reflect important and specific factors shared by all freshwater environments, allowing the selection of bacteria (Zwart *et al.* 2002). The common bacterial groups in freshwater environments detected by the use of denaturing gel electrophoresis analysis (DGGE), are Actinobacteria, Cyanobacteria, Alphaproteobacteria, but the most prevalent bacterial group is Betaproteobacteria (Mueller-Spitz *et al.* 2009). Klammer *et al.* (2002) found that 60% of the total number of DAPI stained cells in the Traun River and Traunsee Lake in Austria belonged to Betaproteobacteria, Alphaproteobacteria and Cytophaga-Flavobacterium. The most abundant groups were Betaproteobacteria in all study environments. Also, Kenzaka *et al.* (2001) used fluorescent in situ hybridization (FISH) to study bacterial community in some rivers across Malaysia and Thailand and found that Betaproteobacteria and Alphaproteobacteria were the most dominant bacterial groups. In the delta of the Sacramento-San Joaquin River in California, Stepanauskas, *et al.* (2003) used terminal restriction fragment length polymorphism (T-RFLP) to determine bacterial structure and found that Actinobacteria were the abundant group followed by Cytophaga-Flavobacterium.

Although some bacteria are distributed in all environments, others can be only found in specific environments. For example, Hugenholtz *et al.* (1998) reviewed 86 phylogenetic studies of the bacteria contributing about 3000 sequences and found that some bacterial phyla are cosmopolitan, such as Proteobacteria and Cytophagales and can be found in a wide range of habitats, while others are limited to grow in specific habitats, such as Aquificales (limited to high-temperature habitats).

1.4 Total bacterial number and total heterotrophic bacteria

How many individual of bacterial cells are found in the target aquatic environment, is one of the most explicit and important questions for researchers. To determine the shifts of microbes in freshwater, total bacterial number has been shown to be a reliable indicator for this purpose (Hyun and Yang 2006; Wang *et al.* 2010). Total bacterial numbers is the basis for the routine assessment of water quality, so any changes in this number must be determined quickly as it may contain pathogenic bacteria (Velimirov *et al.* 2011). In reviewing many surveys of public

water outcomes, Allen *et al.* (2004) concluded that in most cases, the direct impacts on human health of bacteria counted by heterotrophic plate count (HPC) methods were not significant. However, high concentration of bacteria in water can reflect pollution of water. In most areas of microbiology, such as water and natural habitats, it is necessary to determine the total number of bacteria (Lebaron *et al.* 1998). However, the role of bacteria in mineralization and respiration is poorly understood by determining only total bacterial numbers (Davidson *et al.* 2004).

Rapid and simple methods for counting bacteria, such as epifluorescence microscopy, are required for monitoring the quality of water (Ogawa *et al.* 2005). Also, Hammes *et al.* (2008) stated that the complementarity of total bacterial enumeration tools, such as epifluorescence microscopy with culturability measurement tools, such as HPCs is necessary. Compared with attached bacteria, it is easy to count free-living bacteria in the surface water systems (Hobbie *et al.* 1977; Griebler *et al.* 2001). Total biovolume can be calculated from the total number of cells and cell volume. Multiplying a biovolume by a suitable conversion factor can result in biomass (Bolter *et al.* 2002).

Bacteria appear more abundant than other creatures, such as animals and plants. Their numbers on the earth range from 4×10^{30} to 6×10^{30} but they are most abundant in oceanic and terrestrial environments (Horner-Devine *et al.* 2004), for example, in marine waters bacterial numbers range from 0.2 to 2.0×10^{9} cells/L (Turley and Hughes 1992).

Several studies have assessed total bacterial numbers and heterotrophic plate counts in rivers and other freshwaters. These are presented in Table 1.1

Table 1. 1 Total bacterial numbers in rivers, groundwater and lakes.

Total abundance	Total heterotrophic bacteria	Method	Environment	Reference
Ranged from 0.7×10^6 to 22.4×10^6 cells/mL.	Ranged from 0.10×10^5 to 2.41×10^5 CFU/mL (0.13% to 13.1% of total).	EFM and DAPI stain - HPCs (spread plates and casein peptone starch agar).	Hull River, UK	Yamakanamardi and Goulder (1995)
-	Ranged from 1.05×10^5 to 1.26×10^6 CFU/mL.	HPCs (spread plates and plate count agar).	Anacostia River, US	Cavari *et al.* (1981)
Mean = 1.2×10^6 cells/mL. 10% of total DAPI stained cells represented active bacteria.	-	EFM and DAPI stain.	Traun River, Austria	Klammer *et al.* (2002)
Ranged from 3×10^6 to 1×10^7 bacterial cells/mL	19% to 58% of total	EFM and DAPI stain - HPCs (spread plates and R2A medium).	Kelang River basin, Malaysia	Kenzaka *et al.* (2001)
Ranged from 1.6×10^4 to 1.6×10^6 cells/mL.	Ranged from 2.5×10^2 to 1.5×10^4 CFU/mL.	EFM and AO stain - HPCs (spread plates and spread plates and bacto-beef extract).	Ogilvie River, Canada	Albright *et al.* (1980)
Ranged from 1×10^4 to 8.4×10^4 cells/mL.	Ranged from 3.2×10^2 to 1.9×10^3 CFU/mL.	EFM and AO stain - HPCs (spread plates and bacto-beef extract).	Swift River, US	Albright *et al.* (1980)
Mean = 6.7×10^6 cells/mL.	Mean = 531×10^3 CFU/mL.	EFM and AO stain - HPCs (spread plates and iron-peptone agar).	Brda River, Germany	Małecka and Donderski (2006)
-	Ranged from 4.5×10^6 to 8.5×10^6 cells/mL	HPCs (spread plates and plate count agar).	Asa River, Nigeria	Olayemi (1994)

Key symbols: EFM= epifluorescence microscopy, HPCs= heterotrophic plate counts, FCM= flow cytometry, DAPI= 4', 6-diamidino-2-phenylindole, AO= acridine orange.

Table 1.1 (continued) Total bacterial numbers in rivers, groundwater and lakes.

Total abundance	Total heterotrophic bacteria	Method	Environment	Reference
Ranged from 7.7×10^5 to 5.1×10^6 cells/mL, (72.7% of total is free-living bacteria).	-	EFM and AO stain.	Danube River, Europe	Velimirov *et al.* (2011)
Mean = 24×10^6 cells/mL, (24% of total are active cells).	-	EFM and DAPI stain.	Warnow River, Germany	Freese *et al.* (2006)
Total planktonic micro-organisms ranged from 1.6×10^6 to 3.5×10^7 cells/mL.	-	EFM and AO stain.	Berg River, South Africa	Paulse *et al.* (2007)
Mean = 9.2×10^9 cells/L	-	EFM and SYBR green I stain.	Hudson River, USA	Suter *et al.* (2011)
Ranged from 4.4×10^6 cells/mL (mesotrophic habitats) to 10.9×10^6 cells/mL (eutrophic habitats)	-	EFM and DAPI stain.	Different freshwaters	Schiewer *et al.* (2003)
Ranged from 5.9×10^5 to 4.6×10^6 cells/mL, (22.3% of total are viable cells to a depth of 104 m, reaching to 95.7% to a depth of 177 m).	-	EFM and AO stain.	Different groundwaters	Murakami Fujita *et al.* (2002)
Mean = 3×10^6 cells/mL.	-	EFM and DAPI stain.	Different groundwaters	Yamaguchi, Torii *et al.* (2011)
Mean = 1×10^6 cells/mL.	-	FCM and SYBR green I stain.	Lake Zurich	Hammes, Berney *et al.* (2008)
Ranged from 30×10^3 to 3×10^6 bacterial cells/mL.	-	EFM and DAPI stain.	Different lakes	Felip, Andreatta *et al.* (2007)

Key symbols: EFM= epifluorescence microscopy, HPCs= heterotrophic plate counts, FCM= flow cytometry, DAPI= 4', 6-diamidino-2-phenylindole, AO= acridine orange.

1.5 The effects of spatial and temporal variations (biogeography) on bacterial communities

1.5.1 Introduction

Geographical ranges of microbes that can be noticed, recorded and interpreted are called biogeographies. Becking (1934) introduced the first statement in the field of biogeography of microbes, "everything is everywhere, but, the environment selects" (Fierer 2008). However, little is known about the biogeography of microbes and until now books on their biogeographical diversity have not mentioned it (Dolan 2005). Bacteria may exhibit biogeographical patterns in both time and place (Horner-Devine *et al.* 2004). There are three important processes that must be considered when researchers want to explain biogeographical patterns of microorganisms; dispersal, extinction and speciation. The bacteria can be dispersed easily and quickly through water and air due to their small sizes, suggesting that their distributions may be high compared with other microorganisms within environments (Horner-Devine *et al.* 2004). However, Fierer *et al.* (2007) used terminal restriction fragment length polymorphism (T-RFLP) to study the bacterial community across 23 streams in the USA. They have not found any evidence for an influence of geographical distances on the composition of bacteria. Also, a study of the effects of transportation of bacterial cells via the atmosphere (dispersal) on bacterial community dynamics that has been made in two lakes in northern Wisconsin in the USA by Jones and McMahon (2009) and revealed that little or no effects of bacterial cells transferred via the atmosphere on bacterial dynamics in both lakes. The authors suggested that bacterial community dynamics can be influenced and explained by species-sorting depending on abiotic conditions and biotic interactions (Jones and McMahon 2009). Bacterial patterns can be shaped by dispersal, however, this remains less known than is the case for animals and plants (Fierer *et al.* 2007). There are no accurate estimates of the importance of two other processes, extinction and speciation rates on microbes (Fierer 2008). However, the production of some forms during life stages of microorganisms can keep them in extreme environments and reduce extinction rates. For example, in some species of *Bacillus* it has been noticed that these stages enable them to survive and tolerate extreme conditions (Horner-Devine *et al.* 2004). Also, speciation rates of bacteria have been observed on culturable bacteria, but unfortunately, rapid speciation rates have not yet been observed for the majority of natural bacteria (Fierer 2008). In addition, both dispersal and local speciation enhance bacterial speciation through adding new species in a given environment. Bacterial speciation rates may be high compared with other microorganisms. Also, some types of bacteria can receive DNA from other organisms through transfer processes, which may be different from their own DNA sequences and this lead to increases in bacterial speciation (Horner-Devine *et al.* 2004). Bacterial DNA can be also transferred by plasmids, making the conception of bacterial species more complex (Kirk *et al.* 2004).

Dispersal limitations as well as the variations of environmental conditions may shape geographical patterns. Until now, there has been little information published about environmental aspects that may cause spatial and temporal patterns (biogeographical patterns) (Fierer *et al.* 2007), and an important point in the study of biogeography of microbes in aquatic ecosystems is to explain the physical and biological parameters affecting the absence or presence of microbes (Dolan 2005).

Within freshwater ecosystems, there is still a poor understanding of the effects of the variation of space and time on the shifts of bacterial community composition (Lear *et al.* 2008; Nelson 2009; Logue and Lindstrom 2010).

Total coliforms could potentially be used in monitoring pollution in fresh water systems, such as rivers and streams. For example, Lear, Boothroyd *et al.* (2009) concluded that biofilm communities could reveal impacts of human wastes on streams. But this usage is restricted by our limited knowledge of temporal and spatial variations of community composition which can mask any effect of pollution (Sigua *et al.* 2010). Seasonal and spatial variation certainly does occur, but our understanding of their magnitude is also limited (Hahn 2006; Lear *et al.* 2008; Sommaruga and Casamayor 2009).

1.5.2 Spatial factors

Bacteria can be found in environments, such as rivers and lakes that are rich with nutrients and they can also be observed in extreme environments at high temperature and pH. For instance, temperatures between 70˚C and 79˚C are ideal for the growth of *Thermus aquaticus* (Horner-Devine *et al.* 2004). By contrast, low water temperatures at or near 0˚C is ideal for other groups of the bacteria (Gounot 1991). Although, some bacteria are cosmopolitan, there may be some regional types (Yannarell and Triplett 2004).

As with other groups of organisms, habitat heterogeneity may result in increased bacterial diversity. The high heterogeneity of soil is believed to be responsible for its high bacterial diversity, and bacterial community composition can be substantially different on small spatial scales (Horner-Devine *et al.* 2004). By contrast, aquatic ecosystems are much more homogeneous, although water stratification can affect bacterial community composition in lakes (Yannarell and Triplett 2005).

In the Fulda River in Germany, Beier *et al.* (2008) used temperature gradient gel electrophoresis (TGGE) to study bacterial community. They found that bacterial community composition in stream water and sediments was significantly different and correlated with geographical distances and some environmental parameters, such as pH. Betaproteobacteria was the dominant group in both water and sediments. Fischer *et al.* (2009) found that bacterial community composition in nine streams in Sweden was highly different between streams but it was similar to each other within each stream and this was attributed to catchment characteristics. Similar results were found in some streams in Auckland in New Zealand (Lear

et al. 2008; Lear and Lewis 2009; Lear and Lewis 2009). Sekiguchi *et al.* (2002) used denaturing gradient gel electrophoresis (DGGE) and clone library analysis to study bacterial community along the River Changjiang in China. They found gradual shifts of bacterial community composition along the river water. Bacterial diversity decreased as water moves downstream and the common bacteria in the upstream were Betaproteobacteria, while gram positive bacteria, such as Actinobacteria were the common group at downstream sites.

In arctic tundra, Crump *et al.* (2007) found that bacterioplankton community composition in streams and their connected lakes was the same. Dispersal processes played an important role in this similarity. This, however, decreased with distance and also the changes of water chemical conditions. Significantly different bacterial communities were found between unconnected lakes. The same results were found in natural lakes in California, USA (Nelson *et al.* 2009). In thirteen lakes located in north and south of Wisconsin, Yannarell and Triplett (2004) found significant shifts in bacterioplankton community composition along and among lakes. Bacterial diversity was higher and more similar to each other in lakes located in southern Wisconsin than that in northern Wisconsin. Diversity was positively related to water temperature and primary productivity. In six natural lakes from near the Himalayan region and Mount Everest, Sommaruga and Casamayor (2009) found differences in bacterial community composition using denaturing gradient gel electrophoresis across geographical distance that were related to the changes of local environmental parameters. Bacterial richness was negatively related to the ratio of catchment to lake size, while it was positively related to the size of glacier. Common bacterial groups were Alphaproteobacteria, Betaproteobacteria and Actinobacteria.

Much research has been focused to investigate the effects of spatial factors on bacterial community composition and abundance in other aquatic environments, for example, marine environments (Garren and Azam 2010; Zeng *et al.* 2011) and estuaries (Crump *et al.* 2004; Hewson and Fuhrman 2004; Dang *et al.* 2010). These studies observed that spatial factors were responsible for the variations in the abundance and composition of bacterial communities.

1.5.2.1 Land use and bacterial communities

In rivers and other types of surface and groundwater systems, nitrogen and phosphorus have been identified as leading contaminants (Arbuckle and Downing 2001), and their concentrations in catchments are dependent on land use (Carpenter *et al.* 1998). Catchments also can be contaminated with siltation because of the erosion of soils and also toxins. The contamination of catchments also includes exotic species that can be deposited in a catchment and leading to a loss of biodiversity. Cinque (2010) found that the water quality of the Tarago reservoir catchment in Australia was influenced by erosion and surface runoff, leading to increased turbidity, phosphorus and *Enterococcus*.

1.5.2.1.1 Plant-based agricultural practices and bacterial communities

Fluxes of nutrients into freshwater environments can be mainly attributed to agricultural practices (Carpenter *et al.* 1998). In these ecosystems, excessive loads of nutrients, such as phosphorus and nitrates can leach to freshwater by runoff and cause eutrophication (Schindler and Vallentyne 2004; de Figueiredo *et al.* 2007; Piscart *et al.* 2009; Mosley and Fleming 2010). This phenomenon is considered to be the major ecological problem in freshwater ecosystems. Short term effects of eutrophication can be observed through increased phytoplankton production and changes in species composition, while in the long term, it can deplete the oxygen layer in water and cause death to fishes (Carpenter *et al.* 1998). The growth of cyanobacteria can be enhanced by eutrophication (de Figueiredo *et al.* 2007), which can be positively related to phosphate concentration (Okechukwu and Alex 2009). Cyanobacteria produce toxins that affect human health and other aquatic organisms, such as birds and fish (Okechukwu and Alex 2009). Eutrophication also can cause shifts in microbial community composition through its effect on the chemical and physical characteristics of freshwater environments (de Figueiredo *et al.* 2010; Dorigo *et al.* 2010).

Several studies have investigated the effects of agriculture processes on bacterial composition in freshwater, such as in the Murray River in Australia (Mosley and Fleming 2010), four streams in France (Piscart *et al.* 2009), the Morcilla River in France (Dorigo *et al.* 2010), the Okrika creek in Nigeria (Obire *et al.* 2008) and different surface waters in China (Zhang *et al.* 2011).

1.5.2.1.2 Animal-based agricultural practices and bacterial communities

Animal-based agricultural practices represent an important source of surface water contaminants. During rainfall and flow events pathogens can be carried from animal manure into rivers, groundwater and lakes (Sakami *et al.* 2003; Goss and Richards 2008; Sigua *et al.* 2010).

Fernandez-Alvarez *et al.* (1991) found that faecal coliform counts increased in the River Rialto close to cattle, with highest concentrations during rainfall events. Similar results were found in the Pinhal river in Brazil (Sigua *et al.* 2010), different catchments in New Zealand (Buck *et al.* 2004), in the Subin River in Ghana (Obiri-Danso *et al.* 2005), along the south Nation River basin in Canada (Lyautey *et al.* 2010) and the Logan River basin in the US (Harmel *et al.* 2010).

Sousa, *et al.* (2006) found that shrimp farms can have substantial impacts on bacterial community composition and abundance in mangrove swamps and some rivers in Brazil. These environments were dominated by *Vibrio spp.,* Enterobacteriaceae and *Chryseomonas luteola*. Jokinen *et al.* (2010) found that waterfowl were the main source of *Campylobacter* in the Grand

River in Canada. The abundance of this genus was varied between seasons and negatively related to water temperature.

Increased total bacterial numbers between 1976 (mean 1.48×10^6 cells per mL) and 1994 (mean 4.33×10^6 cells/mL) in the Hull River in the UK were attributed to the extension of fish farms along this river (Yamakanamardi and Goulder 1995).

1.5.2.1.3 Urban areas and bacterial communities

Urban rivers are considered to be an important system to humans inhabiting urban areas, and the health of urban rivers can be assessed through measuring physiochemical and biological parameters (Deines *et al.* 2010). Developments without sewage systems, garden fertilizers and construction locations are considered to be major urban nonpoint sources that can contaminate catchments. Urban runoff consists of many contaminants, such as nutrients, bacteria, pesticides and herbicides that can reach water bodies (Carpenter *et al.* 1998). As a result, it was very important to assess the potential health risk in these bodies and one of the common tests is the measure of faecal indicator bacteria. Rivers in urbanized areas normally contain high concentrations of faecal coliform bacteria and *Escherichia coli* compared with other systems (Geldreich 1976; Essahale *et al.* 2010; Kent and Bayne 2010).

Construction of large or small wastewater treatment plants is dependent on the populations inhabiting urban areas. Effluents from these wastewater treatment plants can be discharged into water bodies, such as rivers, causing significant changes in the bacterial communities (Cebron *et al.* 2004; Drury *et al.* 2013) including heterotrophic bacteria (Olayemi 1994; Carter *et al.* 2000; Skorczewski and Mudryk 2009).

Drury, Rosi-Marshall *et al.* (2013) used pyrosequencing to study bacterial communities in two rivers and found that effluents from wastewater treatment plants had substantial effects on bacterial community composition and abundance in the upstream sites of rivers, and that these were significantly different to downstream sites. Bacterial diversity was lower at upstream sites but increased at downstream sites. The common group in the upstream sites was Deltaproteobacteria, while the abundance of other sequences belonging to *Nitrospira* was decreased.

Cebron *et al.* (2004) found that effluent from the Achères wastewater treatment plant was responsible for increasing ammonia-oxidizing (allochthonous) bacteria at downstream sites of the Seine River in Paris. These bacteria were dominated by *Nitrosomonas ureae*.

In the Arga River in Spain, Goni-Urriza *et al.* (1999) found that the bacterial community structure differed at downstream sites below sewage discharge points from that in the upstream. Genera like *Actinobacter spp.* were common at the downstream sites. Also, the total number of heterotrophic bacteria was higher at downstream sites compared with upstream.

In Victoria Harbour in Hong Kong, Thiyagarajan *et al.* (2010) found that bacterial community composition in coastal surface sediments contaminated with untreated sewage were different from that in uncontaminated sites. Seasonal shifts of the bacterial community composition were highly observed at only the contaminated sites. Shifts of bacterial composition were attributed to the variations of total organic carbon, total nitrogen and chlorophyll *a*.

1.5.3 Temporal factors

The principal biogeographical research of microbes has been focused on spatial factors. However, temporal factors of microbial biogeographical patterns should not be neglected. Shifts of biogeographical species composition of animals and plants is slow (species turnover-temporal turnover), with the turnover rate assessed in years or decades. However, species turnover of microbes might be more rapid. The more rapid species turnover rates can make the correlation between environmental parameters and microbial community composition more robust, and vice versa. The turnover rates of microbes can vary across environments, for example, low turnover rates can be observed in microbial communities that tolerate disturbances, living in habitats that have stable conditions and few or no predators (Fierer 2008).

Bell *et al.* (1982) found that the heterotrophic bacterial community differed between seasons in two Canadian rivers. Diversity in summer and autumn was higher than that in winter. The genus *Cytophaga spp.* was common in the Dunbar River, while facultative anaerobic bacteria were common in the River Meduxnekeag. Tirodimos *et al.* (2010) used fluorescent in situ hybridization (FISH) to study bacterial community in the River Aliakmon in Greece. They found that seasonal cycles affected bacterial community composition and diversity. Gammaproteobacteria, Proteobacteria and Firmicutes were the dominant groups in autumn. Similar seasonal changes were found in two temperate rivers using denaturing gradient gel electrophoresis (DGGE) and were attributed to some environmental parameters, such as temperature, flow rate, nutrients and organic matter. Diversity was higher in spring and autumn than other periods. The common bacteria in the two rivers were Betaproteobacteria, Bacteroidetes and Actinobacteria, respectively (Crump and Hobbie 2005). Klammer *et al.* (2002) found that Cytophaga-Flavobacterium varied between seasons in the Tarun River and Traunsee Lake in Austria using FISH technique, with the highest frequency in spring.

Bouskill *et al.* (2010) found that seasonal factors affect microbial diversity in Clark Fork River sediments in Montana, USA, exposed to metal and nutrient contaminations. Microbial activities were negatively related to copper and arsenic and positively related to organic carbon. Less activity of microbes was observed between June and November. Ideal bacterial community composition was recorded in April and November. The common groups in the summer season, as revealed by sequencing from the extracted bands of denaturing gradient gel electrophoresis (DGGE), were Actinobacteria, Deltaproteobacteria and Firmicutes. In the delta of the Sacramento-San Joaquin River in California, Stepanauskas *et al.* (2003) found that the relative abundance of some bacterial phylotypes was significantly different between seasons.

For example, *Microthrix*, which belongs to the Actinobacteria, was common in summer and autumn and was correlated positively with pH but negatively with flow rate, while *Geobacter*, which belongs to the Betaproteobacteria, was common in winter and spring. Febria, Fulthorpe *et al.* (2010) found that bacterial community composition in stream sediments in Ontario in Canada were significantly different between seasons and was attributed to some environmental parameters. Autumn communities were attributed to dissolved organic carbon (DOC), summer communities were attributed to temperature and spring communities were associated with nitrate.

Crump *et al.* (2003) used denaturing gradient gel electrophoresis (DGGE) to study the bacterioplankton community in Toolik Lake in Alaska and found that bacterial composition was shifted in two periods of the year; in spring and early of summer. This was attributed to terrestrial dissolved organic matter deposited into the lake during snow melt in spring, and organic matter provided by phytoplankton at the beginning of summer. The lake contained phyla that are common in other freshwater ecosystems, such as Betaproteobacteria and Alphaproteobacteria. Shade *et al.* (2007) found that the bacterial community composition in eutrophic Mendota Lake in Wisconsin was more similar across years than within years. This was attributed to some environmental factors, such as water temperature, nitrate/nitrite and dissolved oxygen. Wu and Hahn (2006) reported similar seasonal dynamics of *polynucleobacter* (PnecB) in the Mondsee Lake in Austria, and this was attributed to seasonal changes of phosphorus, pH, temperature and chlorophyll a. For example, PnecB abundance was positively related to water temperature but it was negatively related to chlorophyll a. In Crystal Bog and Trout Bog lakes in Wisconsin in the US, Rusak *et al.* (2009) found that diversity and composition of bacterial communities were similar across each lake during 2005, whereas they were different during 2003. Seasonal synchrony in the bacterial community composition was observed during 2003 and was attributed to water temperature. Bacterioplankton community composition in Ria De Aveiro Lake in Portugal, showed significant differences between seasons using DGGE and this was attributed to water temperature. Common bacteria in the lake were Betaproteobacteria and Deltaproteobacteria (Henriques *et al.* 2006). Similar results were found in in Lake Michigan (Mueller-Spitz *et al.* 2009).

Other temporal investigations of bacterial composition have been made in other environments, such as, Gokasho Bay in Japan (Essahale *et al.* 2010) and East Sabine Bay (Moss *et al.* 2006) which found that temporal factors were responsible for the variations in bacterial community composition.

Total bacterial numbers also showed temporal variations in different rivers, such as Słupia River in Poland (Skorczewski and Mudryk 2009), the Ogilvie River in Canada and Swift River in New Hampshire (Albright *et al.* 1980) and Warnow River in Germany (Freese *et al.* 2006).

1.6 The effects of physical, chemical and biological characteristics on bacterial communities

1.6.1 Introduction

Bacterial communities can be influenced by two types of environmental factors: modulators and resources. There is competition between bacteria for the resources, such as nutrients. The modulators, such as temperature, pH and salinity affect the outcome of the competition (Neidhardt *et al.* 1990; Balser *et al.* 2001). Bacteria may exhibit homoeostasis in response to variations of modulators, maintaining their interior environments despite changes in the exterior chemical or physical environments (Russell and Fukunaga 1990). However, bacteria vary in their tolerance to changes in modulators, and increased modulator pressure may alter the composition of bacterial communities, selecting species that are able to tolerate these new environmental conditions (Neidhardt *et al.* 1990).

Conventional competition theory (Tilman 1982; Keddy 2001) has focused on strong competition between pairs of species in which competitive exclusion of one species by another often occurs, but with species coexistence being possible if there is niche differentiation. Early work by Tilman (1977) operationalises this for competition between two planktonic species. The Lotka-Volterra theory provides an overall theoretical framework, with competitive exclusion and co-existence (with limiting cycles) as alternative conditions (Andrewartha and Birch 1953). Even more exotic is the possibility of multiple species co-existing with a pattern of oscillations or chaotic fluctuations (Huisman and Weissing 1999). However, these approaches assume that competition is strong and goes to completion. In the relatively nutrient poor conditions of a freshwater river, bacterial growth will be relatively slow, and competition may be relatively weak. In this context, Hubbell's neutral model (Hubbell 2001) may be more appropriate as a model of multi-species bacterial communities.

The evolution of a community of bacteria in a parcel of water as it moves downstream will depend upon:

1) Population growth rates of the individual OTUs – these may well vary. OTUs with shorter doubling times will make up a greater portion of the community over time.
2) Mortality rates – these may be selective, leading to changes in composition over time or, more likely, be unselective, simply reducing cell density over time. The most likely cause of mortality in planktonic bacteria is grazing, particularly by protists. But in a river, mortality due to grazing is likely to be low, because water flow will prevent the development of dense grazer populations.
3) Addition of bacteria in incoming water (or dilution by incoming water that is low in bacteria).

Assuming that grazing is of limited importance, then the evolution of the community as water moves down a river will depend on inputs of exogenous bacteria and on the relative growth rates of the OTUs present. OTUs are likely to differ in their temperature responsiveness. For

example, an OTU that is resource limited will not increase its population growth rate if temperature increases. So at higher temperatures, it is likely that there will be greater proportional differences between OTUs in population growth rates. In addition, average absolute population growth rates will be higher at higher temperatures. Water velocity is likely to be lower, at least in the UK climate, to a parcel of water that takes longer to move along a given stretch of river. The combined result of these two processes is that the number of cell divisions that occur during the time that it takes for a parcel of water to move downstream will be increased. Therefore, any decline in diversity due to increasing domination of the community by faster growing OTUs will be more important at higher temperatures.

Determining the phylogenetic characteristics of the bacteria in environmental samples and linking these to environmental characteristics has become central to microbial ecology (Dolan 2005; Matcher *et al.* 2011). Also, the main objective in microbial ecology is to determine the main factors affecting bacterial numbers (Selinummi *et al.* 2005).

It is important to characterise the microbial community composition and abundance in water systems as these organisms play key roles in biogeochemical cycles, such as those of nitrogen, sulphur and carbon (Sekiguchi *et al.* 2002; Selinummi *et al.* 2005; Kara and Shade 2009). However, the abiotic and biotic forces that control composition, structure and function of microbial communities are not well known (Lawrence *et al.* 2004; Lindstrom *et al.* 2005; Kent *et al.* 2007). Some studies have investigated the link between richness and composition of bacteria and concentrations of organic matter (Fazi *et al.* 2005), temperature (Hahn 2006) and the role of grazers (Crump *et al.* 2003), but the factors affecting bacterial community composition and dynamics are still poorly understood (Matcher *et al.* 2011).

1.6.2 Physical factors

1.6.2.1 Temperature

Microbes can grow at a wide range of temperatures, from around 0˚C to close to the boiling point of water (Kirschbaum 1995; Kirschbaum 2000).

To understand the bacterial function in any ecosystem, the effects of temperature on bacteria should be determined (Adams 2010). Water temperature is considered to be the key factor driving microbial activity and growth (Sjostedt *et al.* 2012). Freshwater bacteria appear to be more sensitive than other microbes to external factors (Gounot 1991; Shiah and Ducklow 1994; Reche *et al.* 2009).

Shiah and Ducklow (1994) found that the bacterial abundance and growth rate in Chesapeake Bay were positively related to temperature in the range from 3 to 25 ˚C. In temperate lakes in the USA, Hall *et al.* (2009) found that increases in temperature enhanced the positive relationship between bacterial production and bacterial biomass in the summer season.

To determine the biological and chemical role of dissolved organic matter (DOM) in natural cycles, the role of temperature in absorbing DOM should be understood (Kirchman and Rich 1997). Bacterial activities can be controlled and affected by exposure to low water temperature, which interacts with carbon substrates and thereby prevents bacteria from reaching DOM (Adams 2010). So, the slow response by bacteria to the supply of DOM can be observed at low water temperatures (Kirchman and Rich 1997).

Temperature can directly affect bacterial enzymatic activities or indirectly by exchanging bacterial composition (Adams 2010). In arctic streams and lakes, for example, Adams (2010) found that enzymatic activities of the bacterial community were directly influenced by temperature. The productivity of the bacterial community was observed at all temperatures ranging from 6˚C to 20˚C, but its rates were not equal between assemblages. Optimal activities of bacteria have been recorded at 12˚C and 20˚C. High changes in bacterial community composition were also observed and attributed to variations in temperature rather than variations in DOM.

Several studies have investigated the role of temperature in controlling bacterial community composition in freshwater environments. Bacterial community composition differed between winter and summer in three Chinese rivers, with temperature assumed to be the major factor (Zhang *et al.* 2012). Crump and Hobbie (2005) found differences in bacterial community composition in two temperate rivers driven by variations in temperature and other factors like flow rate. Temperature was also the major factor in explaining the variation of bacterial composition and abundance in the River Danube (Winter *et al.* 2007). Lindstrom, Kamst-Van Agterveld *et al.* (2005) observed that the distribution of bacterial taxa belonging to typical freshwater groups in different lakes in Europe was strongly related to variations of temperature and pH. Temperature was the second most important environmental factor explaining the distribution, for example, *Ralstonia pickettii* was most common in lakes with low water temperature. In the Ohio River in the US the abundance of common bacterial groups varied with temperature, for example, Bacteroidetes were most common with high temperatures as revealed by the use of 16S rDNA cloning- sequencing technique (D'Angelo and Nunez 2010).

A positive correlation between water temperature and total bacterial numbers has been reported in the River Hull in the UK (Yamakanamardi and Goulder 1995; Fisher *et al.* 2000), the lower Fraser River in Canada (Albright 1977), Anacostia River in Washington (Cavari *et al.* 1981), Tarun River in Austria (Klammer *et al.* 2002), Brda River in Germany (Małecka and Donderski 2006), Upton Lake in New York (Felip *et al.* 1996) and Tasmanian coastal waters in Australia (Davidson *et al.* 2004). Temperature limited the abundance of bacteria in Upton Lake, despite high concentrations of organic carbon, inorganic and organic phosphorus and nitrogen. (Felip *et al.* 1996). In the River Anacostia, *Aeromonas spp.* was three orders of magnitude more abundant at 25 ˚C than at 4˚C (Cavari *et al.* 1981). However, in the lower Fraser River, degradation of glucose by heterotrophic bacteria was negatively correlated with temperature (Albright 1977).

Significant correlations were also observed between bacterial community composition and temperature in other aquatic environments, for example, the Baltic sea (Degerman *et al.* 2013) in different seawaters (Sjostedt *et al.* 2012) and in Gokasho Bay in Japan (Sakami 2008).

1.6.2.2 Rainfall

Rainfall events can lead to increased transport into rivers of contaminants, organic matter, nutrients and suspended sediment from non-point sources, which may in turn influence bacterial communities (Bae 2013). For example, heterotrophic bacterial counts were positively correlated with rainfall in the Meduxnekeag River, Canada (Bell *et al.* 1982) and Asa River in Japan (Bao *et al.* 2008) and faecal coliforms increased after rainfall events in Bayou Dorcheat in the US (Hill *et al.* 2006), probably as a result of transport from pasture. However, Araujo and Godinho (2008) observed only small fluctuations of the total bacterial abundance between dry and rainy periods in the Pitimbu River and Juqui Lake in Brazil.

1.6.2.3 River flow

River flow rates can be influenced by snowmelt, rainfall events and groundwater flow (Cushing and Allan 2001) and in turn can influence river ecology (Jowett and Richardson 1989; Allan 1995; Poff *et al.* 1997). Flood events can generate spatial patchiness; carry high concentrations of nutrients into the water environment and enable new microbial community dynamics to be established. For example, flood events led to the replacement of 75% of operational taxonomic unites (OTUs) identified using automated ribosomal intergenic analysis (ARISA) in streams in Oman (Abed *et al.* 2011). Similar effects of floods on bacterial composition were observed in South End Greek in Georgia (Kara and Shade 2009) and Santa Ana river in the USA (Ibekwe *et al.* 2012).

Total bacterial numbers increased in the River Brda after flooding (Małecka and Donderski 2006) but in the lowland of Warta River in Poland, river flow was the only one among nine significant parameters that had no correlation with the abundance of bacteria (Wasielewska *et al.* 2009).

1.6.2.4 Total suspended solids (TSS) and free-living bacteria vs. particle-attached bacteria

Total suspended solids (TSS) can comprise both inorganic and organic particles that are small enough to remain in suspension. Their concentrations may increase after disturbance events, potentially leading to increased surface water temperature and reduced light penetration. They may also carry metals, pesticides and other contaminants. All these alterations can impact on

river ecology (Bilotta and Brazier 2008). In the Cértima River in Portugal, bacterial community composition, as revealed by denaturing gradient gel electrophoresis (DGGE), was affected by several factors, including TSS, showing a positive correlation with the abundance of one phylotype of Betaproteobacteria (de Figueiredo *et al.* 2012). In the vicinity of the discharge point of a cassava mill into the Olobshi River in Nigeria, Nwaugo *et al.* (2007) recorded a high abundance of a single bacterial genus such as *Klebsiella spp.* and *Corynebacterium spp.*, attributing this to high TSS. Yamakanamardi and Goulder (1995) found that the total bacterial number as well as total culturable bacteria were both positively related to TSS content in the River Hull in the UK. Suter *et al.* (2011) found that total bacterial numbers in the lower Hudson River estuary in New York, were positively related to turbidity. In addition, in the lowland of the River Warta in Poland, the abundance of bacterioplankton showed strong correlation with TSS (Wasielewska *et al.* 2009).

A key aspect of the ecology of aquatic bacteria is their association with particles (Suter *et al.* 2011). Some studies have shown similarity between bacterial communities associated with different particle sizes. For example, Sinsabaugh *et al.* (1992) used DNA-DNA hybridization to show that the bacterial communities associated with different sizes of fine particulate organic matter in freshwater ecosystems in New York was similar. However, Yeager and Sinsabaugh (1998), using similar methods, found differences in community composition between different particle sizes, and a negative correlation between microbial diversity and particle size. Similar conclusions were reached for Cypress Creek sediments in Texas, with some of the dominant bacteria, such as *Verrucomicrobia-Planctomycetes* being most common on small particles (Jackson and Weeks 2008).

1.6.2.5 Residence time

Residence time is the average time that water spends in a water body. Crump *et al.* (2004) demonstrated that residence time was one of the main factors responsible for the changes of bacterial community composition in the Parker River estuary in Massachusetts, US. It was found by Lindstrom *et al.* (2005) that a short water residence time explained the variability and distribution of bacterial communities in fifteen lakes in Europe.

1.6.3 Chemical factors

1.6.3.1 pH

Variations of water chemistry, such as pH, aluminium and humic acid concentrations play an important role in changing bacterial community composition (Yannarell and Triplett 2004). pH may play an indirect role by altering the chemical speciation of dissolved ions, increasing or decreasing their bioavailability and thus altering growth of taxa which require these as nutrients

or carbon sources (Yannarell and Triplett 2005). Fierer *et al.* (2007) found an interaction between the effects of dissolved organic carbon (DOC) and pH on bacterial community composition in steams and Deanross (1991) showed that pH modulated the effect of Zn on bacteria, with higher total bacterial numbers at low Zn concentration and high pH, and a shift towards more Zn tolerant organisms in high Zn environments.

pH is the environmental variable that has been found to have the greatest effect on freshwater bacterial communities, with greater effects than either temperature or residence time (Lindstrom *et al.* 2005). In 23 streams in the Hubbard Brook catchment in New Hampshire in America, communities were similar at sites with the same pH (Fierer *et al.* 2007). At low pH, Beier *et al.* (2008) found that Acidobacteria and Actinobacteria more common using temperature gradient gel electrophoresis (TGGE), while at higher pH, Proteobacteria are more common.

Kulichevskaya *et al.* (2011) assessed the bacterioplankton community abundance and diversity in some neutral lakes with different trophic status and pH using fluorescent in situ hybridization (FISH). They detected that in neutral lakes where pH = ~ 6.9, natural eutrophic lakes had the highest numbers and diversity of bacterioplankton compared with that in mesotrophic lakes which were highly dominated by phylum Actinobacteria. In acidic lakes with pH = ~ 5.5, the most common bacterial phylum was found to be Acidobacteria.

In different reservoirs in the Pearl River in China (Hong *et al.* 2010) and drinking water distribution systems in Milford (Carter *et al.* 2000) bacterial numbers were positively correlated with pH. However, in some headwater streams in North Carolina, no correlation observed between pH and bacterioplankton concentrations (Palumbo *et al.* 1987).

These results mirror those in soil, where more detailed studies have taken place. Both Rousk *et al.* (2010) and Bartram *et al.* (2013) found that pH values in the range 4 to 8 were positively correlated with abundance and diversity of soil bacteria using denaturing gradient gel electrophoresis (DGGE) and pyrosequencing, with Acidobacteria dominating in acidic soils and Actinobacteria being more common in neutral and alkaline soils.

1.6.3.2 Trophic nutrient status and bacterial communities

One of the most important factors affecting bacterial activities is water trophic status, as organic matter provides carbon sources for microorganisms (Henriques *et al.* 2006; Zeng *et al.* 2011). Organic matter in aquatic environments may be allochthonous (provided by soil and terrestrial plants) and autochthonous (produced by algae and phytoplankton in water) (Fisher *et al.* 2000). Allochthonous organic carbon is often rather recalcitrant, such as humic materials and structural polysaccharides, while labile polysaccharides and proteins are major components of autochthonous DOM (Kirchman *et al.* 2004). In the Hudson River in New York, Kirchman *et al.* (2004) linked changes in the abundance and activity of the major groups of heterotrophic bacteria, such as Alphaproteobacteria and Betaproteobacteria to variations of composition and

concentration of DOM, using fluorescent in situ hybridization (FISH). High phosphatase activity was correlated with the abundance of Betaproteobacteria.

Spring runoff can carry terrestrial organic matter into freshwater, while during summer and autumn, phytoplankton and aquatic plants can produce autochthonous organic matter and support microbial growth and diversity (Fisher *et al.* 2000; Crump *et al.* 2003). Crump *et al.* (2003) found that in lake water, primary production by phytoplankton was much higher than secondary production by bacteria, which represented only 20% of primary production. Bacterial community diversity can be influenced by primary productivity (Kassen *et al.* 2000). Benlloch *et al.* (1995) observed a positive relationship between primary productivity and bacterial community diversity in two coastal lagoons, and others have reported a negative correlation in pristine aquatic sediments (Torsvik *et al.* 1998). In aquatic mesocosms, Horner-Devine *et al.* (2003) and Horner-Devine *et al.* (2004) observed that the relationship between primary productivity and the abundance of dominant bacterial taxa differed between major taxa, with Flavobacteria, Alpha and Beta-Proteobacteria, respectively showing positive, negative, and low correlations.

To understand the role and importance of heterotrophic bacteria in decomposing organic matter in water ecosystems, all aspects of bacterial dynamics, such as bacterial numbers and growth rate should be studied (Barillier and Garnier 1993). The quality of organic and inorganic sources as well as organic carbon is necessary to support and increase bacterial growth rates (Felip *et al.* 1996; Fazi *et al.* 2005). However, responses to these resources by individual bacterial communities are completely different (Ibekwe *et al.* 2012).

Many researchers investigated the correlation between bacterial community composition and diversity and different nutrients, for example, In two Canadian rivers (Meduxnekeag River and Dunbar River), Bell *et al.* (1982) found that ammonia was correlated positively with heterotrophic bacterial diversity. In alpine lakes and reservoirs in the Mediterranean region, Reche *et al.* (2009) found that bacterial production was positively related to dust inputs of particulate matter, with no correlation with bacterial diversity and composition. In aquatic mesocosms, Fisher *et al.* (2000) found that inorganic nitrogen and phosphorus plus carbon had a huge impact on bacterial production, while phosphorus and nitrogen alone had a huge impact on bacterial diversity. De Figueiredo *et al.* (2007) found that trophic status has substantial effects on bacterial diversity in some surface water ecosystems in Portugal using gradient gel electrophoresis (DGGE). Oligotrophic water bodies were dominant by Verrucomicrobia, while Bacteroidetes was the dominant group in mesotrophic and eutrophic waters.

Several studies have found a positive relationship between organic matter and bacterial growth in different environments, for example, different rivers and streams in Québec, France (Comte and del Giorgio 2009), Equatorial Pacific Ocean (Kirchman and Rich 1997), Seine River water, France (Barillier and Garnier 1993) and Warnow River, Germany (Warkentin *et al.* 2011).

Shiah and Ducklow (1994) found that the number and growth rate of bacteria in Chesapeake Bay did not increase, although high concentrations of nutrients were present, and this was attributed to the low water temperature (below 7 ˚C) limiting their growth. However, Kirchman

and Rich (1997) stated that for bacteria to grow effectively in cold water at the same level in warm water, then high concentrations of substrates are needed.

Total bacterial numbers showed a positive relationship with different nutrients, for example, dissolved organic carbon (DOC) in the Ogilvie River in Canada and Swift River in New Hampshire (Albright *et al.* 1980), allochthonous organic matter in the Brda River in Germany (Małecka and Donderski 2006), Chlorophyll *a* in the River Hull, UK (Yamakanamardi and Goulder 1995), Chlorophyll *a* and organic matters in different aquatic environments (Schumann *et al.* 2003), Chlorophyll *a* in the Tarun River in Austria (Klammer *et al.* 2002), total organic carbon (TOC) in drinking water in Milford, US (Carter *et al.* 2000), TOC in different groundwaters in Sweden (Pedersen and Ekendahl 1990), total phosphorus (TP) in six lakes in Canada (Currie 1990) and TP in the Danube River (Velimirov *et al.* 2011).

1.6.3.3 Bacterial tolerance to chemical pollutants

Heavy metals at high concentrations represent a dangerous threat to ecosystem in rivers, and continuous exposure of microbial communities to metals may reduce their diversity and activity, and also change their structure (Vilchez *et al.* 2011). However, some organisms are able to develop to tolerate different concentration levels of heavy metals and may demonstrate a higher abundance than other organisms (Cebron *et al.* 2004). Microbial tolerance to heavy metals represents a very important feature of ecosystems, enabling them to continue their roles in some important processes, such as self-purification and nutrient cycling (Deanross and Mills 1989).

Several studies have investigated the tolerance of some bacterial taxa to heavy metals, such as cadmium, nickel and zinc in the Rémarde River in Paris (Fechner *et al.* 2011), nickel in the Saskatchewan River in Canada (Lawrence *et al.* 2004), zero-valent iron nanoparticles in a the River Thames (a natural river) in the UK (Barnes *et al.* 2010) and lead and copper in Maumee River, St. Mary's River and St. Joseph River in the US (Deanross and Mills 1989).

1.6.3.4 The role of bacterial communities in the bioremediation

Rivers highly loaded with sewage effluents can be inhabited by heterotrophic bacteria (Yamakanamardi and Goulder 1995), and some types of bacteria are able to decompose toxic materials, such as Malathion (pesticide) that is lethal to other organisms (Horner-Devine *et al.* 2004; Dang *et al.* 2010).

Several studies examined the role of some types of bacteria in the bioremediation of aquatic environments, for example, groundwater (Marzorati *et al.* 2006; D'Angelo and Nunez 2010; Vilchez *et al.* 2011), in the River Binlamdoune in Morocco (Essahale *et al.* 2010), in the

Kanzaki River in Japan (Araya *et al.* 2003) and in the Isle River Basin in France (Quemeneur *et al.* 2010).

1.6.4 Biological factors and bacterial communities

Important biotic factors that can influence bacterial survival in water are bacteriophages and protozoa (Pauling and Wagner-Dobler 2006).

Bacteriophages are responsible for about 40% of bacterial mortality rates in freshwater ecosystems, and these rates are higher than that caused by protozoa (Schwalbach *et al.* 2004). Archaea and bacteria are the main hosts to bacteriophages in natural environments (Fuhrman and Schwalbach 2003). However, little is known about their role in regulating bacterial community composition (Fuhrman and Schwalbach 2003; Riesenfeld *et al.* 2004; Wang *et al.* 2010). For example, in a humic lake (bog lake) in northern Wisconsin, Kent *et al.* (2006) reported huge shifts of bacterial composition in the early of summer, and attributed this to changes in the abundance of bacteriophages.

Not all bacterial species within a community can be equally affected by viral infection (Schwalbach *et al.* 2004). Hewson and Fuhrman (2007) found that the abundance of the dominant bacteria in some surface water and sediments was influenced by bacteriophages, giving a chance for the less dominant taxa to grow and coexist. Simek *et al.* (2001) found that different groups of freshwater bacteria showed different responses to viral infection. Also, the abundance of some phylotypes of marine plankton microcosms showed different relationships with the abundance of bacteriophages (Schwalbach *et al.* 2004).

Grazing has been shown in many studies to be the most significant factor affecting the bacterial community composition (Crump *et al.* 2004). The consumption of bacterioplankton by protists is dependent on the sizes of bacteria and also the characteristics of their surfaces (Yokokawa and Nagata 2005). Grazers like protozoa prefer to consume active bacteria rather than inactive (dormant) bacteria (Davidson *et al.* 2004).

Wey *et al.* (2012) examined the effects of grazing by protozoa on bacterial community composition in the Rhine River in Germany, and found a positive link between bacterial richness and flagellates, but this relationship was negative with ciliates. In some surface seawater, Riemann *et al.* (2000) found that three dominant phylotypes of bacteria had disappeared, and this was attributed to the presence of flagellates in high density. Riemann *et al.* (2000) found also that total bacterial numbers decreased by one order of magnitude (from 2.8×10^6 to 7.5×10^5 cells/mL). The negative correlation between predator pressure and bacterioplankton has been shown in different marine microcosms (Gasol *et al.* 1999).

Haynes *et al.* (2007) mentioned that diatoms play an important role in supporting heterotrophic bacteria in estuarine sediments. Polysaccharides and glycoprotein can be produced by them, supporting bacterial growth and changing their composition. However, not all community

members can respond and change. For example, Actinobacteria can be abundant in natural environments when diatoms are present in high density.

1.7 Thesis aims and objectives

As discussed in detail above, molecular approaches have been used much less extensively to characterise bacterial communities in rivers than in either soil or marine environments (Debroas *et al.* 2009).

The main aims of this thesis are: (i) to characterise the bacterial community composition and abundance in a lowland arable catchment using epifluorescence microscopy (EFM), automated ribosomal intergenic analysis (ARISA) and 454 pyrosequencing, and (ii) to determine the effects of spatial and temporal variations and environmental factors on the freshwater bacterial community composition and abundance in an agriculture landscape.

To address these aims, the total bacterial abundance, heterotrophic bacterial counts and bacterial community composition of the River Wensum, a lowland arable catchment in East Anglia, were investigated.

In meeting these aims, the following objectives were undertaken:

- To use epifluorescence microscopy and R2A spread plates to (i) investigate total bacterial numbers in the River Wensum, (ii) to determine spatial and temporal variation and the influence of environmental factors affecting these numbers, (iii) to quantify changes in bacterial numbers as water moves downstream in this lowland arable catchment, and (iv) to determine the relationships between total bacterial numbers and total heterotrophic bacteria.

- To use automated ribosomal intergenic spacer analysis (ARISA) to characterise the bacterial community composition in the lowland arable catchment of the River Wensum and (i) quantify the spatial and temporal variation and impact of environmental factors on this variation, (ii) characterise these changes in terms of both diversity and abundance, (iii) identify the commonest bacterial OTUs and quantify changes in their abundance between sites and times, and (iv) describe the trend of the shift of bacterial diversity and the abundance of the commonest bacterial OTUs when water moves to downstream sites.

- To use 454 pyrosequencing to characterise bacterial communities in the River Wensum including (i) spatial and temporal variations and associations with environmental factors, (ii) to determine the dominant bacterial phyla, (iii) to determine the commonest bacterial OTUs between sites and in time (December 2012), (iv) to describe the trend of the shift of abundance of the commonest bacterial OTUs when water moves to downstream sites, and (v) to identify the taxonomic affinities of the commonest OTUs, based upon the most similar 16S sequences from cultured strains and the most similar

environmental 16S sequences. This is to seek to infer their potential functional significance based on the characteristics of their nearest relatives.

- This research also aimed to use all these findings to characterise the ecological functioning of freshwater bacterial communities in this lowland arable catchment, and (ii) to evaluate whether the results can be used to inform river basin management and the achievement of good ecological status according to the European Water Framework Directive (WFD).

1.8 Thesis outline

This thesis comprises six chapters. The general introduction, Chapter 1, discusses bacterial community composition, structure and abundance and the roles that they play in river ecosystems and the way these are affected by land use, spatial and temporal factors and physicochemical and biological parameters.

Available techniques and methodologies are reviewed in Chapter 2 to identify the most suitable tools and methods for this research, including microscopic and non-microscopic tools for studying bacterial abundance, and fingerprinting and metagenomic techniques for studying bacterial composition.

Chapter 3 investigates total bacterial numbers and total heterotrophic bacterial counts using epifluorescence microscopy and heterotrophic plate counts methods. This provides information on variations in total bacterial numbers and culturable bacteria between sites and times, and the environmental factors driving these variations.

Chapter 4 explores the bacterial composition of the River Wensum using the automated ribosomal intergenic spacer analysis (ARISA) technique. The results of this investigation reflect the spatial and temporal variations of bacterial community composition and the abundance of the common operational taxonomic unites (OTUs). They also provide information on environmental factors affecting these variations.

Chapter 5 examines the bacterial communities in the River Wensum using 454 pyrosequencing. This allows detailed identification of the commonest bacteria and their abundance and taxonomic affinities at all the study sites.

The conclusions from this research and suggestions for future work are presented in Chapter 6.

Chapter Two

A critical review of traditional and molecular techniques used for determining bacterial abundance and composition

The purpose of this chapter is to provide a critical review to identify the most suitable tools and methods for this research, including microscopic and non-microscopic tools for studying bacterial abundance, and fingerprinting and metagenomic techniques for studying bacterial community composition. The suitable tools and methods will be used to characterize bacterial community composition and abundance between sites and times, and also to determine the effect of spatial and temporal factors and environmental parameters on the abundance and diversity of bacterial community.

2.1 Introduction to techniques for determining bacterial abundance

In microbial ecology, a basic task is to quantify the abundance of the microbial community in the targeted environment (Borsheim *et al.* 1990) as the abundance of any microbe is related to its functions and dynamics and to its relationship with other microorganisms. The fluctuations of microbial numbers also reflect the influence of physical and chemical parameters (Daims and Wagner 2007). In many fields, such as water treatment processes and public health areas, it is very important to select fast and accurate techniques for enumerating the total bacterial abundance (Bao *et al.* 2008).

Total cells comprise live (culturable and unculturable) and dead bacteria (Puspita *et al.* 2011). Epifluorescence microscopy gets at total bacterial cells including some that are dead (Seo *et al.* 2010). Live/dead methods seek to identify viable cells (Naganuma 1996). Culturable/unculturable split depends on culture methods and also low stressed organisms are before culturing (Kell *et al.* 1998).

2.2 Culture methods and bacterial abundance

The ability of individual micro-organism, such as bacteria to grow and form distinct colonies on agar medium, is the concept of viability (culturability) (Kell *et al.* 1998).

The culture-based methods, such as most probable number (MPN) and heterotrophic plate counting (HPCs) techniques have long been used as a traditional tool to detect and enumerate

total bacteria in aquatic environment samples (Lepeuple *et al.* 2004). They allow a single bacterium to be grown and shown for a period of time on different kinds of media. But, the selective natures of these media affect the precision of the detection of specific bacterial types (Lemarchand *et al.* 2001).

Different methods culture different fractions of total bacterial numbers. Microbiology traditionally used rich media to pick out faster growers (Wang *et al.* 2010). R2A medium is less rich, but if incubation takes place over a larger period, slower growing operational taxonomic units (OTUs) will be detected (Reasoner and Geldreich 1985). In addition, some bacteria may only be able to grow in mixed species consortia (Schauder and Bassler 2014). Also, some quite important organisms like phosphate precipitators need to be grown in enrichment cultures rather than pure culture (Wang *et al.* 2010).

2.2.1 MPN technique

The most probable number is an indirect tool can be used to determine viable and active populations of microbes in waters and soils. Serial dilutions of replicated cultures should be made and put into tubes or microwell plates and then incubated. These plates and tubes should be pre-inoculated with a suitable test medium, such as pH indicator. The results can indicate the presence or absence of microbes in a dilution series. According to the mathematics of Halvorson and Ziegler (1933), the estimations of the concentration of microbes can be derived. Although the advantage of this method is in terms of reflecting live and active cells, it is a laborious and less precise method compared with microscopic procedures (Halvorson and Ziegler 1933; Oblinger and Koburger 1975; Porter 1979).

The MPN technique has been successfully applied to estimate the levels of faecal coliform and *Escherichia coli* in the Berg River, South Africa (Paulse *et al.* 2007), to count nitrifying bacteria in soil (Papen and von Berg 1998), to determine dilute concentrations of *E coli* in freshwater (Jenkins *et al.* 2009) and to enumerate heterotrophic flagellates in soil (Fredslund *et al.* 2001).

2.2.2 HPC technique

Specific methods that can be used to isolate bacteria through optimizing culture conditions which include the use of different medium contents, incubation time and temperature, normally referred to as heterotrophic plate count (HPCs) (Allen *et al.* 2004). Colonies can be influenced by these conditions due to their sensitivities. As a result, only colonies that are sufficiently grown on the media should be counted (Boulos *et al.* 1999). Different shapes of colonies can be emerged in or on the medium, such as clusters and chains and they can be expressed as colony-forming units (CFU) (APHA 1998).

Public health laboratories have widely used heterotrophic plate counts to determine bacterial concentrations in freshwater specimens and to observe bacterial survival after disinfection at waste water works (Noble *et al.* 1991; APHA 1998). The water quality can be evaluated by measuring heterotrophic bacterial counts in the targeted environment (Carter *et al.* 2000). Several researches have used the HPC technique, for example, to assess water pollution in some subtropical freshwater habitats in Taiwan (Chao *et al.* 2003), to determine the pollution in the Berg River in South Africa (Paulse *et al.* 2007), to assess the quality of the River Danube in Germany (Kavka *et al.* 1996) and to assess the quality of drinking water supplied by Godavari River in India (Rizvi *et al.* 2013). Although HPCs are still the primary tool for evaluating the microbiological quality of aquatic environments, only a small proportion of the total bacterial numbers can be detected by these methods (Hammes *et al.* 2008).

There are three methods used to estimate heterotrophic plate counts, pour plate, spread plate and membrane filtration. There can be variations in HPCs obtained as a result of applying different techniques and media. Of these, the pour plate method is considered to be the simplest one to apply. Up to 2 ml of a sample can be accommodated by it and colonies often do not connect to each other. However, by using this method, bacteria may be exposed to heat shock, affecting and preventing them from growing. Also, the subsequent isolation of these colonies is not easy. The second method is spread plate, in which colonies grow on the surface of the medium and can be easily described. The bacteria are not exposed to heat shock, enabling high numbers to grow. However, a maximum of 0.5 ml of sample can be used, permitting absorption by the medium used. The third method is membrane filtration which it can accommodate large volumes of samples with low heterotrophic bacterial counts. The bacteria are not exposed to heat shock as with the pour plate method. However, filters are expensive and also varied in terms of their quality. In addition, bacteria can be damaged by high filtration pressures (APHA 1998).

2.2.2.1 The media

Four media are in regular use for obtaining microbial plate counts from water; plate count agar PCA (15 g/L agar, 5 g/L tryptone, 2.5 g/L yeast extract and 1 g/L glucose), m-HPC agar (25 g/L gelatin, 20 g/L peptone, 15 g/L agar, 10 ml glycerol), R2A agar (15 g/L agar, 0.5 g/L yeast extract, 0.5 g/L proteose peptone, 0.5 g/L glucose, 0.5 g/L soluble starch, 0.3 g/L K_2HPO_4, 0.3 g/L sodium pyruvate, 0.5 g/L casamino acids and 0.05 g/L $MgSO_4$ anhydrous) and NWRI agar (3 g/L peptone, 0.5 g/L soluble casein, 0.2 g/L K_2HPO_4, 0.05 g/L $MgSO_4$, 0.001 g/L $FeCl_3$ and 15 g/L agar). All these media are varied in terms of their counting results. Both the plate count agar (PCA) and m-HPC agar contain high nutrient concentrations but produce low counts, while R2A agar and NWRI agar contain low nutrient concentrations but produce high counts (APHA 1998; Atlas 2004).

It is very important to design a medium that can recover the highest number of viable heterotrophic bacteria, although there is no specific medium, temperature and incubation time

that can recover all of them from the targeted environment (Reasoner and Geldreich 1985). Allen *et al.* (2004) made some observations in their review of heterotrophic plate count media, one of which is the common employment in the majority of research of the R2A medium for enumerating heterotrophic bacteria.

2.2.2.1.1 Plate count agar (PCA)

The standard plate count method using plate count agar and pour plate (incubation temperature at 35 ˚C for 48 hours) are methods used to enumerate bacteria in waste water and water systems, food and dairy (Reasoner and Geldreich 1985; APHA 1998). It is a nonselective media and suitable for faster growing bacteria (Atlas 2004). However, many researches on water samples have demonstrated that PCA gives low bacterial numbers compared with that obtained using other media and methods, for example, Massa *et al.* (1998) compared PCA and R2A media using spread plates and found that the R2A medium gave 368% higher bacterial counts than those obtained using PCA. The media composition was found to play an important role in deceased or increased bacterial counts, for example, a high concentration of media, such as that used in PCA, can result in a low number of bacterial counts and vice versa (Means *et al.* 1981). Also, Klein and Wu (1974) found that the use of the pour plate method was found to be responsible for the decreases of bacterial counts compared with the spread method, and this was due to bacterial exposure to stresses resulting from the warmed agar.

2.2.2.1.2 R2A media

The failure of the standard plate count using SPA to estimate a high number of heterotrophic bacteria, especially for bacteria that grow slowly, and subculturing for characterization and identification purposes, have led researchers to seek other media to resolve this problem. Reasoner and Geldreich (1985) designed a R2A medium containing low concentration but high diversity of nutrients (0.5 g glucose, 0.5 g yeast extract, 0.5 g soluble starch and 0.5 g casamino acids) than that in plate count agar (PCA). They demonstrated that R2A gave higher counts of heterotrophic bacteria in drinking water compared with PCA. Also, bacterial isolates can be successfully subcultured by the R2A medium which were lost using PCA. Due to the positive relationship between incubation time and total heterotrophic bacterial count and the negative relationship between temperature and heterotrophic bacterial count, authors have recommended a low incubation temperature (20-28˚C) for 5 to 7 days to enable slow growing bacteria to form colonies and give a chance for pigmented bacteria to be appeared. Roszak and Colwell (1987) mentioned that it is often fatal for aquatic bacteria to be exposed to temperatures higher than 20˚C.

Segawa *et al.* (2011) suggested that to succeed in sub-culturing bacteria in the laboratory, a suitable medium should be selected to simulate their real environment. They have used the

R2A and diluted-R2A medium with a spread plate to analyse glacier samples and found that these methods have proven to be the best compared with others, such as LB and xylose agar for obtaining the high bacterial count. They found also that there was low or no differences between bacterial counts on the plates incubated at 4 ˚C and that at 15 ˚C. In addition, the same medium and technique have been used by Kenzaka, Yamaguchi *et al.* (2001) to analyse samples derived from different rivers across Thailand and Malaysia, to discover total culturable bacteria vs. total bacterial numbers, and also to determine the dominant bacterial types responsible for the degradation of organic matter. The R2A medium with seven days incubation time were successfully able to detect the slow growing bacteria, such as pigmented bacteria, which cannot be easily detected by other media, such as PCA (Carter *et al.* 2000). Due to the low concentration of carbon in the R2A medium (e.g. DOC 800 mg /l) compared with other mediums, it is more preferable in studying the quality of drinking water (Hammes *et al.* 2008). The R2A medium with spread plate or membrane filtration applied by Reasoner and Geldreich (1985) on samples collected from water distribution systems have found to be the best techniques for enumerating pigmented bacteria. Those types of bacteria were developed on the R2A medium after 3-5 days at either 35 ˚C or 20 ˚C.

Eutrophs (high substrate concentrations are required for growth) and oligotrophs (low substrate concentrations are required for growth) are two types of freshwater bacteria (Roszak and Colwell 1987). The oligotrophic organisms that live at low concentrations of nutrients in the ecosystems can be recovered through spreading samples onto the R2A medium (APHA 1998). Carter *et al.* (2000) found that R2A medium (low nutrients) recovered highest counts of bacteria compared with plate count agar and sheep blood agar (high nutrients) in samples collected from water distribution systems.

2.2.3 Disadvantages of culture-dependent methods

The main problem of these methods is that they rely on the culturability and viability of microorganisms on the selective media which in turn may lead to an underestimate the total cell counts. For example, due to non-preferable environmental conditions, some microorganisms, such as *Vibrio cholerae* are able to enter a dormant state (non culturable) (Paulse *et al.* 2007). The other problem of traditional methods used to estimate HPC is that serial dilutions of water samples should be made to achieve a countable and acceptable range between 30 to 300 bacterial colonies in each plate, so the dilutions required more plates, media and water samples to be processed (Noble *et al.* 1991). These factors are time consuming, for instance the bacteria need about 3 days to be shown as colonies on agar media incubated at 36 degrees Celsius (Lepeuple *et al.* 2004).

It is known that not all bacteria are able to grow on solid media based on the colony forming cell (CFC) technique (Lisle *et al.* 2004; Skorczewski and Mudryk 2009). However, scientists have long been trying to cultivate as many bacteria as they can through providing them with the same components of nutrients being in their aquatic environment (Wang *et al.* 2010).

Culturable methods are still used due to the fact that culturable bacteria remain the best indicator of the concentrations of organic matter in surface water ecosystems (Skorczewski and Mudryk 2009).

To overcome disadvantages of HPC methods, the development of different techniques, such as epifluorescence microscopy and flow cytometry is needed (Skorczewski and Mudryk 2009).

2.3 Microscopy

The routine tool for determining bacterial cell numbers in aquatic habitats is to use microscopy (Wang *et al.* 2010).

The enumeration of freshwater bacteria is widely measured by fluorescent direct-counting methods (Schallenberg, Kalff et al. 1989). These methods using fluorescence microscopy have enabled scientists to detect more bacterial numbers in water and soil samples by one to four orders of magnitudes compared with that identified by traditional methods like plate-counting methods (Kepner and Pratt 1994). However there are some limitations of these tools, for example, the prolonged uses of the microscopy may affect the operator. Also, dead and living bacteria cannot be distinguished by them (Holm *et al.* 2004). The other limitations that can affect the accuracy of the microscope detection are the uneven distribution of the bacteria on the filter, and the low number of bacterial cells present on filters that should be enumerated (Lebaron *et al.* 1998), and that rare events (single and specific cells) cannot be detected (Lemarchand *et al.* 2001). Also, by the use of microscopy, bacterial numbers may be varied between investigators and this, for example, may result in ambiguity in discriminating between bacteria and particles (Nishimura *et al.* 2006).

Coupling computer image analysis with microscopy has resolved the problems of the use of non-automated counting methods, such as the detection time and operator biases (Daims and Wagner 2007). The more objective and quantitative outcomes of microbial numbers in various habitats can be obtained by automated image analysis (Ogawa *et al.* 2005). For example, Grivet *et al.* (2001) compared automated image analysis and visual analysis in enumerating streptococci and found that just a few seconds were required to count about 600 streptococci in one field using an automated image analyser, while this took 6 minutes when visual analysis was used. Bloem *et al.* (1995) used an automated image analysis system for enumerating soil bacteria and found that in a short time, more quantitative and objective results have been obtained using this system compared with that using traditional visual counting.

2.3.1 Epifluorescence microscopy (EFM)

The advancement of microbial ecology in fresh and marine waters has been supported by the capability and precision of estimating numbers and sizes of bacterioplankton using specific stains and epifluorescence microscopy (Suzuki *et al.* 1993).

Kirchman *et al.* (1982) stated that there are three steps for direct counting of bacteria; using aldehyde to preserve water samples, staining samples with 4',6-diamidino-2-phenylindole (DAPI) or other suitable stains and using polycarbonate filters to collect bacteria during filtration. Then, bacterial numbers can be enumerated under a microscope equipped with blue light excitation or UV light (Bloem *et al.* 1995).

Epifluorescence microscopy has shown to be a successful and accurate tool for estimating the number of bacterial cells (Clarke and Joint 1986; Garren and Azam 2010), sizes of picoplankton assemblages (Sieracki *et al.* 1985), bacterial activity and biomass in freshwater systems (Lisle *et al.* 2004). Researchers generally use EFM with Acridine Orange (AO) and 4',6-diamidino-2-phenylindole (DAPI) stains as standard direct techniques for enumerating total bacterial numbers, sizes and biomass (Hobbie *et al.* 1977; Porter and Feig 1980; Kepner and Pratt 1994; Gasol *et al.* 1999; Yamaguchi *et al.* 2011).

Epifluorescence microscopy can be applied to the most investigated bacteria due to the fact that its optical resolution can meet the sizes of most of them (about 0.2 µm) (Grivet *et al.* 2001), it is also simple, cost effective and is able to be combined with other fluorescence techniques, such as FISH (Nishimura *et al.* 2006). This direct microscopic system is important for determining planktonic populations, which range in size from 0.2 to 2 µm. Also, it produces images that can give good results in numbers and sizes of bacterial populations (Sieracki *et al.* 1985).

Image analysis was firstly used to enumerate bacterial cells in milk after staining with Acridine Orange (AO). Its applications were firstly done by Sieracki *et al.* (1985) who analysed the images from aquatic bacteria produced by epifluorescence microscopy. This image analysis contains an Artek 810 image analyser and Olympus BHT-F EFM and has proven to be an appropriate and rapid technique for bacterial counting, detecting and sizing compared with visual analysis. For example, in this experiment, fifty eight percent of the required time to count bacteria was reduced when images analysis was used.

However, nonbacterial particles present in samples may affect estimates of total cell numbers and reduce the reliability of outcomes, so they should be minimised (Paulse *et al.* 2007). So, in fresh and marine samples that have particles, the enumeration of bacterioplankton by EFM can be affected by the types of systems used, for example Lebaron *et al.* (1998) compared some dyes, such as DAPI and SYBR-II in enumerating bacteria in sea and fresh waters by the use of EFM and FCM tools and found that bacterial numbers obtained by DAPI with EFM were lower than that obtained by other dyes with FCM. Even though more time and labour are needed to meet the microscopy requirements, the improvements of EFM which have been made in terms of, for instance, digital images and various fluorescence stains (Hammes *et al.* 2008), represent

an advance. In addition, Felip *et al.* (2007) stated that the underestimation of total bacterial numbers produced by EFM compared to FCM may be due to the filter pore size used (0.2µm). Bacterial cells smaller than 0.2 µm in size cannot be maintained or collected during sample filtration.

2.3.2 Fluorochromes

For microorganisms to be visualised, staining processes are needed. Under a normal light condition, bacterial cells are not easy to discriminate form the background light. The field of microbiology has been revolutionized by the advent of specific molecular dyes (Bolter *et al.* 2002).

In recent years, valuable and inexpensive techniques for direct counting of total numbers of bacteria in aquatic environments have been developed staining individual cells with fluorochromes (Kirchman *et al.* 1982). These methods are reliable and produce high numbers of targeted bacteria than other methods (Porter and Feig 1980). Bacterial cells to be enumerated under epifluorescence microscopy, one of three different dyes (fluorochromes) is commonly used; SYBR gold (Clarke and Joint 1986; Garren and Azam 2010), 4',6-diamidino-2-phenylindole DAPI (Porter and Feig 1980) and acridine orange (AO) (Hobbie *et al.* 1977). The DAPI and AO are more commonly used to stain and determine number and size of bacteria in aquatic environments (Suzuki *et al.* 1993). The picoplankton communities are routinely estimated by these stains in water environments (Sieracki *et al.* 1985). However, viable, dead and dormant bacterial cells cannot be discriminated by DAPI and AO (Davidson *et al.* 2004).

Kepner and Pratt (1994) reviewed the use of fluorochromes in direct counting of the bacteria and found that during the time between 1940s and 1980s, it was believed that AO was the best fluorochrome when enumerating soil and planktonic bacteria, and was used in approximately 90 per cent of bacterial direct-counting. But since the 1980s, DAPI has replaced AO to determine bacterial abundance as the bacterial stain of choice.

2.3.2.1 DAPI (4', 6-diamidino-2-phenylindole)

The live bacteria compared with other microorganisms are highly and specifically stained with DAPI (Suzuki *et al.* 1993). It is also used to count protozoa in aquatic environments (Bolter *et al.* 2002). Although the common use of DAPI is to enumerate bacterial numbers in marine and aquatic environments, the majority of studies have applied this stain in lakes, ponds and lotic environments (Kepner and Pratt 1994).

The DAPI dye is a specific cytochemical technique for detecting chromosomal DNA does not need specific conditions. The wavelength required for DNA to be visualised is 390 nm; then DNA shows blue but other particles show a slight yellow. As a result, it is easy to discriminate

between bacteria and other particles (Sieracki *et al.* 1985). DAPI binds preferentially with Adenine and Thymine double strand DNA (Bolter *et al.* 2002). So, the DNA sequence that has high percentages of these bases is highly stained with DAPI (Kepner and Pratt 1994). It can also be bound with poly [d (G-C)]. For these sites to be bound with DAPI, pH of the medium should be considered as it plays an important role in controlling this binding (Saby *et al.* 1997). Although, DAPI can be highly excited by xenon or mercury lamps in EFM, it can be also used with other techniques, such as FCM and Scanning Confocal laser microscopy (CLSM) (Bolter *et al.* 2002).

The prior fixation of bacterial cells is not required with DAPI which passes across cell membranes of live and dead bacteria (Hannig *et al.* 2007). More important is that both RNA molecules and particulate matters do not bind with this stain (King and Parker 1988). Although double-strand RNA, protein and cells without nucleotide material (ghost) have been found to interact with DAPI (Zweifel and Hagstrom 1995), DAP does not stain particles and detritus as strongly as AO (Sieracki *et al.* 1985).

DAPI can be combined with tetrazolium dyes; INT [2-(p- iodophenyl)-3-(p-nitrophenyl)-5phenyl tetrazolium chloride] or CTC (5-cyno-2, 3-ditolyl tetrazolium chloride) which detect electron transport activity and then give active cells (King and Parker 1988; Yu *et al.* 1995).

Some researchers compared DAPI with other dyes, such as Backlight and Acridine Orange (AO) and found that DAPI underestimated sizes and numbers of bacteria (Suzuki *et al.* 1993; 2010). However, Davidson *et al.* (2004) revealed that no significant differences between results of total bacterial numbers were obtained using BacLight^{TM} compared with when DAPI was used.

2.3.2.2 DAPI concentration

The optimal concentration of DAPI is required to give accurate estimates of bacterial abundance. The bias in bacterial counts can occur due to different concentrations of DAPI stains (Schallenberg *et al.* 1989). Porter and Feig (1980) found that 0.01 µg/mL of the final concentration of DAPI was enough to stain the bacteria in samples collected from aquatic environments, while the final concentration of AO was 10 µg/mL. However, the increase of particles in samples needs greater concentrations of DAPI than for pure water samples due to the fact that particles can mask the bacteria and prevent them from staining. For example, Schallenberg *et al.* (1989) found that 5.0 µg/mL of DAPI was an optimal concentration to obtain accurate numbers of bacteria in sediments.. Yu *et al.* (1995) demonstrated that 10 mg/L of DAPI is an optimal concentration for staining soil bacteria. They stated that sediments with less water contents but large particles may affect magnification processes when using epifluorescence microscopy and do not give a suitable focus on the targeted bacteria using 100X magnification (Schallenberg *et al.* 1989). However, it is easy to analyse and count bacteria in water samples because the lower background fluorescence in water compared with soil samples (Bloem *et al.* 1995).

2.3.2.3 Ideal time for staining with DAPI

The minimum time for staining with DAPI should be known to reduce the time to be consumed. It is normally longer than the time for Acridine Orange (Kepner and Pratt 1994). For example, Porte and Feig (1980) compared DAPI and AO stains in terms of their proper minimum times for sufficient staining in different water samples and found that the proper minimum time for staining with DAPI was 5 minutes while it was 2 minutes when AO dye is used. Yu *et al.* (1995) found that the required time for staining the total soil bacteria with DAPI was 40 minutes and can give a high number of total bacteria but, it was 8 hours when CTC was used for enumerating active soil bacteria.

2.3.2.4 Factors affecting the stability of DAPI

It is very important to leave samples in the dark after filtration and staining to obtain accurate findings of the total bacteria (Seo *et al.* 2010), because of the fact that the DAPI stain is very light sensitive (Yu *et al.* 1995). Some researchers believe that the ambient light may affect direct counting results, so they prefer to do staining in the dark (Kepner and Pratt 1994).

DAPI can be excited by ultraviolet light and shows blue when binding with DNA (Suzuki *et al.* 1993) The stability of DAPI fluorescence is higher than other dyes exposed to the ultraviolet light using epifluorescence microscopy. For example, Porter and Feig (1980) demonstrated that 3 minutes were a maximum time for DAPI to remain visible under ultraviolet light compared with 1 minute when AO was used, and then dyes can be faded after the continuous exposure to the light beyond these times.

The fluorescence intensity of DAPI-stained bacteria can be reduced through exposing to some physical and chemical factors, such as UV radiation and chlorine. The structure of the DNA can be altered by them, and consequently, bacterial numbers may become underestimated. For example, Saby *et al.* (1997) exposed DAPI-stained bacteria (*Escherichia coli* suspension) to different concentrations of sodium hypochlorite and found that at more than 25mg/L concentration of chlorine, the fluorescence of DAPI-stained bacteria was reduced. The bacteria that have low chromosomal DNA or dead cells and low sizes may weakly stain with 4 '-6-diamidino-2-phenylindole (DAPI), meaning that total numbers of bacteria will be underestimated (Suzuki *et al.* 1993; Seo *et al.* 2010).

Also, the staining property can be influenced by states of the bacterial growth, for example, Berney *et al.* (2007) selected just bacterial cells in the stationary phase to reduce the effects of exponential states of the bacteria on staining properties.

2.3.2.5 Acridine Orange (AO)

Both prokaryotic and eukaryotic cells can be stained with AO (Bolter *et al.* 2002). Bacterial DNA and RNA can be stained with AO but under specific conditions. The emission waves which are required to enable DNA and RNA to be visualised are 436 nm or 490 nm; then DNA of inactive bacteria appears green whereas RNA of active bacteria shows red (Schallenberg *et al.* 1989). Although optimal green fluorescence can be achieved through applying a small amount of AO, its fluorescence will fade quickly at this concentration (Hobbie *et al.* 1977). Detritus, clay and other components of cells can be stained with AO due to the fact that this dye is positively charged (Bolter *et al.* 2002). The main problems of AO use are that detritus and other particles can take up AO dye and so be mistaken for bacteria (Schallenberg *et al.* 1989).

2.3.2.6 LIVE/DEAD BacLight^{TM} kits.

The LIVE/DEAD BacLightTM kit contains two stains, SYTO 9 and propidium iodide PI. Both live and dead cells can be stained with SYTO 9 fluorescence, when used alone, while propidium iodide can only label cells with damaged membrane. The mixture of the two dyes can be binned with bacterial nucleic acid and the total and viable bacterial cells can be easily determined. The bacterial cells with intact membranes are stained with SYTO 9 and show green while bacterial cells with damaged membranes are also stained with PI and show red after mixing with SYTO 9 (Boulos *et al.* 1999; Paulse *et al.* 2007). Active and dead bacterial cells can be discriminated by this kit (Berney *et al.* 2007).

Many researches have applied this techniques for determining bacterial numbers, for example, the BacLight^{TM} kit was applied for the first time to naturally occurring bacteria by Naganuma (1996) and found to give a similar number of intact and dead bacterial cells to that obtained using AO dye. The BacLight^{TM} Kit was applied also by Queric *et al.* (2004) to determine the viability of bacteria in deep-sea sediment. This stain has proven to give reliable and fast data about viable bacteria. It was also able to circumvent the impacts of decompression on the fluorescence intensity. In addition, compared with DAPI, a higher number of total bacteria were obtained and bacterial cells exposed to low activity conditions were easily and highly detected by this kit. Terzieva *et al.* (1996) used the BacLight^{TM} kit to enumerate viable and dead airborne bacteria and also compare it with deferent techniques. The results of nonviable bacteria produced by this kit were in agreement with that for injured bacterial cells produced by a plate count agar tool. Boulos *et al.* (1999) used this kit to enumerate bacteria in drinking water systems and found that a better contrast between two colours (red and green) of bacterial cells, brighter fluorescence and low background fluorescence were obtained. Moreover, it gave the same total bacterial numbers as given by the AO staining tool and also the same viable numbers as given by CTC. However, the viability of bacterial cells was negatively related to some factors, such as fixation with glutaraldehyde and temperature.

The state of a bacterial cell between death and viability is referred to as its intermediate state that cannot be detected by culture-dependent methods, but can be detected by a combination of specific molecules with epifluorescence microscopy or flow cytometry. For example, Berney *et al.* (2007) combined the LIVE/DEAD BacLight^{TM} kit with flow cytometry to assess its role in detecting such stages with known culturable bacteria like *E.coli* recovered from the Glatt River in Switzerland, and found the method to give clear patterns for the description of all physiological states of gram-negative bacteria; live, dead and injured.

However, Boulos *et al.* (1999) concluded that the availability of equipment and qualified workers have limited the BacLight^{TM} kit direct tool from being routinely used.

2.3.2.7 Ethidium bromide (EB)

The DNA of both prokaryotic and eukaryotic cells is easily stained with ethidium bromide (EB). Dead cells can be well indicated using this dye. The permeability of this dye via cell membranes to the cell interior is known to be low. Cell walls and other intracellular contents, clay and detritus cannot be stained with it (Bolter *et al.* 2002). Hannig *et al.* (2007) used this stain to visualise dead bacteria which were shown to be fluorescently orange.

2.3.3 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) can be used to determine microbial numbers, distribution and sizes especially that attached to surfaces (Kenzaka *et al.* 2005). Rapidity and clarity of SEM can be enhanced by the use of filtration to collect bacterial cells before observing them with SEM. The types of filters can play an important role in its rapidity and clarity. For example, Bowden (1977) used SEM to enumerate bacterial numbers have maintained on the surfaces of two types of filters and found that higher numbers of bacteria were obtained when a polycarbonate nuclepore filter was used compared with a cellulose filter.

Several publications have used SEM for estimating bacterial numbers and sizes in different environments. For example, some types of soil bacteria, such as *Bacillus cereus* and *Staphylococcus aureus* have been estimated using SEM by Hagen *et al.* (1968) and bacterial viewing found to be limited when their concentrations were more than 10^7 cells per gram. A new method using automated stage control SEM has been designed and developed by Sanders *et al.* (2012) to enumerate bacteria attached to surfaces and they found to give rapid and accurate estimates of bacterial numbers. This technique also was used by Aoki (2003) to enumerate Testacea (protozoa) in soil and found to give accurate estimates of their numbers after using specific methods to separate them from soil. In the case of bacterial sizes, however, Fuhrman (1981) demonstrated that epifluorescence microscopy has proven to be more accurate than SEM in determining bacterioplankton sizes and this is due to bacterial cell shrinkages that can be produced during the processes of drying bacterial cells before examining with SEM.

However, SEM does not distinguish between live and dead bacteria. It is also labour intensive and requires expensive equipment (Kemp *et al.* 1993; Marie *et al.* 1999).

2.3.4 Flow cytometry (FCM)

The first development of FCM was in the 1960s when it was used by scientists to detect mammalian cells. Since the 1970s, it has also been used in the microbiological field but its popularity and application were hindered by some factors, such as the small size of bacterial cells and the paucity of specific nucleic stains (Wang *et al.* 2010). FCM was considered to be an alternative tool to EFM at the beginning of 1990s to determine numbers of bacteria present in natural ecosystems.

In a short period of time, it is able to enumerate tens of thousands of bacterial cells in a flow system. Reproducibility and objectivity are two of the main features of FCM (Nishimura *et al.* 2006; Felip *et al.* 2007). Flow cytometry is a reliable and multi-parameter technique used to enumerate total numbers of microorganisms and also to assess their physiological states using a wide range of fluorescent dyes. Rapid results can be obtained with FCM compared with microscopy, for example, just five minutes are required per each sample to obtain results compared with approximately twenty minutes using epifluorescence microscopy (Wang *et al.* 2010). Accurate results of certain populations can be achieved by FCM due to the fact that during harsh environmental conditions, some of these populations are able to enter non-culturable states and cannot be detected by other techniques, such as heterotrophic plate count.

The FCM tool has been applied by Holm *et al.* (2004) to enumerate total bacterial numbers in milk and found to have the ability to detect approximately less than 10000 cells per ml. FCM with the Syto 13 stain has been used by Gasol *et al.* (1999) to enumerate and discriminate marine bacterioplankton. Two sub-populations with low DNA and high DNA content were clearly discriminated. The bacteria with low DNA contents corresponded to dead cells, while living cells were represented by the bacteria with high DNA contents. Paulse *et al.* (2007) demonstrated that flow cytometry was the most powerful tool for determining microorganisms in the Berg River in South Africa, it gave higher total numbers of microorganisms than epifluorescence microscopy for the bacteria stained with AO dye (accounted just for 43.08% of the total bacterial numbers identified by FCM). However, Felip *et al.* (2007) compared two techniques, FCM and epifluorescence microscopy in determining bacterial numbers in samples collected from different lakes. Very similar results of total bacterial numbers have been obtained by both techniques, for example, 0.29×10^6 and 0.31×10^6 were the minimum numbers of total bacteria achieved by FCM and EFM, respectively (Felip *et al.* 2007).

However, this technique has some limitations restricting its uses for routine tests, one of which cost in that it requires expensive equipment (Paulse, Jackson et al. 2007). The fewer number of comparative studies on the application of FCM to enumerate microbes in aquatic systems is another issue. A suspension of single cells is required and any clumps and debris in it should

be minimised. Also, FCM data are quite complex (Wang, Hammes et al. 2010). and need well-trained operators to interpret (Nishimura *et al.* 2006).

The microfluidic platform (on-chip flow cytometry) is a direct, fast and automated method that requires just a small volume of sample for bacterial enumeration and also does not need any stains (Bao *et al.* 2008). Because it is a closed device and also many microchips can be easily disposed, so it is biologically not hazardous (Sakamoto *et al.* 2005). The method depends on autofluorescence from the bacterial cell lysate. Then, the cell lysate can be detected by laser (Bao *et al.* 2008). Some researchers have applied this tool to estimate numbers of bacteria, for example, in Neyagawa and Hirano rivers in Japan (Sakamoto *et al.* 2005), in different freshwater samples (Yamaguchi *et al.* 2011) and also with pathogenic bacteria, such as *Escherichia coli* (Bao *et al.* 2008).

2.3.5 Solid-phase cytometry

Although EFM and FCM are commonly used direct techniques in enumerating aquatic bacteria compared with other methods, neither is able to detect rare events (single cells), and this can be performed by the use of a specific stain (Lemarchand *et al.* 2001). For example, Cools *et al.* (2005) used solid-phase cytometry in conjunction with fluorescence dye (carboxyfluorescein ester stain) to detect *Campylobacter jejuni* (viable but non culturable). Also, Lemarchand *et al.* (2001) used this tool in conjunction with SYBER Green II to detect *Escherichia coli*. The authors stated that this tool does not require the large volume of sample to be filtered, Lisle *et al.* (2004) mentioned that this tool is not time consuming and does not overestimate actual bacterial numbers, if bacterial abundance has reduced to less than 10^5. However, the combination of different dyes is impossible because multiple wavelengths of excitation are not available in the solid-phase cytometry tool. It was also applied by Broadaway *et al.* (2003) to count vital bacteria in many water sources, and compered with the R2A agar plate technique. Results of the total number of vital bacteria obtained by solid-phase cytometry in half an hour was identical to that obtained by R2A agar techniques after incubating samples for about 14 days.

2.3.6 Fluorescent in situ hybridization (FISH)

Relative and absolute abundance of bacterial phylotypes can be accurately determined by FISH due to the fact that this technique is not influenced by the variation of the copy numbers of 16S rRNA (a PCR-non-based method) (Stepanauskas *et al.* 2003). Specific bacterial taxa (groups) in freshwater samples can be successfully revealed by FISH using oligonucleotide probes (Bolter *et al.* 2002; Selinummi *et al.* 2005).

Also, the microscopy and flow cytometry tools can be combined with FISH to enumerate fluorescently labelled bacterial cells (Lindstrom *et al.* 2005; Kunin *et al.* 2008).

2.3.7 Other uncommon enumerating methods

There have been several uncommon methods used for enumerating bacteria. For example, the Bioplorer optical system (BP) provided with a blue light source (LED) was applied by Nishimura *et al.* (2006) for enumerating marine bacteria. Also, microbial cells can be enumerated using Immunofluorescence assays through applying antibodies (Daims and Wagner 2007). In addition, the sublimation of Adenine technique was also used by Glavin *et al.* (2004) to enumerate bacterial cell numbers in different natural samples, such as sand and sea water.

2.3.8 Filters and filtration processes

The most reliable types of membrane filters are polycarbonate filters which have been mostly used in enumerating bacteria (Kepner and Pratt 1994). For example, Hobbie *et al.* (1977) compared the efficiency of cellulose and polycarbonate nuclepore filters for direct counting of bacteria. Nuclepore filters retained a large number of bacteria on their surfaces, as they have flat surfaces and uniform pore sizes. In contrast, on cellulose filters, many bacteria cannot be seen because they were held inside the filter, leading to an underestimation of bacterial numbers. To prevent background fluorescence, filters must be stained with black (Kepner and Pratt 1994). The commonly used 0.2 µm pore diameter retains 99% of bacteria on its surface (Hobbie *et al.* 1977). In sediments and freshwater most bacteria are between 0.3 to 0.7 µm in diameter, although, in well water between 0.09 and 0.25 µm represent a significant proportion of bacteria (Kepner and Pratt 1994). Fuhrman (1981) stated that 10% of small bacterial cells can be missed when using 0.2 µm pore size filters. Black filters with pore size of 0.1 µm have recently become available, but filters with a 0.2 µm pore size remain the most commonly used, perhaps because of the paucity of studies using this type of filters to enumerate total bacterial cells (Kepner and Pratt 1994).

It is important when counting bacteria that cells should be visible and remain on the filter surface, and be distinct from other objects on the filter. This can be resolved through the control of staining conditions (Hobbie *et al.* 1977). Numbers of bacteria present on filters affect the precision of counts, and no fewer than 400 cells per filter should be counted (Lisle *et al.* 2004), reducing numbers by dilution to avoid huge numbers of cells per field (Bolter *et al.* 2002).

The minimum numbers of bacterial cells and also numbers of fields that should be counted have been previously investigated (Kirchman *et al.* 1982; Kepner and Pratt 1994; Lisle *et al.* 2004; Chae *et al.* 2008), and also the volume of water sample that should be filtered (Jones and Simon 1975; Kepner and Pratt 1994; Lemarchand *et al.* 2001; Lisle *et al.* 2004; Yamaguchi *et al.* 2011).

To prevent background fluorescence, non-fluorescence immersion oil should be selected (Bolter *et al.* 2002). For example, Wynnwilliams (1985) demonstrated that a lower fluorescence background and increased colour contrast (red, gold and green) between microorganisms was obtained by applying a photo-fading retardant (Citifluor).

2.3.9 Fixation and preservation of water samples

The processing of samples should use sterilized equipment during collection and filtering of samples (Bolter *et al.* 2002). Also, it is preferable to analyse water samples immediately after collection but if not, they should be fixed and stored frozen (Kamiya *et al.* 2007), although this may decrease bacterial counts (Bolter *et al.* 2002). The most commonly used preservative is formaldehyde, but some studies use glutaraldehyde (Kepner and Pratt 1994).

After staining with the dye, slides should be stored at 4˚C and determined no longer than 24 hours later (Yu *et al.* 1995), or kept frozen at -20 ˚C which can prevent changes in total bacterial numbers for up to 70 days (Kepner and Pratt 1994). Bacterial cells can be lost during long storage time of samples (Queric *et al.* 2004).

2.3.10 Researcher bias

The bias of direct counting of bacteria can be produced by different investigators and they should be trained (Kepner and Pratt 1994). However, this remains less than other biases as a result of using different methods, such as numbers of filters and fields that should be counted. For example, Kirchman *et al.* (1982) found that number of fields and filters were responsible for about 80% of the variations of bacterial numbers.

Table 2.1 summarises advantages and disadvantages of some methods used to study bacterial abundance

Table 2. 1 Advantages and disadvantages of some methods used to study bacterial abundance.

Method	Advantages	Disadvantages	References
Plate count agar PCA	Suitable for fast growing bacteria. Inexpensive.	Gives low bacterial counts. Not suitable for subculturing. Underestimate bacterial numbers. Laborious and time consuming. Does not detect unculturable bacteria.	Reasoner and Geldreich (1985) APHA (1998) Massa *et al.* (1998)
R2A agar	Suitable for slow growing bacteria. Gives high bacterial counts. Suitable for subculturing. Inexpensive.	Laborious and time consuming. Does not detect unculturable bacteria.	
Epifluorescence microscopy	Simple, low cost. Can be combined with other tools, such as FISH. Its optical resolution meets the size of most bacteria (0.2 µm).	Laborious and time consuming. Cannot detect rare events.	Nishimura *et al.* (2006) Hammes *et al.* (2008)
DAPI (4',6-diamidino-2-phenylindole)	Prior fixation of bacteria is not required. Does not need specific conditions. Gives higher discrimination between bacteria and particles. Can be combined with other dyes, such as tetrazolium (INT and CTC) and techniques such as FCM.	Very sensitive to light and some chemical materials present in samples such as chlorine.	Sieracki *et al.* (1985) Hannig *et al.* (2007) Kepner and Pratt (1994)
AO (Acridine orange)	Stains DNA and RNA. Stains both Prokaryotic and Eukaryotic cells.	Needs specific conditions. Stains detritus, clay and other components of cells.	Schallenberg *et al.* (1989) Bolter *et al.* (2002)
Flow cytometry	Can enumerate tens of thousands of cells in a short time. Multi-parameter (can enumerate cells and assess the physiological states). Detects rare events. Reliable and reproducible.	Requires expensive equipment. Sophisticated instrumentation and needs well-trained operators. Requires suspension cells with minimum debris.	Felip *et al.* (2007) Wang *et al.* (2010) Paulse *et al.* (2007)

2.4 Molecular-based techniques for studying bacterial communities

2.4.1 Culture-dependent methods

Traditionally, the heterotrophic plate count method was used to study the microbial diversity of freshwaters (Kirk *et al.* 2004) and in the 1980s, based on these culture-dependent methods, it was believed that bacterial species in freshwater habitats were similar to those in soil and marine systems (Hahn 2006). However, many bacterial species cannot be cultivated (Kirk *et al.* 2004) and about 99 % of bacteria in natural environments are not able to grow on widely used media, leading to the overlooking of most species present (Schmeisser *et al.* 2003). Some organisms may inhibit the growth of others, and the spreading of some colonies may hide those with low growth rates or affect their subsequent isolation (Kirk *et al.* 2004). Another drawback is that these methods are not able to differentiate between free-living and particle-associated microorganisms (Plancherel and Cowen 2007).

Culture-based methods can be combined with molecular-based methods to allow detailed characterisation of organisms that can be cultured (Lemarchand *et al.* 2001; Segawa *et al.* 2011). For example, Segawa *et al.* (2011) isolated and characterised 234 of the distinct colonies grown on the R2A agar medium. Also, Hahn (2009) described seven species belonging to the Actinobacteria phylum in freshwaters.

2.4.2 DNA based characterisation of bacterial communities

Microbial communities in the environment are highly diverse (Daims and Wagner 2007), and their full characterisation requires both organism identification and quantification of the abundance of taxa. The limitations inherent with culture-dependent methods have been overcome by the discovery of DNA-based molecular techniques. Huge amounts of data about the taxa and species present in targeted environments can be obtained using molecular methods, particularly those that target 16S rRNA genes, without needing to cultivate individual organisms (Gurtler and Stanisich 1996; Head *et al.* 1998; Ranjard *et al.* 2000). A number of different methods have been used. Cloning and sequencing of 16S genes focuses on organism identification. Fingerprinting methods such as ARISA and DGGE seek to quantify the relative abundance of taxa. Increasingly, the application of high throughput sequencing methods is allowing simultaneous taxon identification and quantification (Fisher and Triplett 1999; Muyzer 1999; Edwards *et al.* 2006; Dall'Agnol *et al.* 2012). In the natural environment, specific microbial taxa can be quantified through designing specific probes (Muyzer *et al.* 1993). These methods give limited information about the functional role of members within a community, but the functional genes can be revealed using shotgun metagenomic (Schmeisser *et al.* 2003) and metatranscriptomic (Gilbert *et al.* 2008; Coll-Lladó *et al.* 2011; Gosalbes *et al.* 2011)

approaches. It has become clear that bacteria in freshwater are taxonomically distinct from bacteria that live in marine or soil environments (Weisse 2006; Mueller-Spitz *et al.* 2009).

2.4.2.1 DNA extraction methods

Most molecular methods are dependent on the extraction of DNA from samples. Methods for this ideally need to be simple, rapid, safe and low cost with good yields of DNA that is free from inhibitors that can reduce the efficiency of PCR amplification (Jara *et al.* 2008). This involves a balance in the selection of cell extraction methods. If the method is too gentle, the lysis efficiency of some microorganisms like gram-positive bacteria may be low. By contrast, harsh extraction methods may shear nucleic acids, meaning that target sequences may not be amplified by PCR (von Wintzingerode *et al.* 1997). Metagenomic techniques, which require high molecular weight DNA, are dependent on DNA extraction and purification approaches. Samples with low DNA content or high concentrations of potentially interfering contaminants may require more elaborate DNA extraction and purification strategies (Seumahu *et al.* 2012).

2.4.3 Non-PCR based methods

2.4.3.1 DNA reassociation

The identification of DNA-DNA similarity is still a standard tool for the description of species (Rossello-Mora and Amann 2001; Kirk *et al.* 2004). DNA is extracted, purified and denatured and the rate of reassociation quantified as the time required for half of the DNA to be reannealed and is expressed by $Cot_{1/2}$ (Kirk *et al.* 2004). This rate is dependent on sequence similarity, and the degree of similarity between two microorganisms can be measured by calculating the difference in thermal denaturation midpoint (Tm) and the relative binding ratio (RBR). If, for example, two DNA samples are mixed and the Tm value is less or equal to 5˚C and the PBR value is equal or more than 70%, the two samples would be classed as belonging to the same species (Rossello-Mora and Amann 2001). This method has been used by Torsvik *et al.* (1990) to study the heterogeneity of soil bacteria by measuring the rate of DNA reassociation in a single (taxonomically heterogeneous) DNA sample. All cultivable prokaryotes can be measured by this method, however, because of the high genetic diversity of the total bacterial populations in soil combined with impurity and degradation of the DNA, an incubation time of several weeks is required to reach 50% DNA reassociation.

2.4.3.2 FISH

Fluorescent in situ hybridization (FISH) uses fluorescently labelled oligonucleotide probes, which hybridise to rRNA in cells that have previously been fixed to increase cell membrane permeability, allowing the enumeration of a particular taxonomic group (Lee *et al.* 1996; Kirk *et al.* 2004; Kunin *et al.* 2008).

The oligonucleotide probes may be designed to target an entire domain, a particular species or any taxonomic level in between. It is, however, time consuming; multiple probes must be used to obtain information on community structure (Lee *et al.* 1996); viability of cells cannot be determined and low rRNA content of cells in oligotrophic environments limits its usefulness outside of eutrophic environments (Kenzaka *et al.* 2001).

2.4.4 PCR-based methods

2.4.4.1 PCR technique

The first application of the polymerase chain reaction (PCR) to environmental samples was to amplify DNA derived from picoplankton in the Sargasso Sea (Giovannoni *et al.* 1990). The diversity of prokaryotes is usually studied using 16S rDNA. Eukaryote communities are often studied using 18S rDNA (Kirk *et al.* 2004) although work on fungal communities often targets the intergenic transcribed spacers (ITS) (Nilsson *et al.* 2009).

When inhibitors are present in environmental samples, DNA may be degraded. A cell lysis buffer is essential for the DNA extraction which can be interfered with and inhibit the polymerase that is necessary for PCR amplification of DNA. It is also important to know that most bacteria produce nucleases that can hydrolyse DNA and prevent it for being amplified (Seumahu *et al.* 2012). To overcome these problems, some researchers have made dilutions of samples before extracting DNA while others have tried to purify nucleic acids (Gutierrez *et al.* 1997). However, these nucleic acids (DNA and RNA) can be lost during purification processes, leading to bias in studies of bacterial diversity (Kirk *et al.* 2004). Also, PCR amplifications of heterogeneous targets are almost inevitably biased, and may give inaccurate estimates of diversity (Yannarell and Triplett 2005).

2.4.4.2 Genetic fingerprinting techniques

The advent of various genetic fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), Temperature gradient gel electrophoresis (TGGE) (Muyzer *et al.* 1993; Muyzer 1999), SSCP (single strand confirmation polymorphisms) (Lee *et al.* 1996),

Amplified ribosomal DNA restriction analysis (ARADA) or restriction fragment length polymorphism (RFLP), terminal restriction fragment polymorphism (T-RFLP) (Liu *et al.* 1997) and automated ribosomal intergenic analysis (ARISA) (Fisher and Triplett 1999), have revolutionized our knowledge of the structure and dynamics of complex microbial communities in freshwater ecosystems and the biotic and abiotic factors affecting them (Yannarell and Triplett 2005; Hahn 2006; Burtscher *et al.* 2009; Humbert *et al.* 2009; de Figueiredo *et al.* 2010). The methods require less effort than clone library construction, allowing more samples to be processed (Ranjard *et al.* 2000). These methods involve PCR amplification of specific genes, such as 16S rRNA genes using specific or universal primers (Muyzer 1999). Then, microbial diversity of the amplified sequences is displayed using gel electrophoresis (for example, RISA or TRFLP or DGGE). The band profiles on the gel will reflect the genetic structure of the microbial community. Excising bands from the gel, cloning and sequencing can be done to establish the phylogenetic groups present (Ranjard *et al.* 2000). However, these methods are subject to PCR biases as primers will not hybridise with equal efficiency to all sequences present (Clement *et al.* 1998; Jones *et al.* 2007).

2.4.4.2.1 RISA/ARISA

RISA and ARISA examine length variation of the intergenic spacer regions (ITS) between 16S rRNA and 23S rRNA. RISA uses manual electrophoresis to quantify fragments present (Kirk *et al.* 2004), whereas ARISA does this using automatic DNA sequencers after labelling the forward primer with a fluorescent dye (Fisher and Triplett 1999). The peak in the electropherogram is sized using a size standard and OTU abundance estimated from peak areas or heights (Cardinale *et al.* 2004). Large DNA fragment sizes of up to 1,400 base pair (bp) in length can be separated by this technique (Fisher and Triplett 1999).

ARISA is inexpensive, rapid and reproducible, and can track and characterize microbial diversity and composition in different environments over temporal and spatial variations (Fisher and Triplett 1999; Brown *et al.* 2005). Because it is automated, bacterial composition, diversity and structure can be easily analysed in a large number of samples (Crump *et al.* 2003; Cardinale *et al.* 2004). The number of OTUs detected per sample by ARISA ranges from 38 to 232 (Fisher and Triplett 1999; Ranjard *et al.* 2000). However, it is limited by PCR biases like other fingerprinting tools (Kirk *et al.* 2004) and it is difficult to identify the organisms responsible for particular ARISA fragments as the majority of ITS sequences deposited in the National Centre for Biotechnology Information (GenBank, http://www.ncbi.nlm.nih.gov/) represent cultivated microorganisms and clinical strains (Brown *et al.* 2005). Also, the same lengths of ITS region can be found in unrelated organisms or multiple ITS lengths may found in the same species or even within different rRNA operons within the same bacterial isolate. However, ARISA patterns are reproducible. Multiple amplifications of the same sample give the same peak intensity and OTU number, and this pattern is not altered by changes in PCR cycle numbers (Brown *et al.* 2005; Kara and Shade 2009) .

Several studies have investigated the efficiency and robustness of ARISA in characterizing bacterial communities in aquatic environments. Diversity and composition of three different communities of freshwater bacteria were evaluated by Fisher and Triplett (1999) who found different patterns in the three communities but the same number of fragment sizes. These results led the authors to propose that ARISA was an effective and rapid tool for estimating bacterial community diversity and for tracking temporal and spatial variations in composition. Schwalbach *et al.* (2004) assessed the changes of the abundance of some marine bacterial phylotypes exposed to different viral treatments using ARISA and TRFLP. Both methods detected changes in composition, but gave the same number of taxa. Lear and Lewis (2009) used ARISA to reveal impacts of different land use on bacterial communities in freshwaters. Danovaro *et al.* (2006) found that ARISA and T-RFLP were equally effective in discriminating between *Pseudomonas* isolates in different aquatic habitats. However, some studies have observed higher abundance and diversity of bacteria using ARISA.

Some modifications of ARISA may increase its sensitivity. Quantitative-ARISA aims to estimate the number of DNA fragment sizes present by making different dilutions of samples. Ramette (2009) used this to investigate microbial community richness in marine sediments. Nested ARISA can be used to test a large number of low volume samples or that might contain a small biomass of microbes. Lear and Lewis (2009) used this to investigate the influence of anthropogenic activities on bacterial community structure in four streams in New Zealand.

1- Intergenic transcribed spacer (ITS) region

Ribosomes are essential for protein synthesis and all bacteria have ribosomal operons containing 16S rRNA genes, 23S rRNA genes and 5S rRNA genes (Brown and Fuhrman 2005; Wolska and Szweda 2012). These contain approximately 1650, 3300 and 120 base pairs, respectively, and are highly conserved during evolution (Rossello-Mora and Amann 2001). The internal transcribed spacer ITS region between the 16S rRNA and 23S rRNA genes can contain 0, 1 or 2 tRNAs (Barry *et al.* 1991), but apart from these sections, it is less conserved than 16S or 23S (Brown and Fuhrman 2005). The ITS region contributes to the correct folding of nascent rRNA as it contains anti-termination motifs (Dall'Agnol *et al.* 2012). The ITS region varies greatly in length and nucleotide sequences because of the fact that the sequences of this region are less conservative among bacteria. This high variability makes it suitable for detecting differences between bacterial strains, and closely related species (Fisher and Triplett 1999; Fisher *et al.* 2000; Brown and Fuhrman 2005). For example, closely related strains of Cyanobacteria, *Prochlorococcus* or *Synechococcus* have been successfully delineated using ITS sequences by Rocap *et al.* (2002) and Dall'Agnol *et al.* (2012). Man *et al.* (2010) investigated the efficiency of three 16S rRNA, ITS and 23S rRNA in identifying and differentiating species and strains of *Campylobacter* and found that the ITS region had the best discriminatory power. They concluded that ITS genes require less effort and time to be amplified, sequenced and assembled compared with others. For example, to amplify and

sequence the complete ITS genes (~ 1000 base pair in length), just one pair of primers could be used compared with 4 and 8 different primers for 16S rRNA and 23S rRNA. However, more than a number of copies can be produced from a single bacterial cell and in turn may lead to the overestimation of bacterial diversity in the targeted environment (Dall'Agnol *et al.* 2012).

Brown and Fuhrman (2005) compared the efficiency of ITS genes and 16S rRNA in revealing the diversity of marine bacteria. They found that ITS genes gave information on fine-scale phylogeny and allow more detailed discrimination of spatial patterns than 16S rRNA gene analysis. Large numbers of bacterial lineages have been identified using ITS genes. They concluded that the ITS region was the best marker for microbial diversity and biogeographical research.

Studies based on RNA are more accurate than those based on DNA in describing the active members of a microbial community. Active cells contain higher concentrations of ribosomes, and RNA is degraded much more rapidly after cell death (Revetta *et al.* 2010). However, ARISA fingerprints cannot be obtained from RNA, as ribosomal RNA is rapidly cleaved from a transcribed spacer after synthesis.

2- Primers

ARISA, like other approaches involving PCR amplification of heterogeneous targets, requires an appropriate choice of primer pairs that efficiently amplify sequences from a broad range of organisms, ideally without taxonomic bias (Gurtler and Stanisich 1996). Cardinale *et al.* (2004) assessed the efficiency of three different ARISA primer sets (1406F/23S, ITSF/ITSReub and S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18). They found that the ITSF/ITSReub primer pair that they had designed generated a higher number of more reproducible peaks, with a wide range of spacer sizes. The ITSF/ITSReub primer set was also more sensitive, and could detect OTUs that represented only 0.1% of the total DNA. Jones *et al.* (2007) examined the efficiency of different primer sets (ITSF/ITSReub, cITSF/ITSReub and 1406f/23Sr) in determining bacterial community composition in Lake Mendota. The different primer pairs generated different community profiles but the conserved ecotypes of the bacterial community were observed and all pairs showed similar patterns. There is however, no universal primer set that can be used to amplify all microbial taxonomic groups by PCR with the same efficiency. As a consequence, no primer sets give an entirely unbiased picture of microbial communities (Jones *et al.* 2007).

2.4.4.2.2 DGGE and TGGE

DGGE and TGGE are similar techniques, introduced by Muyzer *et al.* (1993) and used to characterise complex microbial communities in natural environments. Distinct bands were successfully obtained, allowing them to characterise community diversity and shifts in community composition (Muyzer *et al.* 1993; Muyzer 1999).

16S rDNA regions are amplified by PCR using universal primers. Then, the resulting DNA fragments are electrophoresed on polyacrylamide gels, which either contain a gradient of denaturants, such as formamide and urea, or maintained at a temperature gradient. The DNA fragments are then separated based on their sequences. Bands can be re-amplified, cloned and sequenced to identify the organism that corresponds to each band (Muyzer *et al.* 1993; Muyzer 1999) or used as probes in hybridization methods such as FISH (Kirk *et al.* 2004).

DGGE has been used to study bacterial community composition and diversity in reservoirs (Yan *et al.* 2008), estuaries (Castle and Kirchman 2004), seawater (Riemann *et al.* 2008), sediments (Lai *et al.* 2006) and soil (Gelsomino and Cacco 2006).

DGGE and TGGE are claimed to be rapid, reliable and reproducible, allowing large numbers of samples to be processed (Muyzer 1999). However, they have low sensitivity in detecting rare members of bacterial communities, are subject to PCR and extraction biases and can be affected by the formation of heteroduplex molecules (Muyzer 1999). Primers need to include a 35-40 bp GC clamp to keep the part or most of the DNA as double stranded during separation on the gel and this may produce artefacts during the PCR annealing steps (Lee *et al.* 1996). In addition, different sequences may show similar migration patterns, meaning that one band may represent more than one bacterial species (Kirk *et al.* 2004). Large DNA fragments sizes cannot be separated on DGGE gels, so the methods are limited to DNA fragments between 300 to 500 base pairs long (Vallaeys *et al.* 1997).

2.4.4.2.3 SSCP (single strand confirmation polymorphisms)

Like TGGE/DGGE, SSCP depends on the separation of DNA fragments of the same size, but with DNA sequences on polyacrylamide gels. The separation is based on the formation of secondary structure in single stranded DNA, and the method is simple and does not require the use of radioactivity (Lee *et al.* 1996). SSCP was originally developed to discover mutations in DNA and to determine known and novel polymorphisms (Kirk *et al.* 2004). It can detect small changes in DNA sequences and the low percentage of bacterial population in a whole community can be detected as well. Unlike DGGE, no GC clamp is required and unlike T-RFLP, does not require the use of restriction digestions (Lee *et al.* 1996). It can be used as an alternative to RFLP for improving the discriminatory power of the ITS-PCR method distinguishing DNA fragments with different sequences but having similar lengths (Wolska and Szweda 2012).

However, SSCP can be affected by different factors, and small differences in gel matrix and temperature can alter the resulting fingerprints (Liu and Sommer 1994). It also suffers many of the disadvantages of DGGE, including problems caused by heteroduplex formation (Kirk *et al.* 2004).

2.4.4.2.4 ARDRA (Amplified ribosomal DNA restriction analysis/ restriction fragment length polymorphism (RFLP)

Microbial diversity can be studied using RFLP or ARADA to detect DNA polymorphisms (Kirk *et al.* 2004). After amplifying 16S rDNA and digesting with restriction enzymes, restriction fragment polymorphism is displayed using agarose electrophoresis (Clement *et al.* 1998). The method was first used on cultivated isolates before sequencing became routine. The method can be applied to the whole microbial community, when the RFLP profiles reflect all restriction fragments for the dominant members of the whole community, and common bands can be cloned and sequenced. Alternatively, it can be used to select 16S clones for sequencing, to avoid duplicates of common OTUs (Tiedje *et al.* 1999). Smit *et al.* (1997) used ARDRA to determine microbial community structure of soil environment.

However, variable numbers of fragments per strain can be obtained using ARADA, while with T-RFLP, one fragment per unique ribosomal operon can be obtained, which is often equivalent to one per strain (Clement *et al.* 1998; Tiedje *et al.* 1999).

2.4.4.2.5 Terminal restriction fragment length polymorphism (T-RFLP)

Complex microbial communities can be detected by T-RFLP, originally described by Liu *et al.* (1997). 16S rDNA is amplified using fluorescently labelled primers, and the products digested with two to four restriction enzymes, such as *Hha*I and *Rsa*I. The labelled fragments are detected on an ABI sequencer, sized and quantified (Liu *et al.* 1997; Clement *et al.* 1998; Tiedje *et al.* 1999). This has marked similarities to ARDRA, but as only terminal fragments are labelled, the resulting fingerprints are simpler (Kirk *et al.* 2004).

Many different species can contain the same TRF length produced by the same restriction enzyme, so multiple TRFLPs produced by different restriction enzymes are usually combined (Clement *et al.* 1998).

However, as other fingerprinting methods, it is biased by the PCR amplification (Clement *et al.* 1998) and the use of different DNA extraction methods (Tiedje *et al.* 1999).

Moeseneder *et al.* (1999) compared T-RFLP and DGGE in terms of their sensitivity and precise detection of OTUs when applied to determine microbial communities. The method produced the same clustering of samples but T-RFLP detected more OTUs. However, it is much harder

to obtain DNA sequences corresponding to particular sized fragments than it is for the DGGE (Thiyagarajan *et al.* 2010).

T-RFLP has been used to study bacterial community composition in various environments, including lake sediments (Zhao *et al.* 2012), sea water (Stoica 2009), estuaries (Moss *et al.* 2006), river water (Ibekwe *et al.* 2012) and soil (Kuske *et al.* 2002).

2.4.4.2.6 Repetitive extragenic palindromic-PCR (Rep-PCR)

Rep-PCR is a fine-scale fingerprinting tool that can be used to discriminate strains (Tiedje *et al.* 1999). The high repetition of short DNA sequences on gene locations (between 1 to 10 base pairs in length) can be found in many types of prokaryote. These locations on the gene are referred to as microsatellite regions. Their sequences can be excised using *rep*-PCR, making it possible to fingerprint the bacterial diversity and find out the differences between bacterial strains (Kirk *et al.* 2004). Different primer sets can be used for PCR amplification of each one of the three repetitive and conserved DNA sequences, repetitive extragenic Palindromic (REP) (35 to 40 bp), enterobacterial repetitive intergenic consensus (ERIC) (124 to127 bp) and the BOX DNA sequence (154 bp). Then, DNA can be separated on a gel, stained and interpreted. The bacterial isolates can be discriminated at species, subspecies and strain level using this method and can also be applied for human epidemiological research. The *rep*-PCR tool is fast, reproducible, easy to use and not expensive (Wolska and Szweda 2012).

This method is most useful for identification of individual isolates (but it cannot be used on a complex community) (Tiedje *et al.* 1999). In addition, microsatellite sequence should be known before selecting suitable primers (Fisher and Triplett 1999; Kirk *et al.* 2004). Also, isolates are required in this test.

Table 2.2 summarises advantages and disadvantages of some methods used to study bacterial composition.

Table 2. 2 Advantages and disadvantages of some methods used to study bacterial composition.

Method	Advantages	Disadvantages	References
ARISA (Automated ribosomal intergenic spacer)	Rapid. Reproducible. Inexpensive. Robust. Automated so it can analyse large numbers of samples simultaneously. Compares different microbial communities.	Still subject to PCR biases. Difficult to identify the organisms responsible for a particular ARISA fragment size.	Fisher and Triplett (1999) Cardinale *et al.* (2004) Brown *et al.* (2005) Kirk *et al.* (2004)
DGGE and TGGE (Denaturing/temperature gradient gel electrophoresis)	Reproducible. Reliable. Automated so it can analyse large numbers of samples simultaneously. Compares different microbial communities.	Low sensitivity to detect rare members. PCR biases. Can be affected by the formation of heteroduplex molecules. More than one species can be represented by one band. Large DNA fragments cannot be separated on the gel. GC clamp is required.	Muyzer (1999) Kirk *et al.* (2004) Vallaeys *et al.* (1997)
T-RFLP (Terminal restriction fragment length polymorphism)	Automated so it can analyse large numbers of samples simultaneously. Reproducible. Compares different microbial communities.	Biases by PCR and different DNA extraction methods. Difficult to identify the organisms responsible for particular TRFLP fragment size.	Kirk *et al.* (2004) Tiedje *et al.* (1999) Thiyagarajan *et al.* (2010)
SSCP (Single strand confirmation polymorphisms)	Simple. Can detect small changes in DNA sequences. No GC clamp is required (unlike DGGE). Radioactivity is not required. Restriction digestions are not required (unlike T-RFLP). Microbial communities can be identified.	Resulting fingerprints can be affected by small differences in gel matrix and temperature. Can be affected by the formation of heteroduplex molecules. PCR biases.	Kirk *et al.* (2004) Lee *et al.* (1996) Liu and Sommer (1994)

2.5 Bacterial communities identification using next generation methods

2.5.1 16S rRNA gene marker, cloning and sequencing

Microbial diversity as one of many aspects in microbial ecology can be highly explored by amplifying, cloning and sequencing of 16S rRNA genes (Muyzer 1999; Tiedje *et al.* 1999). The 16S rRNA gene is considered to be a standard marker that has long been utilized for rapid identification of microbial phylogeny including all phyla of bacterial communities (Dall'Agnol *et al.* 2012). The length of 16S rRNA is (~ 1500 bp) and approximately ubiquitous in all bacterial members (Man *et al.* 2010). A large database of microbial 16S rRNA sequences is available to meet the requirements of comparison studies among bacteria and more than 1 million sequences are available for classification purposes (Steven *et al.* 2012; Jaziri *et al.* 2014).

On the 16S rRNA gene, sequences of bacterial species are highly conserved at 5' and 3' ends of sequence locations. It also contains nine hypervariable regions (V1-V9). Although, many studies have demonstrated that hypervariable regions can be used as a measure for the taxonomic classification of microbes in environmental samples, sequence analysis of entire conserved regions of 16S rRNA is still considered to be the standard tool (Matcher *et al.* 2011). Because the conservative nature of sequences of 16S rRNA genes between bacteria, those genes of different bacterial species can be amplified by one pair of universal primers (Wolska and Szweda 2012).

To create a library through cloning of 16S rRNA, a specific region of 16S rRNA is amplified, and inserted into transformants (plasmid vectors based on plasmid that is found naturally in *E. coli*) so it could be cloned, screened and sequenced. The sequences can then be used to apply phylogenetic analysis (Osborn and Smith 2005). However, cloning takes a long time to process (Clement *et al.* 1998), with low throughput but can easily identify the clones that represent the dominant sequences (Osborn and Smith 2005).

Complete sequencing of 16S rRNA has become routine and popular and it is more preferable than using the 23S rRNA gene due to its large nucleotides length (3300 nucleotide) (Rossello-Mora and Amann 2001). Different methods are used for DNA sequencing, such as Sanger and next generation sequencing. Sanger sequencing is a traditional method based on the modification of normal nucleotides to dideoxy nucleotide triphosphate (ddNTPs) (Sanger *et al.* 1977). The sanger method was first described by Sanger, Nicklen *et al.* (1977) and applied to the study the DNA of bacteriophage. Since then, a few hundred cloned sequences per sample can be analysed by capillary electrophoresis, but only the dominant phylotypes within a given community will be known (Matcher *et al.* 2011). The next generation approaches, such as 454 pyrosequencing and Illumina are different from the Sanger method. They represent low cost per base and high throughput, performing massively parallel sequencing (Jia *et al.* 2013).

However, after intensive work to amplify, clone and sequence 16S rDNA in order to determine its diversity, just the quantitative information about microbial community composition can be achieved. Species that represent a small fraction of the total community cannot be detected by this technique (Muyzer *et al.* 1993). As 16S sequences evolve relatively slowly, it is of limited use to characterise bacterial community diversity at species and subspecies levels (O'Sullivan *et al.* 2002). 16S rRNA can be influenced by PCR biases and the so number of copies can vary between bacterial species as a result (Kunin *et al.* 2008). Phenotypic diversity of microorganisms cannot be inferred using this marker as well (Dall'Agnol *et al.* 2012). The cloning of 16S rRNA genes is laborious and expensive, so it is not suitable for monitoring successional shifts of complex microbial communities over time and places. Genetic fingerprinting techniques, such as DGGE are needed to achieve this purpose (Muyzer 1999).

2.5.2 Metagenomic approaches

Metagenomic is the use of culture-independent methods to analyse the sequences and functions of genomes from mixed communities recovered from environmental samples. They are also referred to as shotgun sequencing or environmental DNA libraries (Riesenfeld *et al.* 2004; Petrosino *et al.* 2009). Metagenomic approaches study the abundance and identity of bacteria. Next generation approach methods, such as 454 pyrosequencing and Illumina, play a key role in the development of metagenomic studies, making the possibility of metagenomic to be applied in many areas (Jia *et al.* 2013).

Metatranscriptomics are techniques used to study the genes expression, retrieving mRNAs from microbiota. However, it is not easy to determine the gene expression of prokaryotic due to the fact that mRNAs in these creatures are difficult to isolate, they have a short half-life and they represent just small portion of the total RNA (Gosalbes *et al.* 2011).

Metagenomic has been applied to assess microbial community in different environments, such as terrestrial and aquatic environments, oral cavities and faeces (Healy *et al.* 1995; Handelsman *et al.* 1998; Riesenfeld *et al.* 2004; DeLong 2005). However, freshwater ecosystems have received only little attention in metagenomic researches compared with marine and soil ecosystems (Debroas *et al.* 2009). For example, Alos *et al.* (2005) studied bacterial diversity in Delaware River, and Pope and Patel (2008) studied toxic cyanobacteria blooms in freshwater.

However, metagenomics do not provide the equal sequencing of all genes present in environmental samples (Kembel *et al.* 2011). Metagenomics also cannot indicate which genes can express only viable cells or whole cells (Gosalbes *et al.* 2011). The development of metagenomic analysis is not equal for all targeted environments. For example, the method is slow in soil compared with seawater due to the matrixes or inhibitors that can be found in soil and may be affected in the cloning of the DNA. The difficulty of sequencing and assembly processes can increase with increases in species richness per ml (water) or gram (soil) in the targeted environment. The sizes of metagenomic libraries are also dependent on this richness

which will be, for example, 500 Gbp in terms of the soil environment (4000 species richness), making the interpretation of the data from metagenomic libraries more difficult (Riesenfeld *et al.* 2004). Databases of metagenomics (shotgun metagenomic data) still need to be developed (Steven *et al.* 2012).

2.5.3 Next generation sequencing

Recently, a number of high throughput sequencing technologies have been developed and used to characterise microbial communities, with 454 sequencing (also known as pyrosequencing) being the most common approach (Amend *et al.* 2010). This is an alternative to traditional Sanger sequencing, able to sequence up to a million DNA molecules simultaneously. Read lengths have steadily increased over time, and the technology currently has a model read length of 700 bp per molecule (output is 0.7 Gb per run within 24 hours) (Harbers and Kahl 2012).

Pyrosequencing does not require cloning and can be applied directly to environmental samples (Kunin *et al.* 2008) and PCR-amplified DNA. This allows identification of the dominant phylotypes but also reveals rare members of the bacterial population. It can also be used to determine the relative abundance of bacterial phylotypes (Matcher *et al.* 2011). The read abundance derived from 454 pyrosequencing data normally reflects the abundance of taxon. This abundance can be used as a helpful measure for quantitative comparisons of the similarity within a bacterial community. The dominant members of any community should dominate the pyrosequencing data. However, it should be taken into account that ribosomal gene copy numbers can be varied among microorganisms due to the innate structure of DNA sequences. For example, one order of magnitude in the variation of ribosomal gene copy numbers has been recorded between bacterial species. Consequently, the relative abundance of genes quantified by 454 pyrosequencing reads may be altered and biased (Amend *et al.* 2010). However, due to the lack of meiotic recombination within bacterial species compared with that in fungi, ribosomal gene copy numbers within bacterial species are more stable and can be quantitatively used as a measure of read abundance (Amend *et al.* 2010). Bacterial abundance using 454 pyrosequencing can be biased by PCR amplifications, types of primers used and different DNA extraction methods (Amend *et al.* 2010). 454 pyrosequencing is also expensive and inherited by homopolymer errors (Liu *et al.* 2012).

The 454 pyrosequencing technique can be carried out using the pair of primers 27F/338R which was used by Suzuki and Giovannoni (1996) to study a mixture of different 16S rRNA gene templates retrieved from marine bacteria and found to give a good quantity of information. The 27F/338R primers targeting hypervariable regions (V1 and V2) of 16S rRNA genes have also been chosen by Lauber *et al.* (2009) to assess soil bacterial community structure along pH gradients. The reason of this choice was due to the fact that this targeted region amplified by 27F/338R primers has proven to give accurate taxonomic classification of microbes compared with other 16S rRNA positions (Liu *et al.* 2007).

To allow the large scale genotyping of different samples in a single sequencing run, barcodes (tags) can be used with 454 pyrosequencing and these can be subsequently detected by suitable software (Jones *et al.* 2009). The unique tags should be first added to primers. Each known tagged primer can then be used to amplify a specific sample. Multiple samples can be pooled together and sequenced by 454 pyrosequencing. Then, the specific generated sequences of each sample can be detected based on its unique tag (Binladen *et al.* 2007). Parameswaran *et al.* (2007) described a 454 pyrosequencing-tailored barcoding method and used it to resolve ambiguity during DNA sequence assignments. They found that the low mis-assignment rates of sequences (less than 0.005%) obtained as a result. Barcode approaches, however, are limited by the number of unique tags they utilize and the ability of these tags to identify sequencing errors, which may then affect subsequent samples assignments. However, Hamady *et al.* (2008) constructed error-correcting barcodes and used these to process more than 1,500 freshwater samples together in a single sequencing run. They found that about 92% of sample assignment errors have been corrected using this constructed approach.

454 sequencing relies on the detection of pyrophosphate to terminate DNA chain amplification. The first single-stranded DNA molecule is prepared by the denaturation of DNAs and adapters. Single DNA is captured by amplification beads, and the polymerase is elongated using dNTPs. If the dNTP is incorporated, pyrophosphate is released (equal to the incorporated nucleotide) and it is then converted into ATP by sulfurylase. ATP is used by luciferase to oxidize luciferin and generate light. The light appears as peaks representing each one of nucleotides incorporated, enabling the whole sequence to be read. If, however, dNTP is not incorporated, no pyrophosphate will be released and no light will be generated (Liu *et al.* 2012).

The first study conducted using pyrosequencing in environmental samples was performed by Edwards *et al.* (2006) to study microbial communities in water and sediments at two sites in Soudan Mine groundwater in the US. The technique revealed different microbial communities between the two sites which were distinct from that reported previously. Thompson *et al.* (2011) used the pyrosequencing technique to analyse the V6 hypervariable region of 16S rRNA genes obtained from bacterial communities in coastal and brackish samples in Latin America and found that about 80 % of total OTUs (134,197 high quality sequences) belonged to Proteobacteria, Bacteroidetes, Cyanobacteria and Actinobacteria. The use of this high-throughput technique in this study also revealed new taxa that have not been described previously. In the Amazon River Ghai, *et al.* (2011) utilized 454 pyrosequencing tool to analyse water samples from sites free of contaminants and human influences and with natural pH. The results of this technique revealed that the most dominant phyla in the river water were Actinobacteria and Proteobacteria. The Actinobacteria phylum was dominated by the acI lineage which is known to be common in freshwater environments. This study also demonstrated that freshwater environments contain common and similar taxa which are less similar to that in marine and terrestrial environments. The use of the 454 pyrosequencing in this study has also contributed knowledge about freshwater metagenomes that have not been described previously. Bowers, *et al.* (2009) used the pyrosequencing technique to study bacterial community composition in the snow and air samples in Colorado, USA. In all samples about 4,864 sequences were retrieved. The study revealed the ability of this technique to

discriminate between communities being in the snow and air. For example, the snow bacterial communities were found to be varied based on the relative humidity, while that in the air were very similar to each other. Matcher *et al.* (2011) also used this method to assess bacterial diversity along the Kariega Estuary in South Africa. In all samples, > 27,000 sequences were retrieved with most belonging to Bacteroidetes and Actinomycetes. Matcher *et al.* (2011) concluded that 454 pyrosequencing is a sufficient method for bacterial diversity identification in the Kariege Estuary. They concluded that valuable data about the function and health of aquatic ecosystems can be deduced.

Sequences of different species can be incorrectly assembled during PCR amplification and produce chimeric, which can mistakenly be interpreted as a new biodiversity present in environmental samples. However, chimeric sequences can be discriminated by sequencing many clones and also applying many PCRs amplifications (Amann *et al.* 1995). Wang and Wang (1997) assessed the occurrence and frequency of chimeras as a result of applying different PCR amplification cycles on mixtures of bacterial genomes, and found that when cycles of PCR were increased the frequency of chimeras increased as a result. For example, as a result of applying 35 cycles of PCR amplification, a frequency of 15.5% of chimeras were detected compared with 8.8% as a result of applying 25 cycles of PCR amplification. Chimeric sequences can be determined by some computer programmes, such as CHECK-CHIMERA (Larsen *et al.* 1993) and more recently the Uchime algorithm (Edgar *et al.* 2011). However, chimeric sequences represent more than 85% similarity and cannot be determined easily (Kopczynski *et al.* 1994).

Illumina sequencing is another next generation method released in 2006 and depends on the mechanism of sequencing by synthesis. Recent Illumina HiSeq 2000 and HiSeq 2500 are becoming methods of choice and can output 200 to 600 Gb per run, respectively, which achieve more than 454 pyrosequencing outputs per run (0.7 Gb per run within 24 hours). They are lower cost reagents than 454 pyrosequencing and thousands of samples can be handled by this tool simultaneously. However, they provide short read lengths (100-150 bp) and their run takes between 5 to 11 days (Liu *et al.* 2012). Illumina has been applied to study the diversity of bacteria in different environments, such as freshwater and marine sediments (Wang *et al.* 2012) and soil (Gittel *et al.* 2014).

Analysing sequences of DNA retrieved immediately from environmental samples using culture-independent methods has enabled us to gain a deeper understanding about the function and diversity of microbial communities (Amend *et al.* 2010). The interpretation of the sequence data can be enhanced through the collection of physiochemical data, such as temperature and pH; and spatial and temporal data (Kunin *et al.* 2008). Recently, advances in statistical analysis of phylogenetic diversity using 16S rRNA gene markers have facilitated the quantification of the relative importance of some factors, such as dispersal and environmental parameters, which are believed to be responsible for shifts in microbial community structure (Kembel *et al.* 2011).

Although, each of the different sequencing approaches is able to reveal differences between microbial communities, each can give different results with the same community under study. For example, Steven *et al.* (2012) compared three different sequencing approaches; 16S rRNA

pyrosequencing and other shotgun metagenomic techniques for studying soil microbial communities and found that each revealed different characterizations of the composition of the same microbial community under investigation. The other disadvantage of sequencing is that the cost is still high and so limits its popularity for routine uses, although there has been a remarkable decreases in cost during the last two decades (Wolska and Szweda 2012).

Table 2.3 summarises advantages and disadvantages of some methods used to characterise bacterial communities.

As discussed previously comparing fingerprinting and sequencing methods, there is no universal method for typing the bacteria present that can carry all features and has no drawbacks. Differences exist in terms of their reproducibility, level of resolution, expense and ease of use (Wolska and Szweda 2012).

2.6 Tools and methods used in this research

From this critical review of molecular techniques used for determining bacterial abundance and composition, the research presented in this thesis applied the epifluorescence microscopy and heterotrophic plate counts to investigate total bacterial numbers, and also to determine spatial and temporal variation and the influence of environmental factors affecting these numbers. ARISA also was applied to characterise bacterial community composition in the River Wensum, and also to quantify and examine the spatial and temporal variation and impact of environmental factors on this variation. The 454 pyrosequencing techniques was applied to characterise bacterial communities in the River Wensum, with the results interpreted in comparison with spatial and temporal variations and associated with environmental factors.

The next three chapters present the results and discussion of the application of these methods to samples collected from the River Wensum.

Table 2. 3 Advantages and disadvantages of some methods used to characterise bacterial communities.

Method	Advantages	Disadvantages	References
Sanger sequencing	Gives long read length (400 to 700 bp). High quality.	Low throughput. High cost. Cloning is required. Produces about 84 Kb per run.	Harbers and Kahl (2012) Liu et al. (2012)
454 pyrosequencing	Fast. Gives high read length (700 bp). Produces 0.7 Gb per run. Cloning is not required and can be applied directly to environmental samples. Reveals dominant phylotypes and rare members of bacterial community.	High cost. Homopolymer errors. Biases by PCR, different DNA extraction methods and primers.	Harbers and Kahl (2012) Kunin *et al.* (2008) Matcher *et al.* (2011) Amend *et al.*(2010) Liu *et al.* (2012)
Illumina HiSeq 2000 and HiSeq 2500	Cloning is not required and can be applied directly to environmental samples. High throughput. Produces 200 to 600 Gb per run. Thousands of samples can be handled simultaneously.	Gives short read length (100-150 bp). Long run time (5-11 days per run).	Harbers and Kahl (2012) Liu *et al.* (2012)

Chapter Three

Determining total bacterial numbers in the River Wensum using epifluorescence microscopy and heterotrophic plate counts

3.1 Introduction

In microbial ecology, it is a fundamental task to enumerate microbes in the targeted environment because fluctuation of their numbers can reflect their functions, dynamics and their interactions with other microorganisms (Daims and Wagner 2007) and help understand their role in regenerating nutrients (Selinummi *et al.* 2005). The total bacterial number is proposed as a reliable indicator of quality of freshwater ecosystems (Wang *et al.* 2010) and fluctuations in bacterial abundance may reflect changes in pathogenic bacteria numbers (Velimirov *et al.* 2011).

Culture-dependent heterotrophic plate counts (HPCs) have long been used to characterise bacteria in water (Lepeuple *et al.* 2004), resulting in counts of colony-forming units (CFU) (APHA 1998; Boulos *et al.* 1999). Considerable effort has been exerted to optimize culture conditions, including choice of medium, incubation time and temperature (Allen *et al.* 2004). For example, the R2A medium was introduced by Reasoner and Geldreich (1985) to resolve problems arising from the use of standard plate count agar (PCA), including recovery of low numbers of heterotrophic bacteria and difficulty in subculturing them. R2A medium contains lower concentrations of nutrients than PCA and allows the growth of a higher number of heterotrophic bacteria. Incubation times between 5 and 7 days at temperatures between 20 to 28 ˚C allow slow growing bacteria to be detected (Reasoner and Geldreich 1985). The R2A plates have successfully been used to enumerate bacteria in drinking water (Reasoner and Geldreich 1985; Carter *et al.* 2000), glaciers (Segawa *et al.* 2011) and rivers (Kenzaka *et al.* 2001). However, methods of culturing total bacterial numbers have revealed that culture-dependent techniques only recover a small proportion of the bacteria present in the natural environment (Skorczewski and Mudryk 2009).

Rapid and precise techniques for enumerating total bacterial numbers in water are required (Bao *et al.* 2008), and microscopy can achieve this purpose (Wang *et al.* 2010) by detecting up to 10,000 times more bacterial cells than culture-based methods (Szewzyk *et al.* 2000). As a result, epifluorescence microscopy (EFM) with AO or DAPI staining (see Chapter 2) have

become standard tools used to determine total bacterial numbers (Hobbie *et al.* 1977; Porter and Feig 1980; Kepner and Pratt 1994). EFM also allows the estimation of biomass (Lisle *et al.* 2004) and the determination of the sizes of aquatic bacteria (Sieracki *et al.* 1985; Grivet *et al.* 2001). It is simple, inexpensive and can be combined with tools such as FISH (Nishimura *et al.* 2006). EFM has been widely used to enumerate bacteria in different environments including freshwater (Jones and Simon 1975; Garabetian *et al.* 1999), frozen and heat-treated foods (Rodrigues and Kroll 1989), raw milk (Pettipher *et al.* 1980), seawater (Peele and Colwell 1981), sediments (Liao *et al.* 2012), bacteria attached to coral mucus (Garren and Azam 2010) and estuarine water (Clarke and Joint 1986).

3.2 Aims

As introduced in Section 1.7, the aims of the research presented in this chapter are to use epifluorescence microscopy and R2A spread plates to (i) investigate total bacterial numbers in the River Wensum, (ii) to determine spatial and temporal variation and the influence of environmental factors affecting these numbers, (iii) to quantify changes in bacterial numbers as water moves downstream in this lowland arable catchment, and (iv) to determine the relationships between total bacterial numbers and total heterotrophic bacteria.

3.3 Materials and methods

3.3.1 Study sites

Samples were collected monthly from the River Wensum catchment and sub-catchment areas in Norfolk, UK, from June 2011 to February 2013. This period encompassed contrasting hydrological conditions with 2011 being dominated by low flow (drought) conditions and 2012/13 by high flow (flood) conditions. This study was conducted at 26 sites, 20 in the wider catchment (1 to 18, 20 and 21) (Figure 3.1) and 6 in the Blackwater sub-catchment (A-F) (Figure 3.2). The sites were divided into 9 groups based on catchment characteristics (Table 3.1). The Upper Wensum (UW) is highly dominated by chalk groundwater. The Wensum at Swanton Morley (WSM) and Wensum at Costessey Mill (WCM) are on the main river downstream. The Blackwater sub-catchment sites are on the area that dominated by intensive arable agriculture practices.

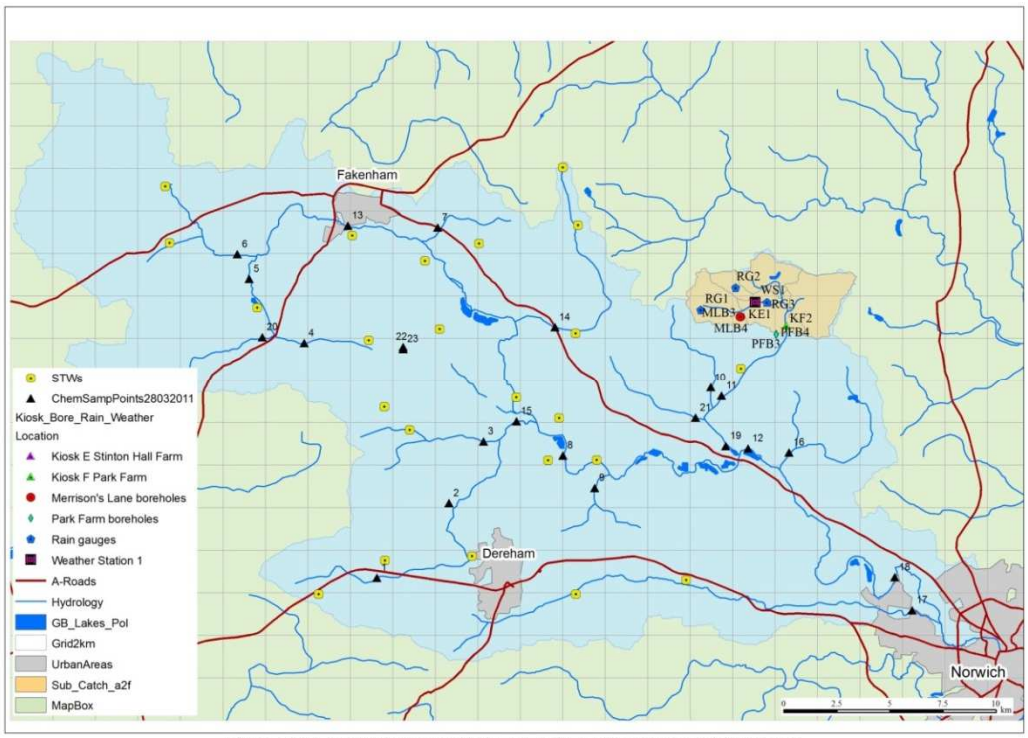


Figure 3. 1 Sampling sites (1-18, 20 and 21) in the River Wensum catchment, Norfolk, UK showing the location of sewage treatment works. Map obtained from Wensum Alliance (River Wensum Demonstration Test Catchment (DTC) Project).

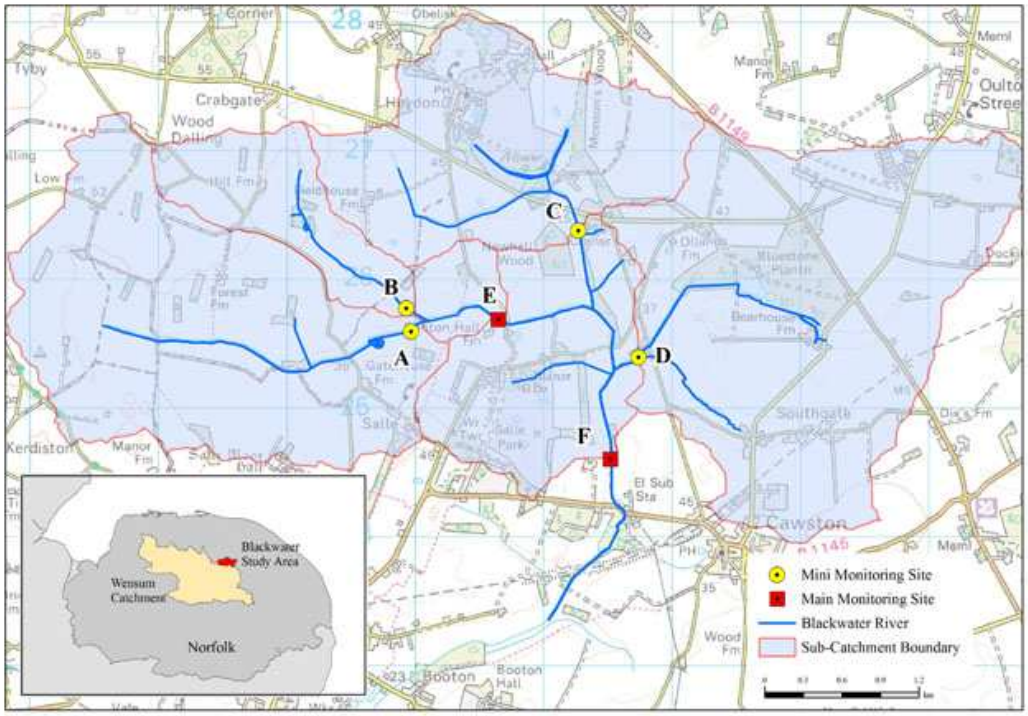


Figure 3. 2 Sampling sites (A-F) in the Blackwater sub-catchment of the Wensum, Norfolk, UK. Map obtained from Wensum Alliance (River Wensum Demonstration Test Catchment (DTC) Project).

Table 3. 1 Organisation of individual sites, groups and their symbols and numbers in the River Wensum, Norfolk, UK.

Individual sites	Group name	Group symbol	Group number
S4, S5, S6, S13 and S20	Upper Wensum	UW	1
S1, S2, S3 and S15	Wensum Beck	WB	2
S7 and S14	Mid catchment tributaries	MCT	3
S8	Wensum Swanton Morley	WSM	4
S10, S11, S12 and S21	Blackwater catchment	BC	5
S9 and S16	Lower catchment tributaries	LCT	6
S17 and S18	Wensum Costessey Mill	WCM	7
SA, SB and SE	Blackwater sub-catchment 1	BSC1	8
SC, SD and SF	Blackwater sub-catchment 2	BSC2	9

National Grid Reference, sampling date and times for all sites in the River Wensum are presented in Appendix Tables A 3.24 and A 3.25.

3.3.2 Total bacterial numbers

3.3.2.1 Sample collection for measuring total bacterial numbers

Water samples were collected from a depth of about 10-20 cm below the water surface at each site to avoid potential contamination from the water surface (Araya *et al.* 2003; Ibekwe *et al.* 2012). Sites were deep enough to collect samples, especially during the wet year 2012 and the beginning of the year 2013. Water samples (20 ml) for analysing total bacterial numbers presented in this chapter and that for determining bacterial community composition (500 ml, see section 4.3.2 in chapter 4) were collected at the same time.

20 ml of river water were collected in sterile 50 ml centrifuge tubes (Scientific Fisher, UK). Samples were placed on ice and transferred to the laboratory and stored at 4 ˚C until further analysis, usually within a maximum of 8 hours.

3.3.2.2 Sample collection for physiochemical measurements and other environmental data

Water temperature was measured in the field at the same time as sample collection using a mercury thermometer (Fisher scientific, UK). Samples for other chemical and physical measurements were collected at the same time and physicochemical measurements made at the

University of East Anglia (UEA) as part of the River Wensum Demonstration Test Catchment (DTC) Project (see www.wensumalliance.org.uk). Chemical and physical data provided by the project include pH, total nitrogen (TN), total phosphorus (TP), total carbon (TC), total organic carbon (TOC) and total suspended solids (TSS). All these parameters were analysed in the Analytical Facilities Laboratory at UEA. Monthly rainfall data for north Norfolk were obtained from Wensum Alliance (location of rain gauge NGR TG 108 257). Monthly flow data for the River Wensum at the downstream outlet at Costessey Mill were obtained from the Environment Agency (location details of gauging station NGR TG 176 127). The DTC project also provided data on land use (percentages of arable land, improved grassland, other grassland and urban area upstream of each sampling point, and the locations of sewage treatment works (STWs). All land use data were obtained from Land Cover Map 2007 (LCM2007). In addition, stream order data were derived from a digital map (scale 1:50,000 Ordnance Survey Digimap) of Norfolk, UK. Stream order is a method of classifying waterways based on the number of joining tributaries. A stream with no tributaries (headwater stream) is given a first order. When a first order stream joins another first order stream, the waterway downstream of the confluence is considered a second order stream. When the second order stream joins another second order stream, a third order stream is formed, and so on. Thus, the stream ordering system continues towards the downstream section within a drainage network (Strahler 1952). In the River Wensum, stream orders are presented in Appendix Table A 3.21. Streams are ordered from 1 (upstream) to 4 (downstream). Some streams in different areas share the same order, for example, sites S4, S6, S20, S1, SE and SC are all 2nd order streams, while sites S8, S12, S15 and S18 are all on 4th order streams.

Based on the review of the effects on bacterial community abundance and composition and the availability of data without missing values, the above 15 environmental parameters were selected for inclusion in detailed analysis.

3.3.2.3 DAPI staining and filtration

10 µl of 4',6-diamidino-2-phhenylindole (DAPI, Molecular Probe, Invitrogen, UK), to give a final concentration of 0.25 µg/mL, was added to 50 mL Eppendorf tube (Fisher Scientific, UK) containing 20 mL of water sample (Porter and Feig 1980). The tube was vortexed using a Vortex Genie 2 (Myconostica Ltd, UK), stored in the dark for ~15 min at 4˚C (Schallenberg *et al.* 1989), and filtered through a 0.2 µm (47 mm diameter) black polycarbonate filter (Whatman, UK). A microscope slide was prepared by adding a small amount of immersion oil to its centre. The black filter was transferred onto the centre of the microscope slide, and left for ~10 minutes in the dark until dry. The drop of immersion oil was placed on the centre of a filter, and a cover slip placed on the filter. Bubbles and wrinkles were removed by passing gently on the cover slip. A scalpel was used to cut the edges of the filter. Slides were wrapped in aluminium foil to minimise exposure to light, labelled and stored at 4 ˚C until further analysis, usually within one day (Kepner and Pratt 1994).

The vacuum pressure for filtration was between 50 to 70 mm Hg (Lisle *et al.* 2004), in order to the avoid rupture of bacterial cells that has been observed at pressures above 80 mm Hg (Kepner and Pratt 1994). Minimal quantities of immersion oil were used to avoid floating of bacterial cells on the filters (Turley and Hughes 1992).

3.3.2.4 Quantifying total bacterial numbers

The stained bacterial cells were counted on an Olympus BX40 epifluorescence microscopy equipped with a D400 dichroic mirror. Stained cells were viewed with a 100X magnification objective lens, under UV light with a 358-461 excitation filter. Five fields were photographed per filter (an example, see Appendix Figure A 3.23) and cell numbers counted using the Volocity image analysis software version 6.3 (PerkinElmer, UK). To avoid fading of the fluorescence , the UV shutter was closed except when acquiring a fluorescence image (Sieracki *et al.* 1985). Bacterial cells per ml in each sample were calculated using the following equation:

$$\text{Total bacterial cell numbers (per mL)} = \frac{\left(\frac{\text{filter area}}{\text{view area}}\right) \times \text{ average number of cells per photo}}{\text{total volume of samples filtered (20 ml)}}$$

(Kirchman *et al.* 1982; Ibekwe *et al.* 2012).

3.3.3 Total heterotrophic bacterial counts

R2A spread plates were used to count the total heterotrophic bacteria in water samples (Reasoner and Geldreich 1985) using standard methods (APHA 1998).

3.3.3.1 Medium preparation

R2A medium was made up with deionised distilled water following the manufacturer's recommendations (Lab M Limited, UK), autoclaved, poured into sterile petri dishes and stored following standard methods (APHA 1998).

3.3.3.2 Sample collection and dilution

Water samples were collected in 100 mL sterile glass bottles from a depth of 10 cm at each of the 26 sites in February 2013. Samples were stored on ice and transferred to the laboratory and processed within 2 h after collection to reduce changes in the bacterial community.

Serial dilutions of; 0.1mL, 0.01mL and 0.001mL were carried out using sterilise deionised water. A separate sterile pipette was used with each sample and dilution. Each sample and dilution was mixed well to ensure the bacteria were well distributed. 1 ml of sample volume was then pipetted into a new tube containing sterile deionised water, with the remaining dilutions treated in the same way.

3.3.3.3 Spread plate method

0.1 ml of each sample or dilution was pipetted onto the surface of predried R2A agar medium and spread using a glass rod. Plates were incubated at 20˚C for 5 days (Reasoner and Geldreich 1985).

Colonies were counted using a Quebec colony counter, plates with colony numbers between 30 and 300 were used to calculate total heterotrophic bacterial numbers as colony-forming units (CFU) per ml using the equation:

$$\text{Total culturable bacteria (CFU/mL)} = \frac{\text{(colonies number (must be between } 30 - 300 \text{ colonies)}}{\text{sample volume plated (mL)x dilution used}}$$

3.3.4 Statistical analysis of bacterial abundance and total heterotrophic bacteria

The statistical package SPSS, version 18 (IBM, UK) was used to analyse all the collected data.

Parametric statistical methods assume that the data are approximately normally distributed. (Bolter *et al.* 2002). Normality of data was examined using Kolmogorov-Smirnov tests and all variables that were not normally distributed were square root transformed.

Two-way ANOVA was applied to test for significant differences in bacterial numbers between sites and sampling times. Spearman's rank correlations (*rs*) were used to examine relationships between bacterial numbers and environmental parameters. Multiple linear regression was performed, and included all environmental parameters that showed significant correlations with total bacterial numbers, using stepwise regression and hierarchical partitioning analysis to identify the most important factors (Field 2009).

3.4 Results

3.4.1 Total bacterial numbers

3.4.1.1 Spatial variation of total bacterial numbers

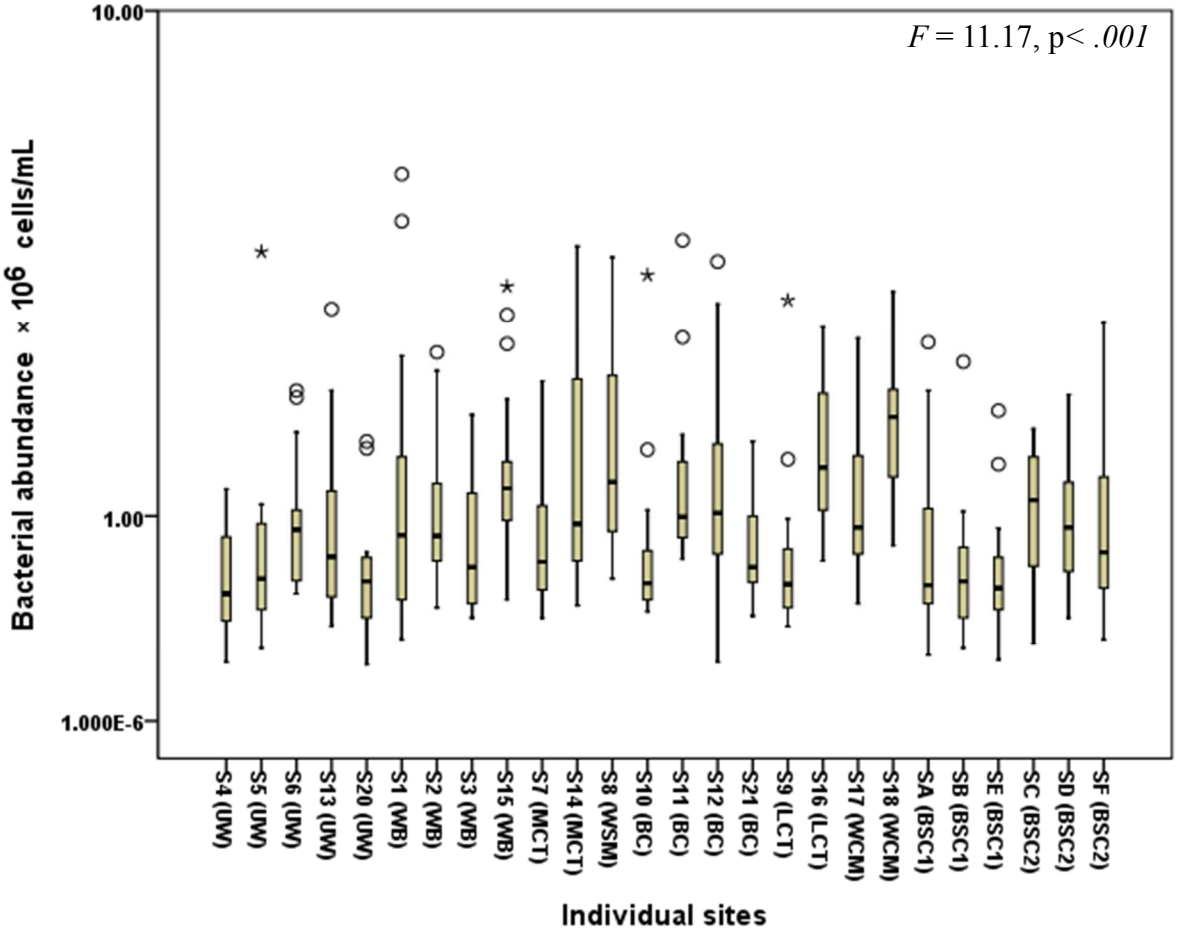
Total bacterial numbers ranged from (0.21×10^6 cells/mL) at site S20 in February 2013, to (5.34×10^6 cells/mL) at site S1 in December 2011, with an overall mean of 1.1×10^6 cells/mL.

Table 3.2 and Figure 3.3 show the spatial variation of total bacterial numbers in the River Wensum from June 2011 to February 2013.

Two-way ANOVA analysis showed a significant variation between sites ($F = 11.17$, $p < 0.001$). The highest bacterial numbers were at the WCM and WSM regions in the downstream sites of the river, with highest values at sites S8 and S18 (4^{th} order), respectively. The lowest numbers were in BSC1 and UW regions, with lowest values at the upstream sites S20 and SE (2^{nd} order) (Table 3.2). However the differences between sites are not large. Total bacterial numbers increase as water moves to downstream sites (3^{rd} – 4^{th} order) of the river (Figure 3.9). Bacteria can multiply and increase at these sites, with the majority located in urban areas in the vicinity of sewage treatment works.

Table 3. 2 Mean, standard error, minimum and maximum of total bacterial numbers (cells/mL) in the River Wensum by sites from June 2011 to February 2013.

Individual site	Mean ± standard error	Minimum and maximum
S4	0.85 ± .046	0.22 - 1.19
S5	0.83 ± .042	0.28 - 3.88
S6	1.06 ± .042	0.54 - 2.05
S13	0.92 ± .039	0.38 - 3.02
S20	0.71 ± .042	0.21 - 1.57
S1	1.25 ± .042	0.32 - 5.34
S2	1.00 ± .039	0.47 - 2.47
S3	0.88 ± .042	0.42 - 1.81
S15	1.48 ± .042	0.51 - 3.34
S7	0.98 ± .042	0.42 - 2.15
S14	1.49 ± .042	0.48 - 3.97
S8	1.47 ± .039	0.62 - 3.79
S10	0.85 ± .042	0.45 - 3.51
S11	1.36 ± .042	0.73 - 4.07
S12	1.20 ± .039	0.22 - 3.72
S21	0.88 ± .042	0.43 - 1.57
S9	0.83 ± .042	0.38 - 3.14
S16	1.55 ± .042	0.72 - 2.78
S17	1.22 ± .042	0.49 - 2.64
S18	1.89 ± .042	0.81 - 3.26
SA	0.92 ± .042	0.25 - 2.59
SB	0.78 ± .042	0.28 - 2.36
SE	0.74 ± .042	0.23 - 1.85
SC	1.14 ± .042	0.30 - 1.68
SD	1.10 ± .042	0.42 - 2.01
SF	1.02 ± .042	0.32 - 2.84



Key symbols: UW = Upper Wensum, WB = Wensum Beck, MCT = Mid Catchment Tributaries, WCM = Wensum Swanton Morley, BC = Blackwater Catchment, LCT = Lower Catchment Tributaries, WCM: Wensum Costessey Mill, BSC1 = Blackwater subcatchment 1, BSC2 = Blackwater subcatchment 2.

Figure. 3 Box plot of spatial variation of total bacterial numbers (cells/mL) in the River Wensum (sites as individuals and groups) from June 2011 to February 2013. Note that bacterial abundance is plotted on log scale.

A box plot is a useful graphical visualization for the data containing statistical measures and explaining the distribution of the data. Box plots presented in this thesis include the lower 25% quartile, the median separating the box into two parts, and the upper 75% quartile. Therefore, between the bottom and top of the box represents 50% of the observations. The two whiskers on the back of the box plot extend from the minimum value to the lower quartile and from the upper quartile to the maximum value. The Whiskers therefore represent the spread of the data. Points above or below the two whisker lines are called outliers and are plotted separately on the figure. Outliers are extreme values that are distant from other values. Outliers do not always include the minimum and maximum values.

3.4.1.2 Temporal variation of total bacterial numbers

Table 3.3 and Figure 3.4 show temporal variation of total bacterial numbers in the River Wensum from June 2011 to February 2013.

Two-way ANOVA showed significant differences between months ($F = 39.93$, $p < 0.001$), with greater variation between months than between sites, accounting for 50.5% and 18.6% of the overall variance in bacterial numbers, respectively.

Bacteria were highest in summer 2011 (warm months and during low river flow), with the highest values in June and August. Numbers were lowest in winter (cold months and during high river flow), with lowest values in February 2013 and December 2012 (Table 3.3). These results demonstrate the independent effects of temperature and river flow on bacterial numbers. River flow is high in winter, and temperature is low, and the strong effect of temperature partially hides the effect of river flow (Table 3.4).

Table 3. 3 Mean, standard error, minimum and maximum of total bacterial numbers (cells/mL) in the River Wensum by months from June 2011 to February 2013.

Year	Month	Mean ± standard error	minimum and maximum
2011	June	2.91 ± .089	2.26 - 3.79
	July	2.01 ± .056	1.01 - 2.59
	August	2.70 ± .040	1.20 - 4.41
	September	1.64 ± .035	0.37 - 3.57
	October	1.44 ± .035	0.22 - 3.52
	November	0.99 ± .034	0.49 - 1.69
	December	1.14 ± .034	0.57 - 5.34
2012	January	0.66 ± .034	0.35 - 1.78
	February	0.68 ± .034	0.42 - 1.39
	March	0.90 ± .034	0.54 - 2.02
	April	0.83 ± .034	0.50 - 1.89
	May	0.80 ± .034	0.35 - 1.80
	June	1.01 ± .034	0.50 - 2.06
	July	1.15 ± .034	0.58 - 2.48
	August	0.80 ± .034	0.39 - 2.17
	September	0.73 ± .034	0.40 - 1.57
	October	1.05 ± .034	0.40 - 2.85
	November	0.57 ± .034	0.34 - 0.99
	December	0.51 ± .034	0.23 - 0.97
2013	February	0.51 ± .034	0.21 - 1.05

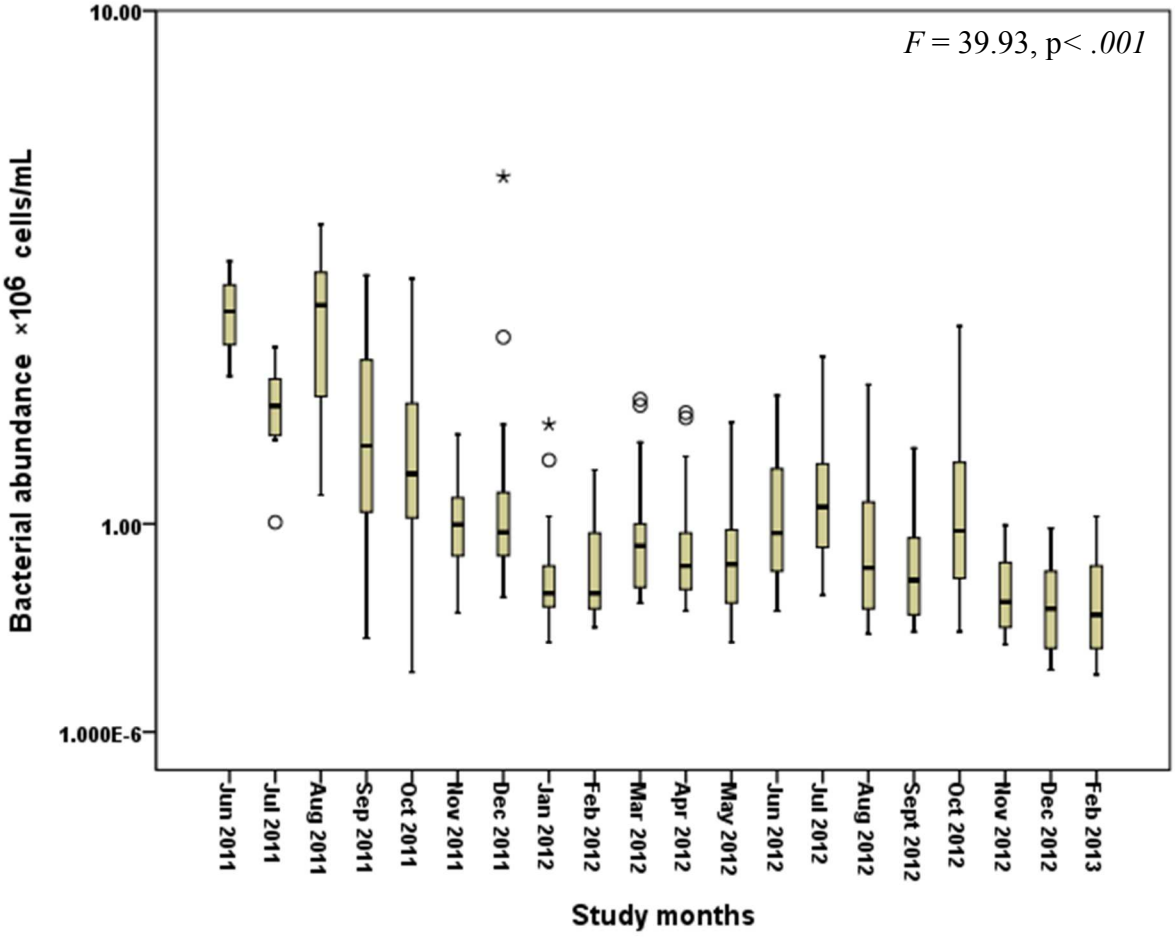


Figure 3. 4 Box plot of temporal variation of total bacterial numbers (cells/mL) in the River Wensum (by month) from June 2011 to February 2013. Note that bacterial abundance is plotted on log scale.

For an explanation of the presentation of the box plot, see Figure 3.3.

3.4.1.3 Environmental parameters and total bacterial numbers

The mean, standard error, minimum and maximum of environmental parameter results are all presented in Appendix Tables A3-1 to A3-22.

Table 3.4 shows the correlations between environmental parameters and total bacterial numbers in the River Wensum from June 2011 to February 2013 using spearman's rank correlation and the results of the stepwise multiple regression analyses.

Spearman's rank correlation analysis revealed that total bacterial numbers were positively related to temperature, stream order, TOC, TP, STWs, urban area, improved grassland and other grassland, respectively, while they were negatively related to flow rate, TC, arable land, TN, and TSS, respectively.

However, among these significant parameters, stepwise multiple regression analysis revealed that the most significant parameters correlated positively with total bacterial numbers were temperature (Figure 3.5), TP (Figure 3.7), TOC (Figure 3.12), stream order (Figure 3.9), STWs (Figure 3.10), while the most significant parameters correlated negatively with total bacterial numbers were TC (Figure 3.6), flow rate (Figure 3.11) and TN (Figure 3.8). All of these environmental parameters explain approximately 52% of the variation of total bacterial numbers.

Overall, bacteria enter the river from different sources including land drainage (agricultural, particularly at upstream sites and urban at the downstream sites), sewage treatment works (particularly downstream sites). Bacteria increase as water moves downstream of the river (3^{rd} – 4^{th} order). Hierarchical partitioning showed an independent effect of temperature and flow. The highest numbers are in summer 2011 when water temperature is high and during periods of low river flow when residence time is long. However, bacterial numbers are lower during cold months when water temperature is low and also during periods of high flow when residence time is short. The abundance of bacteria showed a strong negative relationship with TC. TC increased during the wet year 2012 compared with the dry year 2011 (Appendix A 1.3).

Table 3. 4 Relationships between environmental parameters and total bacterial number (cell/mL) in the River Wensum from June 2011 to February 2013 using Spearman's rank correlation and stepwise multiple regression analysis.

Environmental parameter	Spearman's rank correlation	Stepwise multiple regression
Temperature (˚C)	.437, $p < 0.001$	.309, $p < 0.001$
pH	N.S.	-
Total nitrogen TN (mg/L)	- .284, $p < 0.001$	- .143, $p < 0.001$
Total phosphorus TP (µg/L)	.263, $p < 0.001$	.208 , $p < 0.001$
Total carbon TC (mg/L)	- .344, $p < 0.001$	- .398, $p < 0.001$
Total organic carbon TOC (mg/L)	.284, $p < 0.001$	.204, $p < 0.001$
Total suspended solid TSS (mg/L)	-.103, $p < 0.05$	-
Arable land (%)	- .296, $P < 0.001$	-
Improved grassland (%)	.188, $p < 0.001$	-
Other grassland (%)	.140, $p < 0.001$	-
Urban area (%)	.226, $p < 0.001$	-
Rainfall (mm)	N.S	-
Sewage treatment works (n)	.253, $p < 0.001^c$	.101, $p < 0.01$
Stream order (n)	.336, $p < 0.001^c$	.181, $p < 0.001$
River flow rate (m^3/s)	-.499, $p < 0.001^c$	- .278, $p < 0.001$

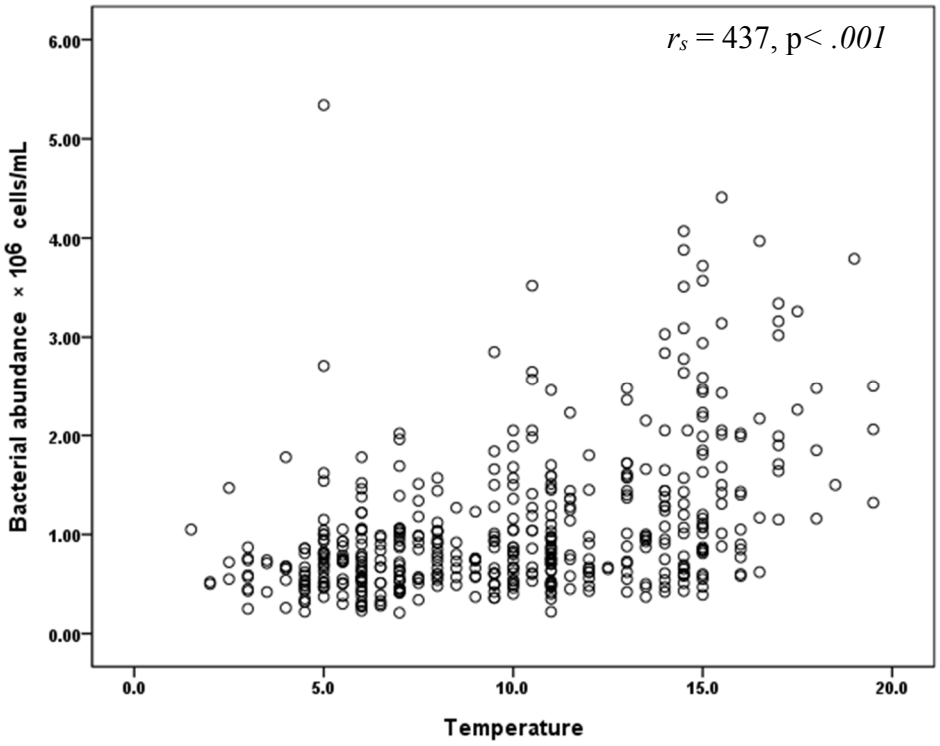


Figure 3. 5 Relationship between total bacterial numbers (cells/mL) and temperature (˚C) from June 2011 to February 2013 for all sites.

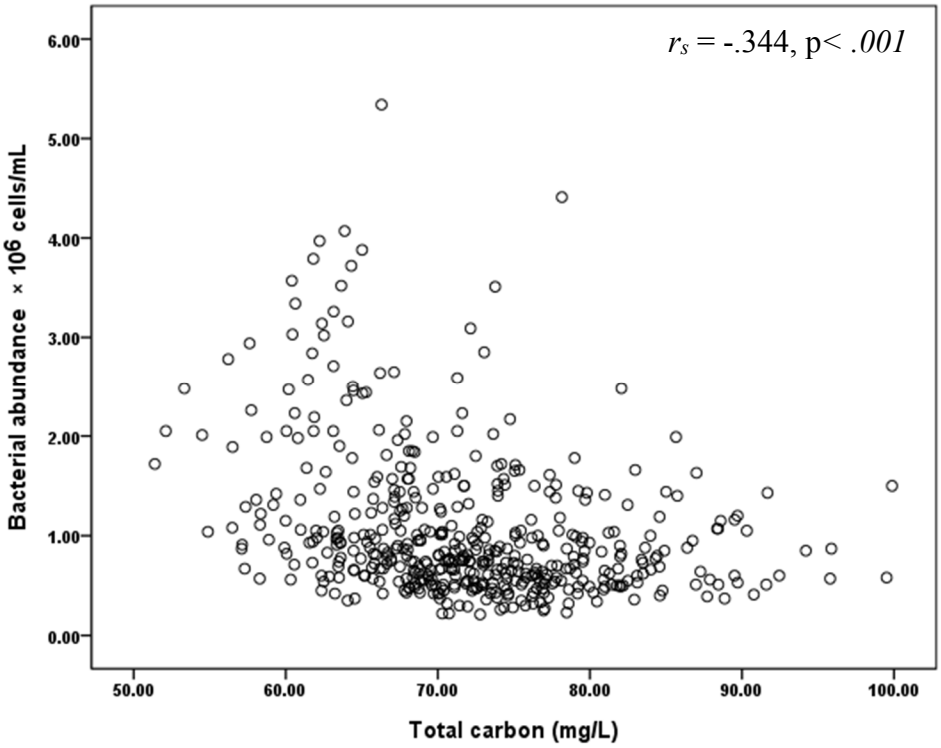


Figure 3. 6 Relationship between total bacterial numbers (cells/mL) and total carbon (mg/L) from June 2011 to February 2013 for all sites.

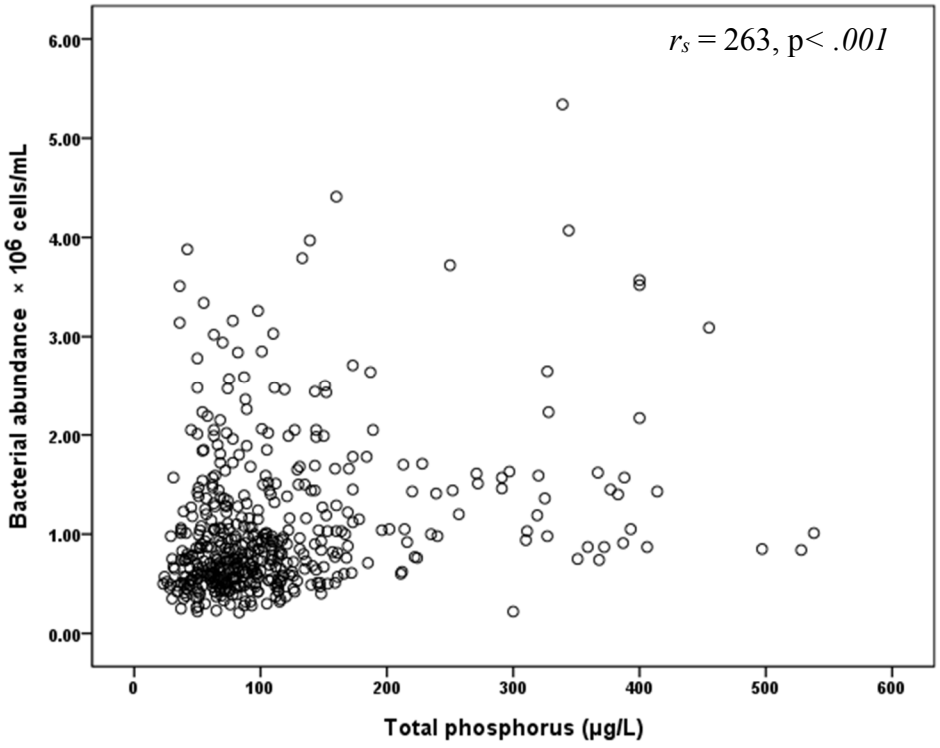


Figure 3. 7 Relationship between total bacterial numbers (cells/mL) and total phosphorus (µg/L) from June 2011 to February 2013 for all sites.

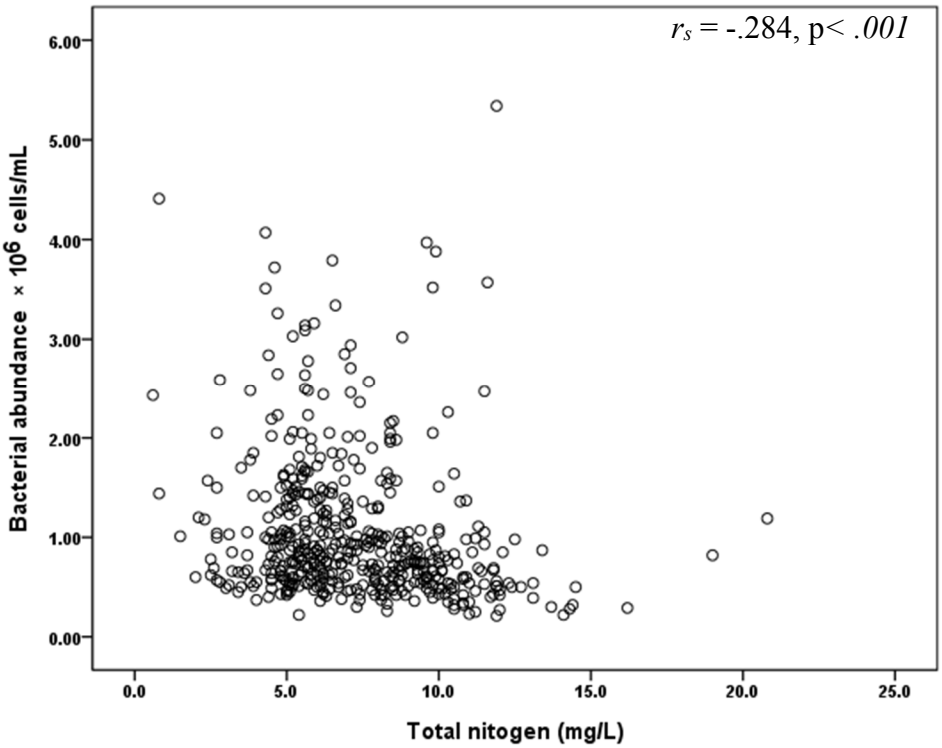


Figure 3. 8 Relationship between total bacterial numbers (cells/mL) and total nitrogen (mg/L) from June 2011 to February 2013 for all sites.

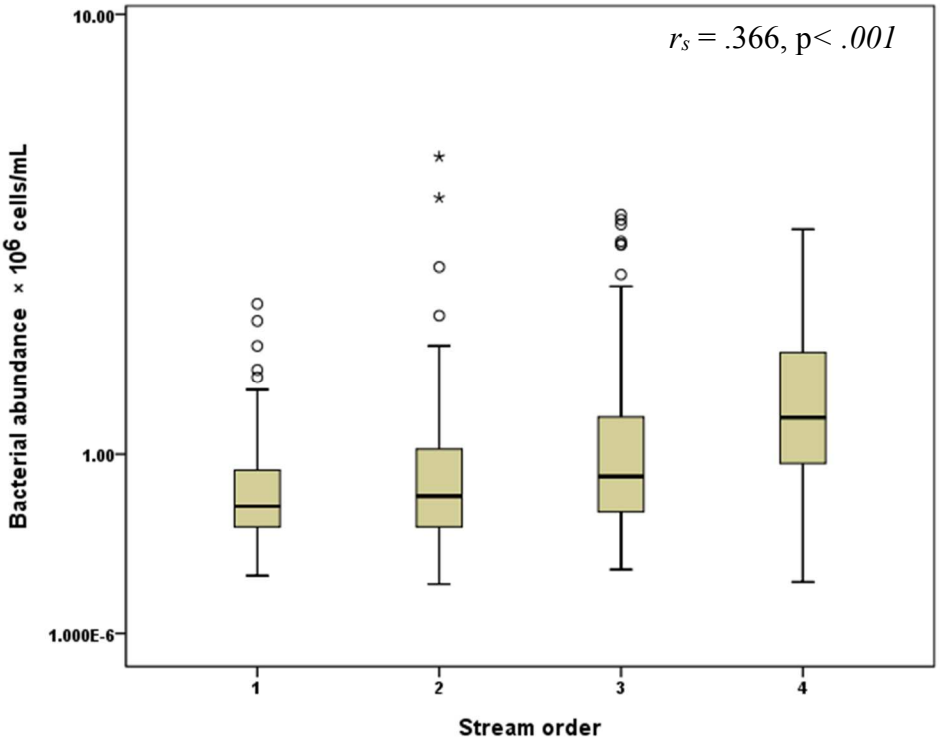


Figure 3. 9 Box plot of the relationship between total bacterial numbers (cells/mL) and stream order (number) from June 2011 to February 2013 for all sites. Note that bacterial abundance is plotted on log scale.

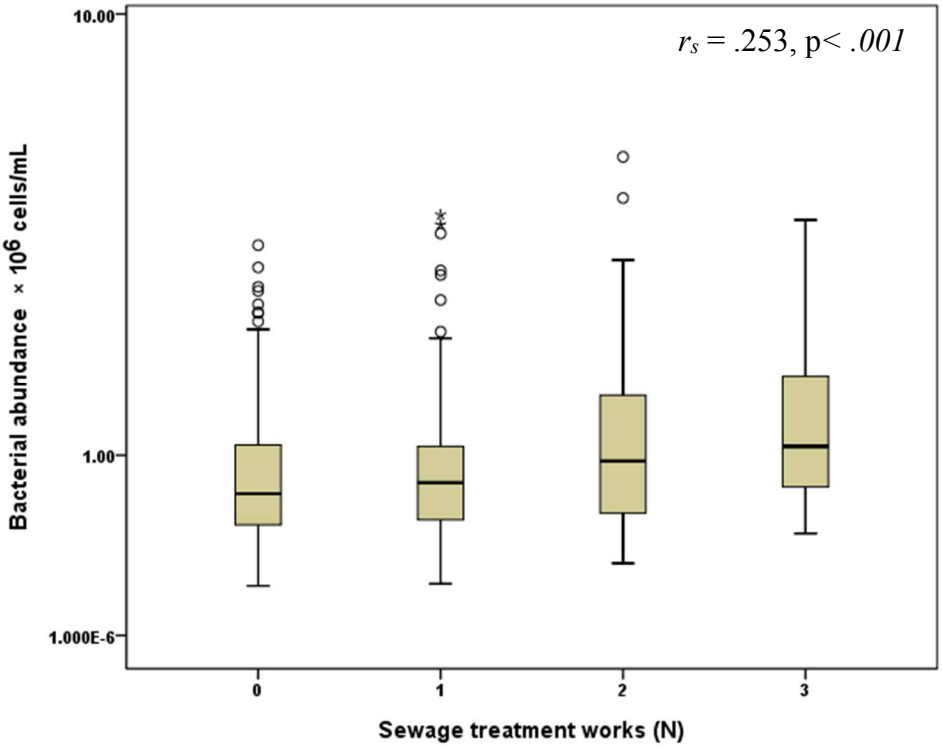


Figure 3. 10 Box plot of the relationship between total bacterial numbers (cells/mL) and sewage treatment works (number) from June 2011 to February 2013 for all sites. Note that bacterial abundance is plotted on log scale.

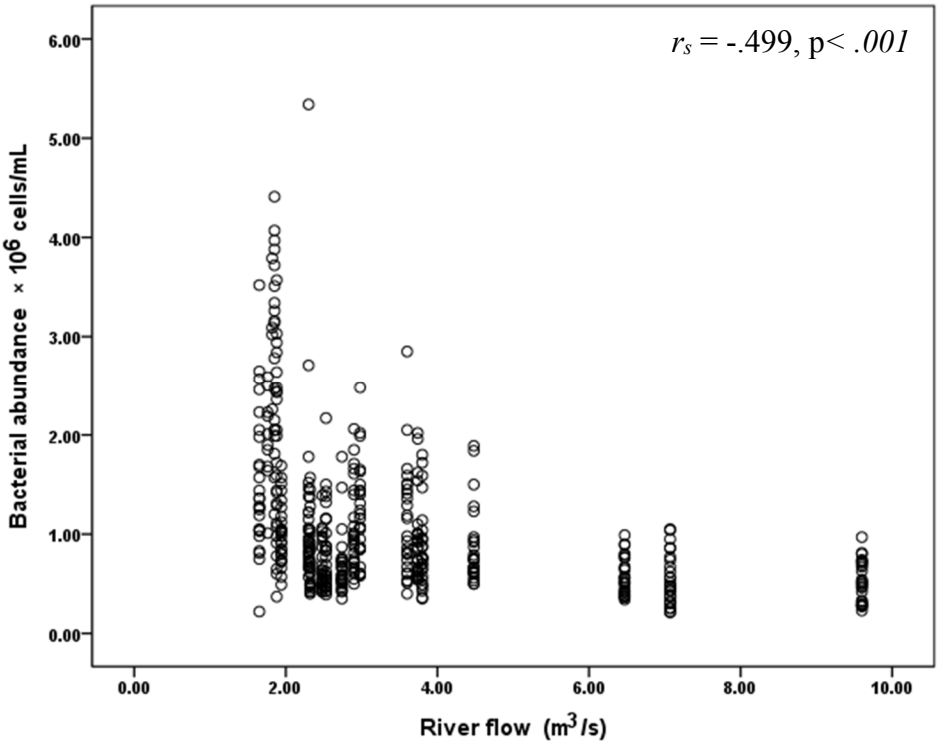


Figure 3. 11 Relationship between total bacterial numbers (cells/mL) and river flow (m^3/s) from June 2011 to February 2013 for all sites.

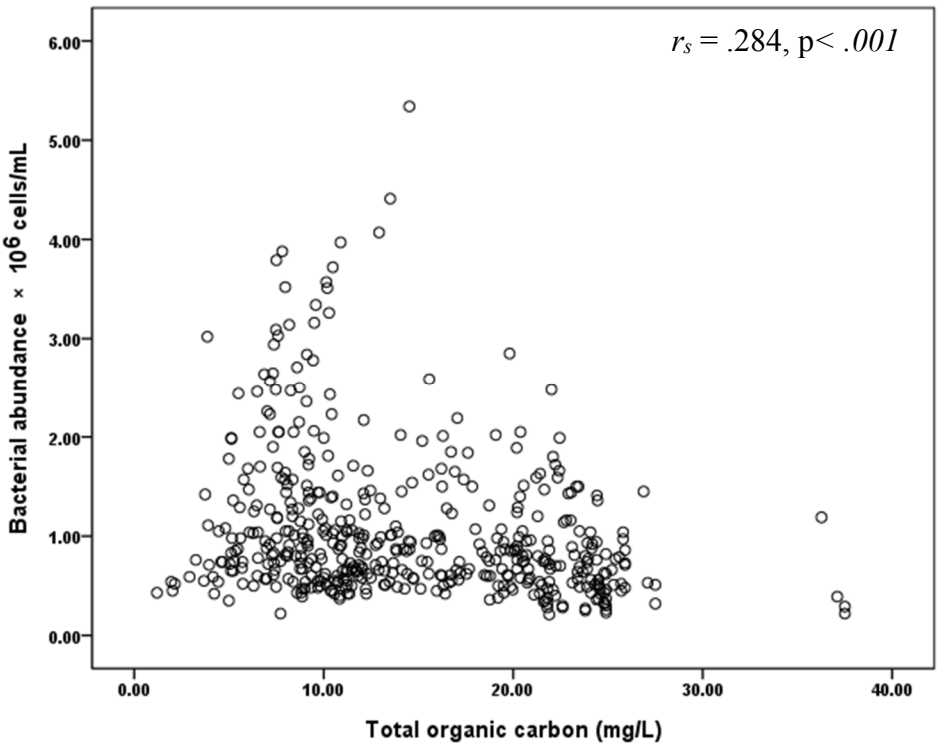
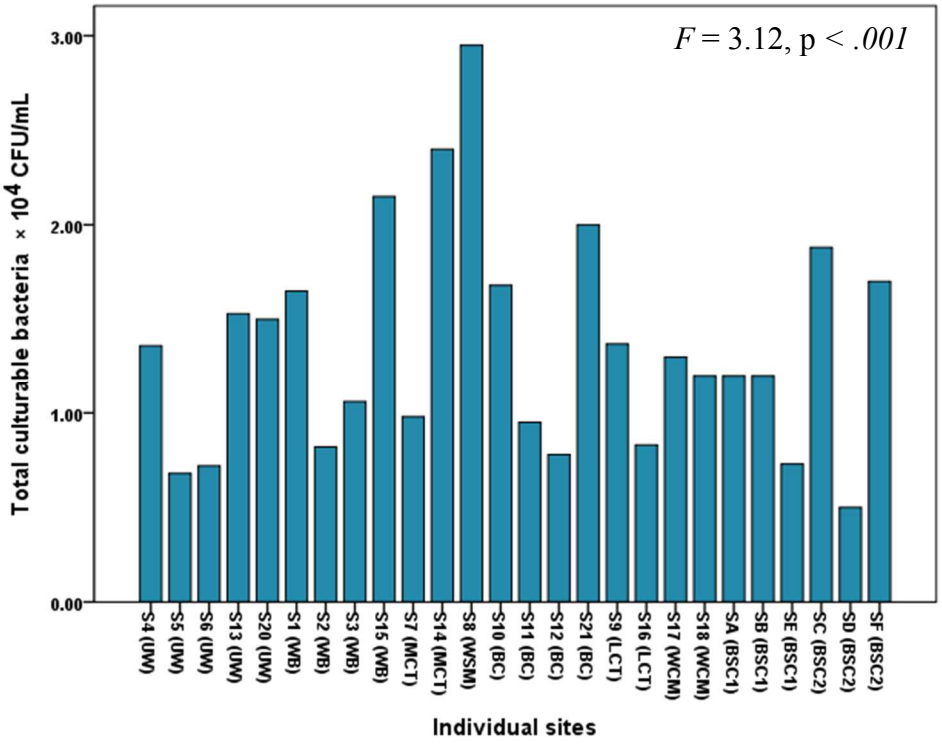


Figure 3. 12 Relationship between total bacterial numbers (cells/mL) and total organic carbon (mg/L) from June 2011 to February 2013 for all sites.

3.4.2 Total heterotrophic bacteria numbers

The mean number of heterotrophic bacteria in the River Wensum in February 2013 was 1.35 × 10^4 CFU/mL) with a range from 0.50 × 10^4 to 2.95 × 10^4 CFU/mL. There were significant differences in numbers between sites (F = 3.12, p< .001; Figure 3.13).

Numbers show an increase as water moves downstream in the river (3rd – 4th stream order). The highest numbers were at sites S8 (4th order), S14 (3rd order) and S15 (4th order), while the lowest numbers were recorded at site SD. No significant effects of sites as groups were observed on the shifts in total heterotrophic bacteria (F = 1.39, p> .05).

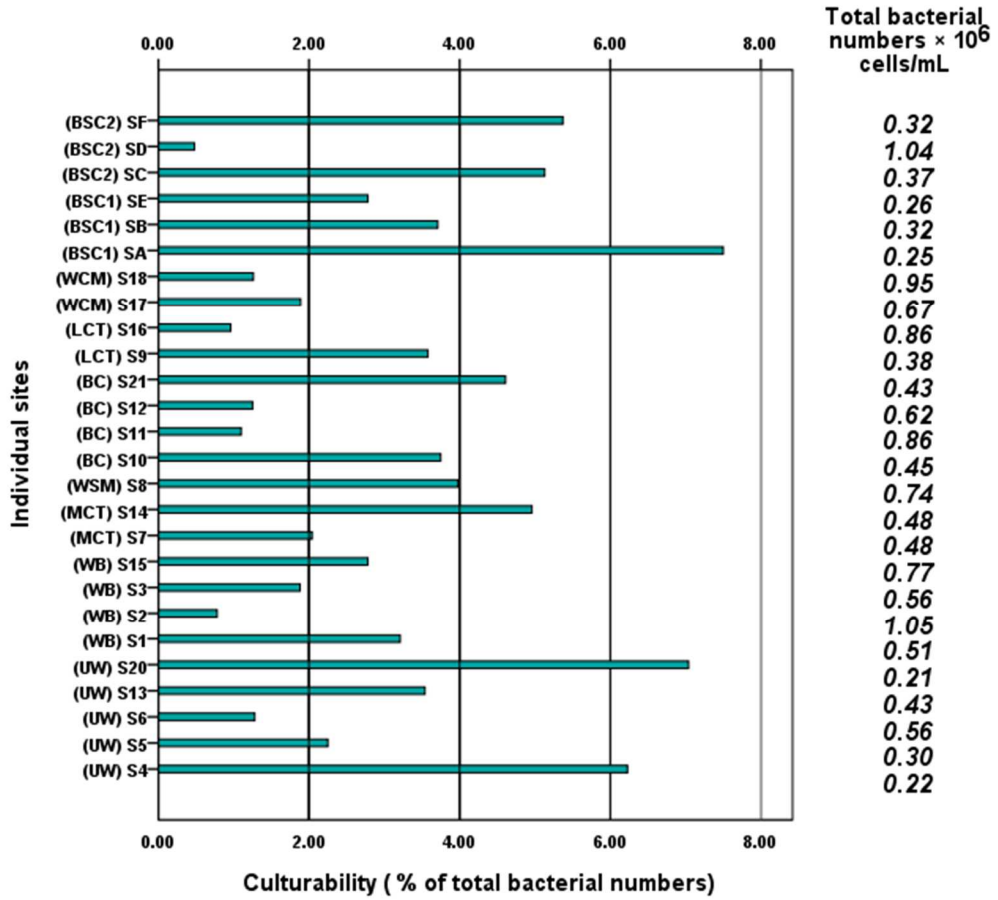


Key symbols: UW = Upper Wensum, WB = Wensum Beck, MCT = Mid Catchment Tributaries, WCM = Wensum Swanton Morley, BC = Blackwater Catchment, LCT = Lower Catchment Tributaries, WCM: Wensum Costessey Mill, BSC1 = Blackwater subcatchment 1, BSC2 = Blackwater subcatchment 2.

Figure 3. 13 Total culturable bacteria (CFU/mL) in the River Wensum (as individual sites and groups) from February 2013.

The percentages of total bacterial numbers that were culturable varied from 0.48% (site SD) to more than 7% at sites SA and S20 (Figure 3.14). Culturability was strongly negatively correlated with total bacterial numbers (Figure 3.15; $r_s = -.795$, $P < .001$). In the River Wensum, the abundance of some bacterial taxa increase as water moves downstream. This presumably reflects the fact that those bacteria are actively growing in the river, and diluting the abundance of other taxa including that are heterotrophic.

No significant correlations ($P > .05$) were found between total heterotrophic bacteria and any of the environmental parameters measured, but the number of data points is much smaller than for the total bacterial counts.



Key symbols: UW = Upper Wensum, WB = Wensum Beck, MCT = Mid Catchment Tributaries, WCM = Wensum Swanton Morley, BC = Blackwater Catchment, LCT = Lower Catchment Tributaries, WCM: Wensum Costessey Mill, BSC1 = Blackwater subcatchment 1, BSC2 = Blackwater subcatchment 2.

Figure 3. 14 The percentages of total culturable bacteria relative to total bacterial numbers in the River Wensum (as individual sites and groups) from February 2013.

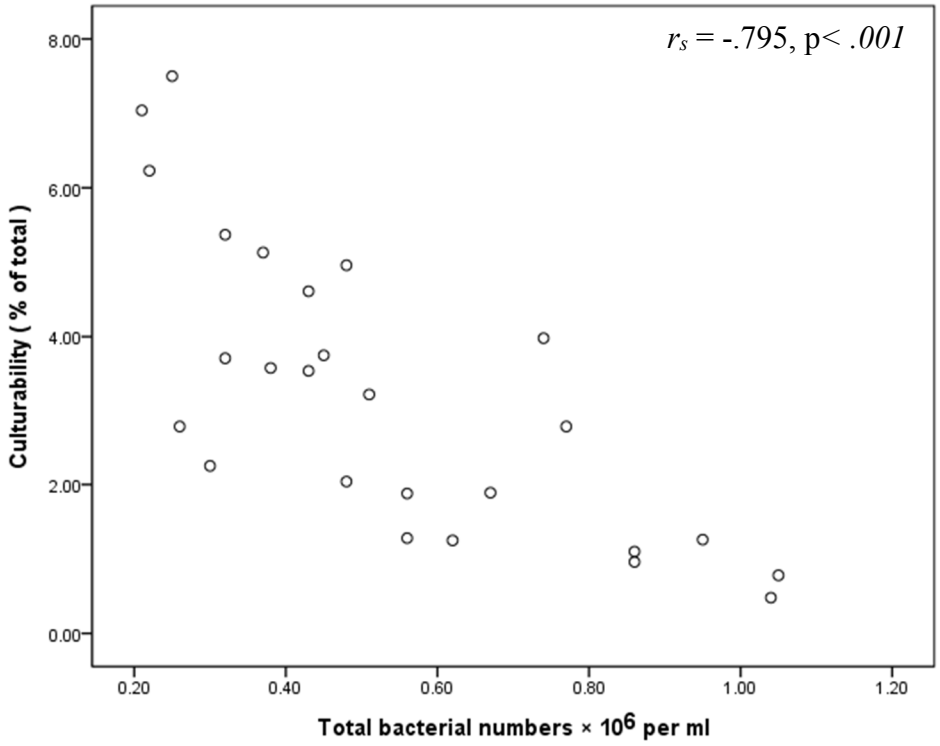


Figure 3. 15 Relationship between percentages of total culturable bacteria and total bacterial numbers in the River Wensum in February 2013.

3.5 Discussion

3.5.1 Total bacterial numbers

This research conducted in the Wensum catchment using epifluorescence microscopy (EFM) with DAPI staining as a standard technique (Hobbie *et al.* 1977; Porter and Feig 1980; Kepner and Pratt 1994). This technique was found to be suitable for the sizes of bacterioplankton populations investigated (Grivet *et al.* 2001).

Total bacterial numbers in the River Wensum range from 0.21 – 5.34 × 10^6 cells/mL and are similar to those found in freshwaters worldwide. Hobbie *et al.* (1983) reported bacterial numbers in the River Kuparuk, US of 0.3 × 10^6 cells/mL to 2.7 × 10^6 cells/mL with the highest abundance in the summer season. Also, Velimirov *et al.* (2011) reported 7.7 × 10^5 to 5.1 ×10^6 cells/mL in the River Danube, with numbers increasing as the river approached the sea. Castillo *et al.* (2004) recorded similar numbers of bacteria (0.6 × 10^6 and 0.8 × 10^6 cells/mL) in several lowland rivers of the Orinoco basin over a two year period, with increases during periods of low flow. The mean bacterial abundance in the River Traun in Austria was 1.2 × 10^6 cells/mL (Klammer *et al.* 2002). Freese *et al.* (2006) recorded the maximum numbers of total bacteria 24 × 10^6 cells/mL in the River Warnow, Germany. They attributed the high values to its eutrophic status and the presence of large amounts of organic matter in the river. In the River Hull, UK and three smaller water courses, Yamakanamardi and Goulder (1995) reported bacterial numbers between 0.7 and 22.4 × 10^6 cells/mL, with a mean of 4.3 × 10^6 cells/mL, with highest values in spring and summer.

Schumann *et al.* (2003) found that bacterial abundance ranged from 4.4 × 10^6 cells/mL in mesotrophic water habitats to 10.9 × 10^6 cells/mL in eutrophic water habitats. Raw water abstracted from Lake Zurich contained 1 × 10^6 cells ml^{-1} (Hammes *et al.* 2008) and in eighty natural lakes distributed through the Pyrenees mountains between France and Spain, bacterial numbers were between 3 × 10^4 to 3 × 10^6 cells/mL (Felip *et al.* 2007).

In this study, total bacterial abundance generally varies more temporally than spatially. This is due to the variation of water temperature and river flow as revealed by hierarchical partitioning analysis. The highest numbers were recorded in summer, while lowest numbers occurred in winter. This is in agreement with other studies. For example, in the Ogilvie and Swift rivers, Yukon Territory, Canada, heterotrophic counts and total bacterioplankton numbers changed seasonally, with higher numbers in the spring and summer and low numbers in the winter (Albright *et al.* 1980). In the Ogilvie River, the average numbers of heterotrophic bacteria and total bacterioplankton were 2.5 x 10^2 and 1.6 x 10^4 cells/mL, respectively in winter (average temperature, 0 ˚C) compared with 7 × 10^3 and 8.4 × 10^5 cells/mL, respectively in summer (average temperature, 12˚C). In the eutrophic River Warnow, total counts were 24 × 10^6 cells/mL in the summer season at an average water temperature of 22˚C, while the average

total numbers in spring were 6×10^6 cells/mL when the average water temperature was 8˚C (Freese *et al.* 2006).

The highest numbers of bacteria in the river water were recorded at sites S18 and S8 in the 4th order downstream section of the river, suggesting that bacteria increase in abundance as the water moves downstream. There may also be contributions from urban area runoff (urban area represents 4.9% of the sub-catchment area draining to S18 and 3.1% to S8 and discharges from sewage treatment works (3 STWs upstream of S8 and 2 STWs upstream of S18).

3.5.1.1 Environmental variables and total bacterial numbers

Environmental parameters explain 52 % of the variation in total bacterial numbers.

Water temperature, which ranged from 1.5 ˚C to 19.5 ˚C (mean = 9.9 ˚C), showed the strongest relationship with total bacterial numbers, and the mechanism behind this is not well understood. This is in agreement with several studies in freshwater environments. Yamakanamardi and Goulder (1995) reported a positive link between temperature (range 2-20 ˚C) and bacterial numbers in the River Hull. In the oligotrophic Tarun River, Austria, temperature showed a positive relationship with bacterial numbers among several chemical and physical parameters. For example, in January (average temperature, 4˚C), bacterial numbers were 7.9×10^5 cells/mL, while there were 1.6×10^6 cells/mL in September when water temperature was 20˚C. Felip *et al.* (1996) found that a low water temperature at 4˚C played an important role in limiting bacterial growth and abundance in Upton Lake, UK, with the presence of high concentrations of organic carbon, inorganic and organic phosphorus and nitrogen in the lake water. Felip *et al.* (1996) stated that this phenomenon can cause negative impacts on an aquatic ecosystem through accumulations of these nutrients in the winter and in turn influence the dynamics of bacterioplankton in the later spring and summer seasons. In the Anacostia River in the US, Cavari *et al.* (1981) recorded low numbers of *Aeromonas spp.* (mean 5.5×10^2 cells/mL) when water temperature was low (4˚C) compared with a high number (mean 5.5×10^5 cells/mL) when water temperature was 25 ˚C. Low numbers of bacteria were attributed to their inability to uptake nutrients and maintain their cellular needs at low water temperatures.

This study also found that total bacterial numbers were negatively related to river flow, which probably reflects dilution by rainwater (Poff *et al.* 1997; Mihailova *et al.* 2013). This result is in agreement with other studies that found a negative correlation between number of bacteria and water flow (Donlan and Pipes 1988; Ragazzo and Nardo 2002). In the River Brda, Poland, river flow played an important role in decreasing the percentage of total psychrophilic bacteria to about 26.8% (Małecka and Donderski 2006). However, Wasielewska *et al.* (2009) did not find any correlation between bacterial numbers and river flow in the lowlands of Warta River in Poland.

Nutrients were also correlated with total bacterial numbers in the River Wensum. Similar positive correlations with P concentrations have been reported in other studies. Velimirov *et*

al. (2011) found that total bacterial numbers in the Danube river were positively related to only phosphorus among other chemical factors. Wasielewska *et al.* (2009) found that numbers of bacterioplankton in the lowlands of the River Warta, Poland were significantly and positively related to environmental parameters such as TP, dissolved oxygen and conductivity. In some temperate lakes in Denmark, Sondergaard and Danielsen (2001) reported a strong positive correlation between DAPI-stained bacteria and total phosphorus. In 36 US and Canadian lakes, total phosphorus showed a strong relationship with bacterial and algal abundance (Currie 1990). In 20 stratified lakes in the US, Cole *et al.* (1993) found that total phosphorus was strongly and positively correlated with total bacterial abundance, while bacterial cell size was suggested to be regulated by other processes.

Bacterial numbers were negatively related to total nitrogen (TN) in the Wensum. This is in agreement with Reche *et al.* (2009) who reported a negative correlation between bacterial abundance and TN in one alpine lake in Sierra Nevada, Spain but no noticeable correlation between total bacterial abundance and TN in three other investigated waters (reservoirs). However, in 24 lakes in Sweden, bacterial numbers were positively related to total nitrogen, total phosphorus and dissolved organic carbon (Anesio *et al.* 2004). Rees *et al.* (2005) found no link between bacterial numbers and TN in the Murray River, Australia, with total numbers related to the concentration of chlorophyll *a*.

Total organic and inorganic carbon (TC) was also found to be negatively correlated with total bacterial numbers in the Wensum. Total organic carbon was positively correlated with bacterial numbers. There are no studies examining the link between TC and TOC and total bacterial abundance in any freshwater systems. In the River Wensum, TC increased during the wet year 2012 compared with the dry year 2011. During the wet year 2012 and after events of high flow rates, discharge from terrestrial areas and ground water may play an important role in the increases of TC (organic and inorganic), limiting bacterial abundance. However, during the dry year 2011 when resident time and water level are low, TC could be biologically up-taken in groundwater. Low flow is dominated by groundwater baseflow.

Results for the Wensum show that total bacterial numbers are positively related to the number of STWs. River water can be contaminated by discharges from STWs which may carry biological and physicochemical pollutants, such as bacteria, nutrients and organic matter (Cebron *et al.* 2004). Water bodies near human populations are exposed to a wide range of contaminants through discharge processes from different sources, such as sewage treatment works (Olayemi 1994). These discharges can contribute to increases in the abundance of total bacteria, heterotrophic bacteria and total coliforms (Geldreich 1976; Goni-Urriza *et al.* 1999). Along the River Wensum, the majority of sewage treatment works are found at downstream sites of the river (3^{rd} and 4^{th} stream order).

The changes in the bacterial community as water moves downstream in the River Wensum depends on inputs of exogenous bacteria (from agricultural areas, urban area and sewage treatment works) and on the relative growth rates of the bacterial species. Total bacterial numbers increase as water moves downstream but this results from population growth of only a subset of bacterial OTUs (as explained in Chapter 4). The relative abundance of some

bacterial species increases and shows positive correlation with some nutrients, such as TP. These bacteria presumably grow faster than others at the effect is particularly marked during periods of higher water temperatures or low flow (presumably reflecting higher average population growth rates and longer residence times respectively). However, the mechanisms of the responses of bacteria to water temperature (direct or indirect) are not well understood. Although, there are significant differences in total bacterial numbers between sites, these differences are not large. Also, there are significant correlations between total bacterial numbers and environmental variables but these are not strong enough to reflect the impact of environmental conditions in each specific site on the bacterial abundance. As a result, total bacterial abundance in this research is not able to give a detailed assessment of ecological status of the river water.

3.5.2 Total heterotrophic bacteria and their percentages of total numbers

Abundances of heterotrophic bacteria in the Wensum were found to be between 0.5 and 2.95 $\times 10^4$ CFU/mL, representing between 0.48% and 7.5% of total counts. Several studies have investigated heterotrophic bacterial numbers in freshwater systems. In the River Słupia, northern Poland, Skorczewski and Mudryk (2009) found that the mean of heterotrophic bacteria numbers was 2.8×10^4 CFU/mL in the winter, while it was 5.1×10^4 CFU/mL in spring. In the River Brda in Germany, the average number of total heterotrophic bacteria was 5.31×10^5 CFU/mL, with the majority being ammonifying bacteria, representing about 83% of total counts. The average total heterotrophic bacterial numbers in winter were 6.64×10^4 CFU/mL when the water temperature was between 2 and 5 ˚C, while there were 14.3×10^5 CFU/mL in spring when the water temperature was between 13 to 19 ˚C (Małecka and Donderski 2006). Masuda *et al.* (1980) found that heterotrophic bacterial counts in the River Ogilvie in Canada were between 2.5×10^2 and 1.5×10^4 CFU/mL, while they were between 3.2×10^2 and 1.9×10^3 CFU/mL in the River Swift, US. In the heavily contaminated River Asa in Nigeria, Olayemi (1994) found that numbers of heterotrophic bacteria were high and ranged from 4.5×10^6 to 8.5×10^6 CFU/mL, with highest values during rainfall events.

Yamakanamardi and Goulder (1995) found that total heterotrophic bacterial counts in the River Hull ranged between 0.10 and 2.41×10^5 CFU/mL, representing between 0.13% and 13.1% of the total numbers. In two eutrophic rivers, Kenzaka *et al.* (2001) found that total counts of culturable bacteria in Kelang River in Malaysia were between 1.3 and 6.9×10^3 CFU/mL, representing between 19% and 58% of the total counts, while in the Chao Rhraya River in Thailand, they were between 0.4 and 0.8×10^3 CFU/mL, representing between 5% and 8% of the total. In four streams, Lemke and Leff (2006) found that total bacterial numbers were between 0.3 and 0.5×10^6 cells/mL. The proportions of the culturable bacteria of the total were small at all times, and were 0.35% in November, 0.33% in February and 0.36% in April.

The percentages of total culturable bacteria in the River Wensum were strongly and negatively correlated with total bacterial numbers. The reason is that in the River Wensum, the common

OTUs become more abundant as the water moves downstream. This presumably reflects the fact that these bacteria are actively growing in the river, and reducing the abundance of other taxa including those that are heterotrophic. Results in Chapters 4 and 5 indicate that not all bacterial types increase as water moves downstream. Some bacteria are able to grow in these sites and may reduce the abundance of the other types including heterotrophic bacteria. This result is in contrast to the study conducted by Lemke and Leff (2006) who found a strong positive linear relationship between total bacterial abundance and total culturable bacteria in four investigated streams.

Some of the differences in the abundance of total culturable bacteria may be due to culturing factors, such as the type of media and temperature of incubation but they could be also due to the variation of culturable bacteria between streams (Lemke and Leff 2006).

The highest numbers of culturable heterotrophic bacteria in the Wensum were recorded at downstream sites; S8 (4^{th} order), S14 (3^{rd} order) and S15 (4^{th} order). This observation may be attributed to the high numbers (3 each) of upstream sewage treatment works, which may discharge high amounts of nutrients and bacteria into these sites (Cebron *et al.* 2004). Sewage treatment works can deposit a high amount of nitrogen and also ammonium-oxidizing bacteria into streams (Dang *et al.* 2010). Chemical data revealed that these sites experience high mean concentrations of nitrogen (S14 = 7 mg/L, S8 = 7.2 mg/L and S15 = 7.7 mg/L). Also, 454 pyrosequencing data (Chapter 5) revealed that these sites have a high abundance of common members belonging to the family of Comamonadaceae and the class of Betaproteobacteria (S14 = 4.07% (OTU2), S8 = 5.14% (OTU5) and S15 4.41% (OTU5) of the total abundance). Species of this class are known as ammonium-oxidizing bacteria and can be discharged from treated sewage into streams (Newton and Madison 2008). Skorczewski and Mudryk (2009) found that the highest numbers of total heterotrophic bacteria (mean 37.23×10^3 CFU/mL) were recorded at a site downstream of the sewage treatment works on the River Słupia, Northern Poland, compared with two other investigated sites (site 2, mean 8.39×10^3 CFU/mL and site 3, mean 4.17×10^3 CFU/mL). In addition, Skorczewski and Mudryk (2009) stated that effluents from sewage treatment works caused increases in the size and abundance of metabolically active bacteria.

3.6 Summary

In the research reported in this chapter, total bacterial numbers showed significant spatial and temporal differences. These differences are related to different environmental parameters, and multiple regression analysis showed that these parameters explain about 52% of the variation of total bacterial numbers. The remaining percentages that can affect the variation of total bacterial numbers may be related to other chemical and physical parameters and also biological factors, such as grazing by large flagellates and protozoa. Total bacterial abundance was shown to increase as the water moves to downstream sections of the river. Total bacterial numbers that were heterotrophic showed significant differences between individual sites and were not

related to any of the environmental parameters. Numbers of heterotrophic bacteria as a percentage of total bacterial numbers in the Wensum may reflect the potential for recovering many bacteria when developing suitable methods like the R2A medium.

Some methodological considerations should be taken into account before and during the enumeration of bacteria to avoid underestimation of total bacterial numbers. First, increasing particles in water samples may mask bacteria and prevent their fluorescence with DAPI (Paulse *et al.* 2007), although this stain has been shown to be less influenced by particles compared with other staining methods such as acridine orange (AO) (Sieracki *et al.* 1985). Secondly, long exposure time to light during staining with DAPI and then examination by microscope can result in the fading of this stain (Yu *et al.* 1995). Thirdly, during filtration, the vacuum pressure should be between 50 to 70 Hg to avoid penetration of many bacterial cells (Kepner and Pratt 1994). Finally, a small amount of immersion oil added to the filters is recommended to prevent bacterial cells from floating and becoming lost (Turley and Hughes 1992).

Chapter Four

Investigating bacterial community composition in the River Wensum using automated ribosomal intergenic spacer (ARISA)

4.1 Introduction

Work based on culture-dependent methods has concluded that aquatic and soil bacterial communities have a similar taxonomic composition (Hahn 2006). However, culture based methods detect only a small proportion of the bacterial diversity present (Schmeisser *et al.* 2003; Kirk *et al.* 2004; Zeng *et al.* 2011).

Fingerprinting techniques, such as T-RFLP (Liu *et al.* 1997), DGGE (Muyzer *et al.* 1993; Muyzer 1999) and ARISA (Fisher and Triplett 1999) have revolutionized our view about the composition of freshwater bacteria (Hahn 2006) and allowed the study of changes of bacterial community with time and between sites (de Figueiredo *et al.* 2010), and the biotic and abiotic factors that may be responsible (Humbert *et al.* 2009).

ARISA is inexpensive, rapid and reproducible and is able to provide a good description of the composition of bacteria in large numbers of samples (Fisher and Triplett 1999; Crump *et al.* 2003; Brown *et al.* 2005) that allows temporal, spatial and geographical effects on bacterial community composition to be monitored in detail (Fechner *et al.* 2010). It examines the most variable region of rRNA, the ITS region between 16S rRNA and 23S rRNA (Brown and Fuhrman 2005), and is able to detect a large number of peaks per bacterial profile (Fisher and Triplett 1999; Ranjard *et al.* 2000). Many studies have demonstrated that ARISA is a robust and efficient tool in describing bacterial communities (Fisher and Triplett 1999; Ranjard *et al.* 2000; Schwalbach *et al.* 2004; Brown *et al.* 2005; Yannarell and Triplett 2005; Lear and Lewis 2009).

ARISA has been used successfully in a range of environments, including lake water (Fisher and Triplett 1999), sea water (Mapelli *et al.* 2013), soil (Ranjard *et al.* 2001), estuaries (Hewson and Fuhrman 2004), catfish ponds (Arias *et al.* 2006), and the rumen of lactating dairy cows (Palmonari *et al.* 2010).

4.2 Aims

As introduced in Section 1.7, the aims of the research presented in this chapter are to use automated ribosomal intergenic spacer analysis (ARISA) to characterise the bacterial community composition in the lowland arable catchment of the River Wensum and (i) quantify the spatial and temporal variation and impact of environmental factors on this variation, (ii) characterise these changes in terms of both diversity and abundance, (iii) identify the commonest bacterial OTUs and quantify changes in their abundance between sites and times, and (iv) describe the trend of the shift of bacterial diversity and the abundance of the commonest bacterial OTUs when water moves to downstream sites.

4.3 Methods and materials

4.3.1 The study sites

Samples were collected from 26 sites in the Wensum catchment and sub-catchment areas from June 2011 to December 2012 as detailed in Section 3.3.1.

4.3.2 Sample collection and filtration

Water samples were collected in sterile 500 ml bottles from a depth of 10-20 cm (see section 3.3.2.1 in chapter 4) below the water surface at each site. Samples were placed on ice and transferred to the lab. Filtration was carried out immediately after sample collection. Samples were inverted and shaken to ensure suspension of all bacterial cells. Water samples were filtered through a 0.22 µm cellulose membrane filter (Millipore, Germany). Filters were then transferred into 15 mL centrifuge tubes and stored at -80 ˚C, prior to DNA extraction.

4.3.3 DNA extraction

DNA was extracted using a NucleoSpin® food kit (Macherey-Nagel, Germany), based on the recommendation of Jara *et al.* (2008). Following the manufacturer's protocol with some modifications: 10 µl of proteinase K was added to the cellulose membrane filter and mixed gently for 2-3 s. 1000 µl of lysis Buffer CF (preheated to 65˚C) was then added to the tube and mixed for 15 s before placing into a water bath at 65˚C for 30 min. The tube was then vortexed for 1 min and centrifuged (500 g) for 4 min and stored in a freezer at -80 ˚C for 30 min. After freezing, the tube was transferred into a water bath at 65 ˚C for 30 min and again stored in the

freezer at -80 ˚C for 30 min. These extra steps of heating and thawing were important because the amount of DNA and cells lysis is enhanced by 2 fold when using an additional thermal shock step of three rounds of heating in a water bath at 65 ˚C and cooling at -80 ˚C (More *et al.* 1994). The tube was placed in the water bath at 65 ˚C for 30 min for the final heating, and then vortexed for 5 min to break the filter. 1000 µl of binding Buffer C4 and ethanol were added to the tube to adjust DNA binding conditions, with the mixture then vortexed for 30s. 700 µl of the mixture was next transferred onto the NucleoSpin® food column tube to bind the DNA. The tube was centrifuged (11000 g) for 1 min. These steps were repeated to load all the amount of supernatant. To wash the silica membrane, 400 µl of wash Buffer CQW was added into the NucleoSpin® food column. The tube was centrifuged (11000 g) for 1 minute, 200 µl of wash Buffer C5 was added into the NucleoSpin® food column, the tube was centrifuged (11000 g) for 1 min, and then repeated. The NucleoSpin® food column was transferred into a new 2 ml centrifuge tube and DNA eluted with 2 × 100 µl of Elution Buffer CE (5 mM Tris/HCl, pH 8.5) (preheated to 70˚C). DNA was stored at – 20 ˚C, prior to analysis. Quantification of the DNA was performed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, UK).

4.3.4 PCR amplification

ARISA was conducted using two primers. ITSF-Hex (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') (Cardinale *et al.* 2004), synthesised and supplied by Applied Biosystems.

The extracted DNA templates were amplified in a 25 µl mixture containing 12.5 µl of BioMix™ (Bioline, UK), 1 µl of ITSF-Hex primer and 1 µl of ITSReub primer (final concentration of primers: 10 pmol/ml), 9.5 µl of ultrapure sterile water (MilliQ water) and 1 µl of DNA (5 ng DNA). The amplification of the DNA templates was carried out in a StepOne™ Real-Time PCR System (Applied Biosystems, UK). PCR cycling conditions were: initial denaturation at 95 ˚C for 5 min, followed by 35 cycles of denaturation at 95˚C for 30 s, annealing at 55˚C for 30 s, extension at 72˚C for 1 min and a final extension step at 72˚C for 10 min, with samples held at 4˚C.

Agarose gel electrophoresis was used to check the success of PCR amplification as follows: 1% of agarose gel was diluted with 1% of Tris-Borate EDTA buffer (TBE, pH = 8.0) and stained with ethidium bromide (10 mg/ml) (Sigma, UK). 5 µl of each of the PCR products and also a DNA ladder (Fisher Scientific, UK) were mixed with 5 µl of loading dye (Sigma, UK). The mixture was loaded onto 1% agarose gel and run for 40 minutes at 100 V. The DNA bands were checked under a UV transilluminator. The results were photographed using a Polaroid camera (an example, see Appendix Figure A 4.22).

1 µl of each of the successful PCR products was pipetted into a 0.2 ml tube of a 96 well PCR plate containing 10 µl of Hi-di formamide and 0.2 µl of LIZ 1200 labelled size marker (Applied Biosystems, UK). Separate pipette tips were used to avoid cross contamination. PCR products

were sent to the John Innes Centre, Norwich, UK for fragment sizing on a 3730® DNA Capillary Analyser (Applied Biosystems, UK).

4.3.5 Analysis of ARISA fingerprints

ARISA Profiles were assembled using the Peak Scanner™ programme, version v1.0 (Applied Biosystems, UK), downloaded from the website http://www.appliedbiosystems.com. Fragment sizes less than 50 bp and peak heights with an intensity of less than 50 fluorescence units were removed from the analysis (Fisher *et al.* 2000; Jones *et al.* 2007). Calculated peaks sizes and heights were exported into an Excel spreadsheet and peaks aligned across samples using T-Align (http://inismor.ucd.ie/~talign/) (Smith *et al.* 2005), using a bin size of 0.5bp.

The consensus file from T-Align was imported into Primer V6 (Clarke and Gorley 2006) for statistical analysis. Multivariate methods including multidimensional scaling (MDS) and cluster analysis were used, following Field, Clarke *et al.* (1982) after square root transformation to reduce the influence of abundant species (Field *et al.* 1982). The Bray Curtis coefficient (Bray and Curtis 1957) was used to measure similarity between samples, as this has been proven to be a robust tool in analysing microbial communities (Rees *et al.* 2004) and gives meaningful information on data sets containing large numbers of absences, as joint absences do not contribute to similarity. Samples with the same species composition represent 100% similarity (similar samples), while a similarity value of 0% represent no shared species (Clarke and Warwick 2001).

MDS is a non-parametric method used to carry out the dimensional ordination of samples. MDS produces a graphical display of the similarity or dissimilarity between samples in which high distances between samples reflect low similarity of composition (Chatfield and Collins 1980). The stress value measures the extent to which multidimensional scaling captures distances between communities, with values less than 0.05 indicating good representation of underlying distances (Field *et al.* 1982; Clarke 1993; Rees *et al.* 2004). MDS has proven to be a robust tool in discriminating microbial communities in different environments in river sediments (Rees *et al.* 2004) and in sites exposed to heavy metal contaminations (Austen and McEvoy 1997).

The analysis of similarity (ANOSIM) was applied to determine the significant spatial and temporal differences in bacterial community composition (Clarke 1993; Rees *et al.* 2004).

SIMPER (similarity percentages) analysis was performed to identify the taxa contributing to differences in the community between sites and dates (Clarke and Warwick 2001).

Abundances of the commonest OTUs were also imported into SPSS v18 and the same as on the statistical analyses on the bacterial counts (see section 3.3.5.). Data that were not normally distributed were square root transformed. Two-way ANOVA was used to test for differences in abundance between sites and study months, and estimated marginal means calculated to resolve inequality of samples sizes. Relationships between the abundance of commonest OTUs

and environmental parameters were examined using the Spearman's rank correlation coefficient (r_s), and stepwise multiple linear regression analysis and hierarchical partitioning analysis were used to identify the most important factors responsible for differences in their abundance. All graphs of the abundance of the common OTUs were produced using SPSS v18, while MDS plots were produced using Primer v6. Principal component analysis PCA was performed using SPSS to evaluate the changes of bacterial OTUs over spatial and temporal scales. PCA is a measure of ecological distance by ordination diagrams (changes of bacterial species between sites and times) which can transform a single data set (original variables) to a new coordination system called principal components (synthetic variables). In the PCA output, the rows of the new synthetic variables represent the species matrix, while the columns indicate the principal components. The first two or three principal components (new loadings) account for the largest components explaining the variations of the original data set (Ramette 2007).

In addition, canonical component analysis (CCA) was carried out to map the variations of bacterial OTUs over spatial and temporal variations, and also to detect the relationships between environmental parameters and bacterial community composition. CCA (also an analysis of ecological distance) determines the variations of two data sets in which one can explain the other. Unlike PCA, it describes the variations of two data sets (e.g. species changes vs. environmental parameters) rather than a single data set (Hotelling 1936; Ter Braak 1986). Canonical analysis of principal coordinates (CAP) was carried out in R using the Capscale command in the Vegan package and significance testing of relationships between OTUs and environmental parameters was tested using permutation based Anova (using the Adonis command) in the same package. This involves canonical correlation between the principal coordinates produced from OTUs and environmental factors, similar to canonical coordination analysis CCA (Anderson and Willis 2003; Sinkko *et al.* 2011).

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4.4 ARISA Results

4.4.1 Overall bacterial community composition

4.4.1.1 Spatial variation of bacterial community composition.

Figures 4.1-4.3 show 2-D MDS for the bacterial community composition in the River Wensum for individual sites from June 2011 to December 2012.

There is substantial overlap of the community composition between sites, and the large number of data points makes it difficult to observe clear patterns in Figure 1. However, ANOSIM revealed that there were significant differences in bacterial community composition between sites (R= 0.294, p= 0.1%) and calculating mean positions on the MDS plot displays these differences (Figure 4.2). The greatest differences of bacterial community composition were

between the upstream sites SA, SB and SE (Blackwater sub-catchment 1) and the downstream sites S8 (Wensum, Swanton Morley) and S18 (Wensum, Costessey Mill) (Figures 4.2 and 4.3), ANOSIM (R= 0.793, *p*= 0.1%). If data from these sites alone are examined within the MDS results, there is no overlap between the two groups (Figure 4.3).

SIMPER analysis (Appendix Table A 4.19) showed that 14 OTUs (702.09, 795.42, 806.71, 591.97, 810.12, 572.13, 575.27, 713.78, 718.52, 565.24, 724.25, 817.97, 559.55 and 694.57) made substantial contributions to the average dissimilarity between these two groups at upstream and downstream sites. 10 out of these 14 taxa increase in abundance as they move to downstream sites. SIMPER also indicated that bacterial diversity at the upstream sites was higher than at downstream sites (Figure 4.8).

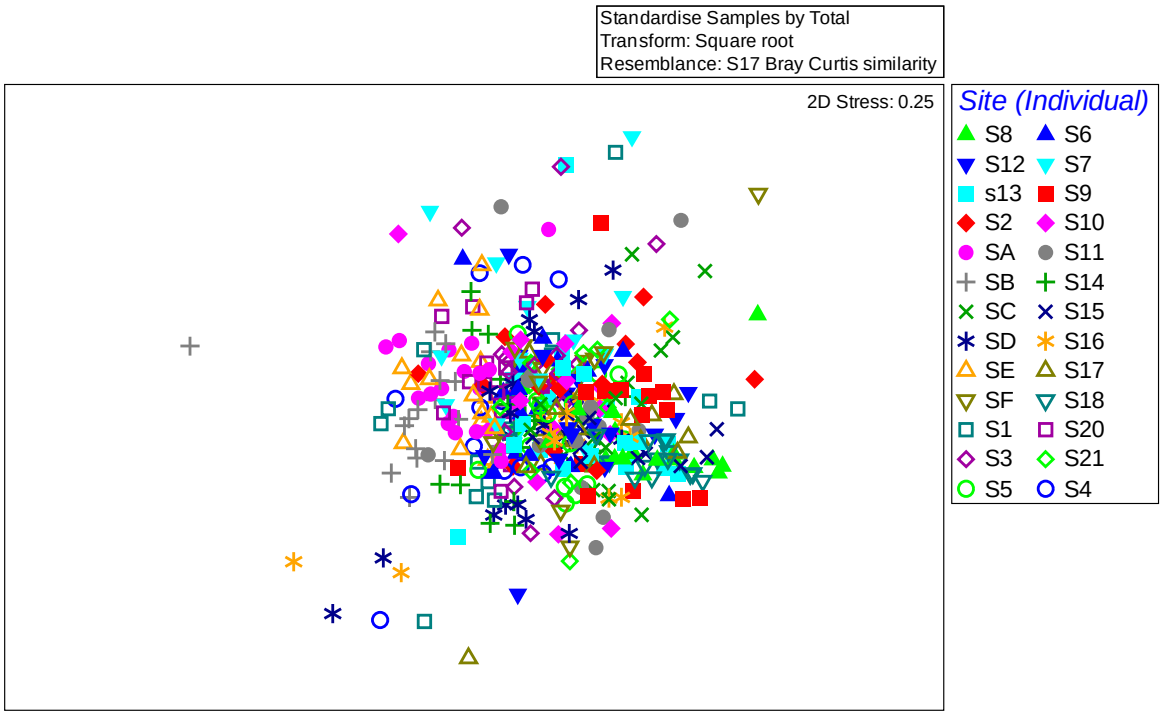


Figure 4. 1 Multi-dimensional scaling ordination (all MDS values) of bacterial composition in the River Wensum between individual sites from June 2011 to December 2012.

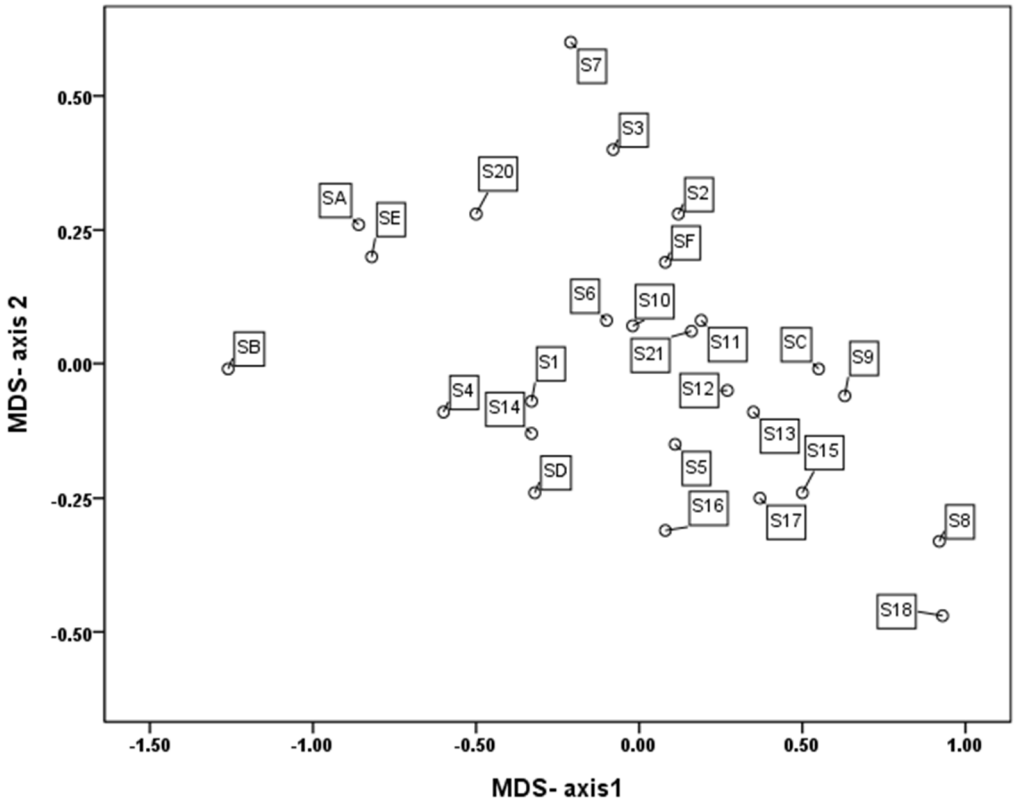


Figure 4. 2 Multi-dimensional scaling ordination (mean of MDS values) of bacterial composition in the River Wensum between individual sites from June 2011 to December 2012.

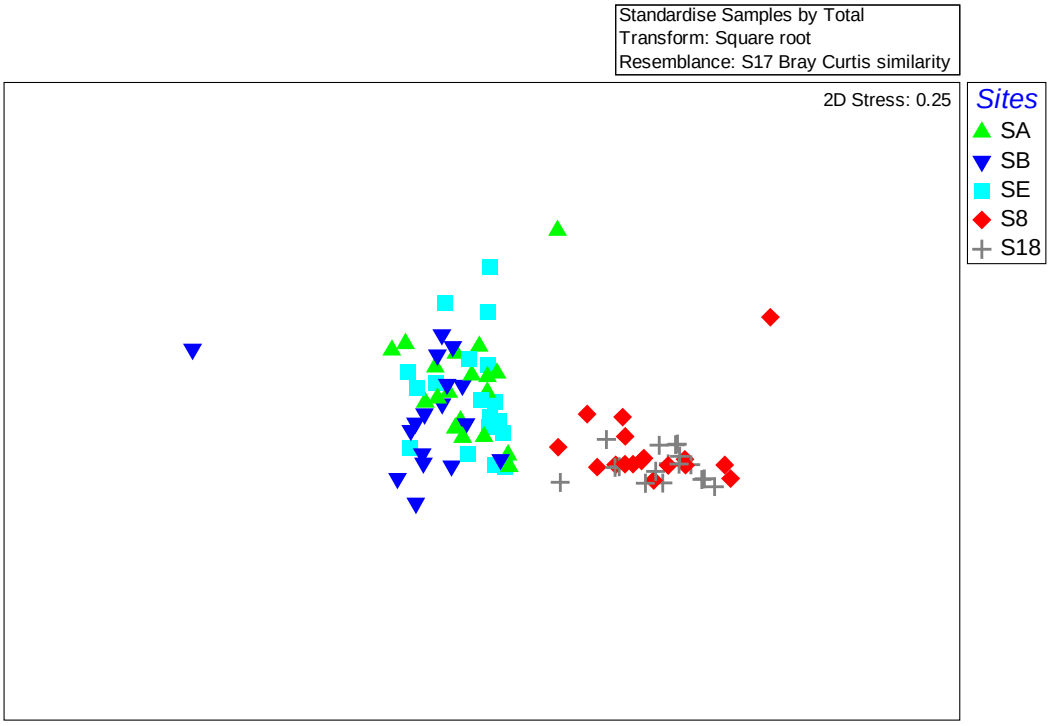


Figure 4. 3 Multi-dimensional scaling ordination (all MDS values) of bacterial composition in the River Wensum between upstream (SA, SB and SE) and downstream (S8 and S18) sites from June 2011 to December 2012.

4.4.1.2 Temporal variation of bacterial community composition

Figure 4.4 and 4.5 show multidimensional scaling ordination of the bacterial community composition in the Wensum between June 2011 to December 2012. As with the pattern of spatial variation, there is considerable overlap of community composition between sampling times, but ANOSIM shows significant differences in bacterial community composition between months (R= 0.255, *p*= 0.1%) and the monthly mean positions on the MDS plot (Figure 5) display these differences.

It can be seen that large differences in community composition can occur in the same month for different years. For example, Figure 4.6 shows the differences of bacterial community composition between December 2011 and December 2012. Mean water temperature was the same (5.7 ˚C) in both years, but water flow was very different (mean = 2.30 m^3/s and 9.60 m^3/s respectively).

SIMPER analysis (Appendix Table A 4.20) showed that 17 OTUs (638.7, 705, 702.09, 683.31, 729.94, 724.25, 848.48, 756.76, 779.95, 782.92, 718.52, 795.42, 557.13, 806.71, 727.07, 776.61 and 694.57) made substantial contributions to the dissimilarity in bacterial community composition between December 2011 and December 2012. 9 out of these 17 taxa decrease in abundance in December 2012. SIMPER also showed that bacterial diversity in December 2012 was higher than that in December 2011 (Figure 4.9).

Another example is between September 2011 and September 2012 (mean temperatures were 14.2 ˚C and 11.6 ˚C and mean water flows were 1.88 m^3/s and 2.32 m^3/s, respectively). (Figure 4.7; significant difference between years, R= 0.464, *p*= 0.1%).

SIMPER analysis (Appendix A 4.21) showed that 16 OTUs (702.09, 795.42, 806.71, 756.76, 572.13, 591.97, 727.07, 718.52, 711.23, 742.92, 162.59, 565.24, 753.56, 697.29, 678.11 and 638.7) made substantial contributions in the dissimilarity of bacterial community composition between September 2011 and September 2012. 8 out of theses 16 taxa decrease in abundance in December 2012. SIMPER analysis also showed that bacterial diversity in September 2012 was higher than that in September 2011 (Figure 4.9).

Bacterial community composition was very similar in the months of spring 2012. In the summer months June, July and August 2011, bacterial composition was similar to that in June, July and August 2012. Also, bacterial composition in October and November 2011 was very similar to that in October and November 2012 (Figure 4.4 and Figure 4.5).

There were several common OTUs that occurred in all periods at all sites that were commoner in the downstream sites, presumably because they are able to multiply and grow in the river (702.09, 795.42, 591.79, 806.71, 817.79, 572.13 and 718). By contrast, three of the commonest OTUs were more common in the upstream sites, particularly during periods of high rainfall and river flow rates.

The results show that the overall bacterial community composition in the River Wensum changed significantly between sites and months. Hierarchical partitioning analysis showed that variations of water temperature, flow and the presence of arable land were the important factors affected the shift of bacterial community composition between sites and months. The bacterial community showed changes in both diversity and abundance, with bacteria originating in different sources, in drainage from different land uses (agricultural and urban areas) and with variation in stream flow runoff contribution. Bacterial diversity increased in the upstream sites of the river especially when water temperatures are low and rainfall and river flow are high, but decreased as the water moves downstream (3^{rd} -4^{th} order streams) (Figures 4.8 and 4.9). Bacterial abundance, on the other hand, increased as water moves downstream sites and only some of the commonest OTUs are able to multiply and grow at these sites. The commonest OTUs contributed the most to dissimilarity of bacterial community composition between months and sites (Figure 4.12 and Table 4.5).

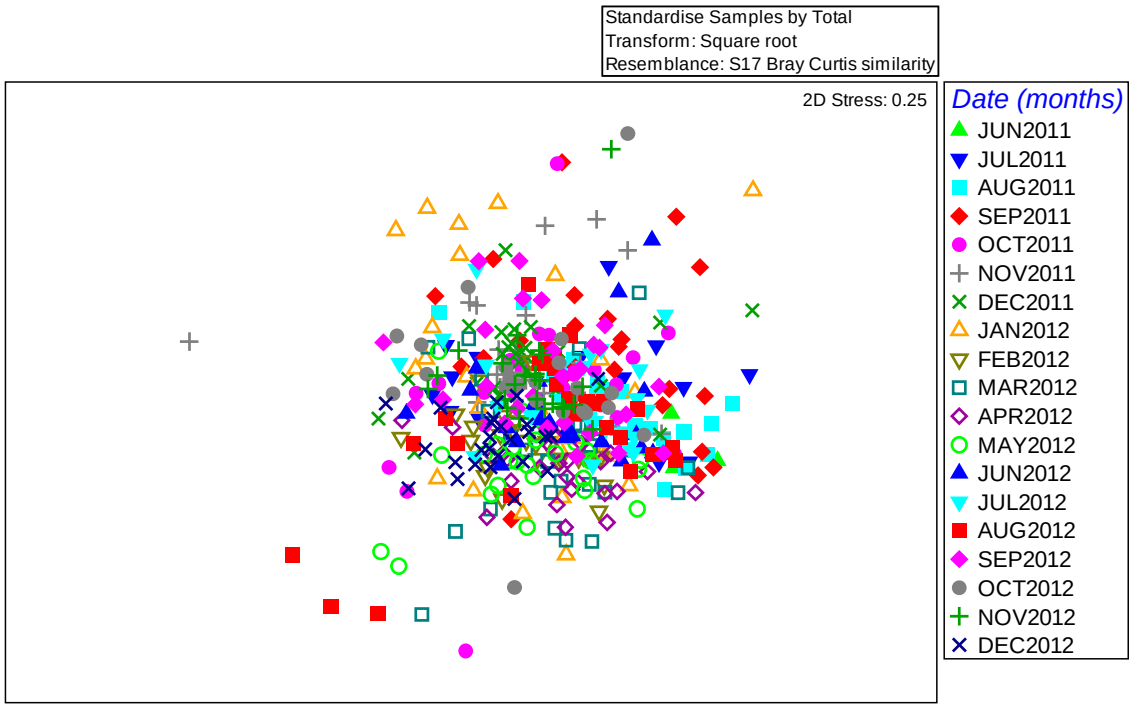


Figure 4. 4 Multi-dimensional scaling ordination (all MDS values) of bacterial composition in the River Wensum from June 2011 to December 2012 (monthly data).

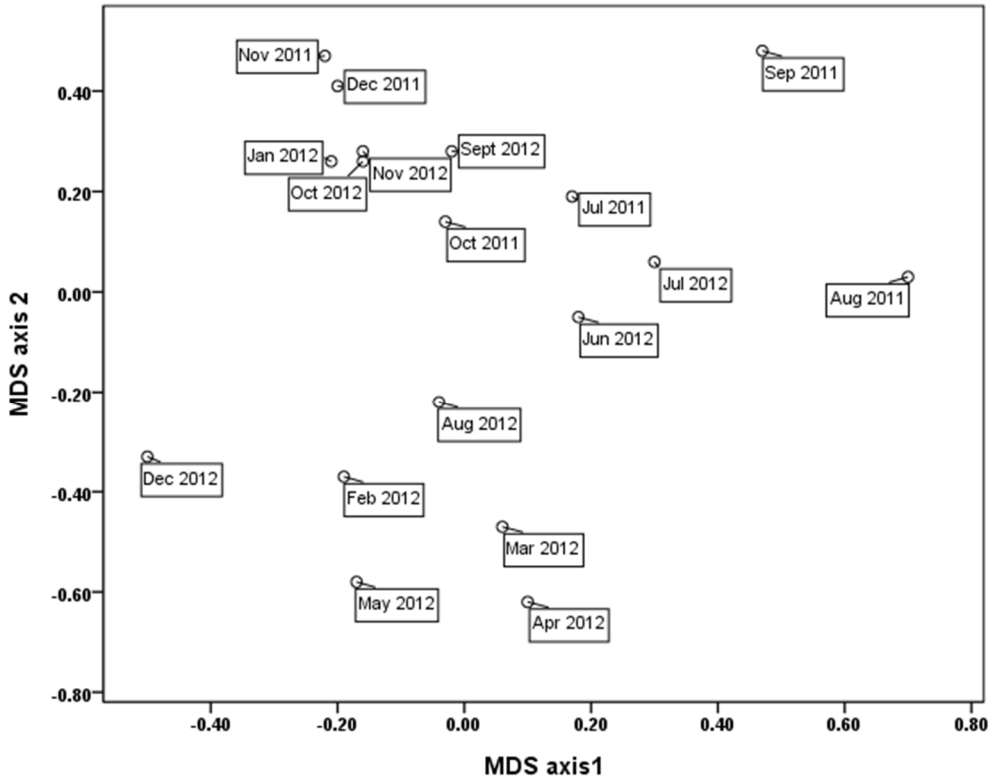


Figure 4. 5 Multi-dimensional scaling ordination (mean of MDS values) of bacterial composition in the River Wensum from June 2011 to December 2012.

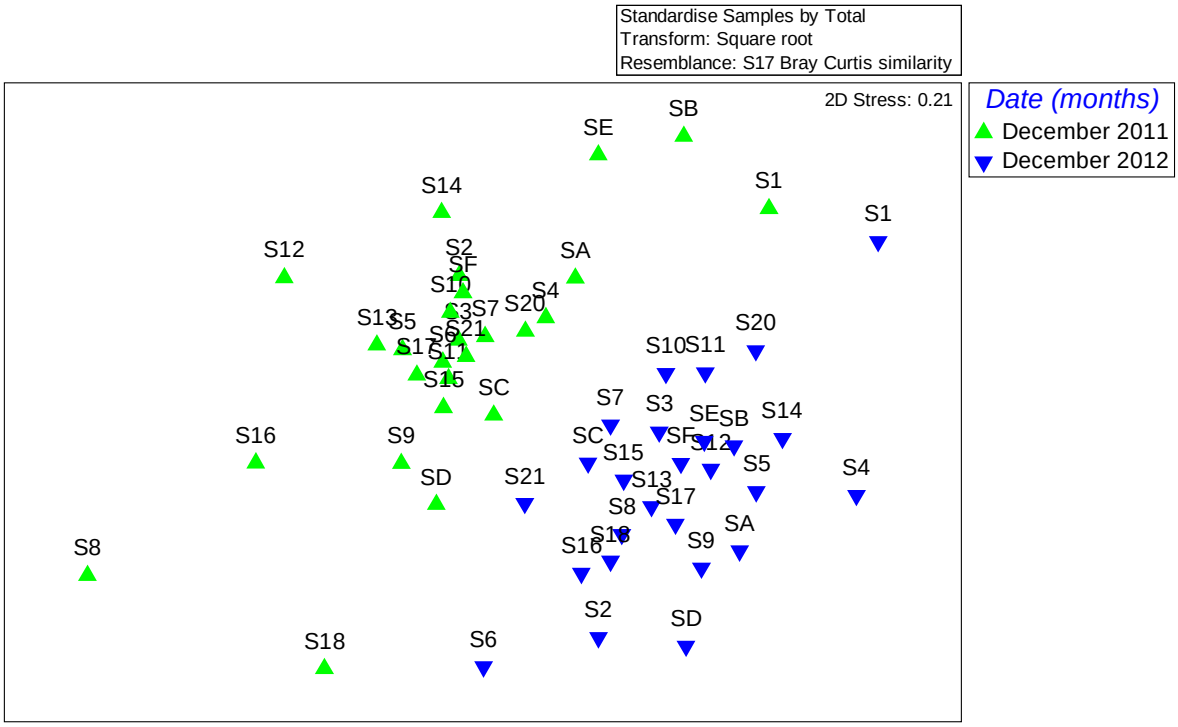


Figure 4. 6 Multi-dimensional scaling ordination (MDS) of bacterial composition in the River Wensum from December 2011 and 2012.

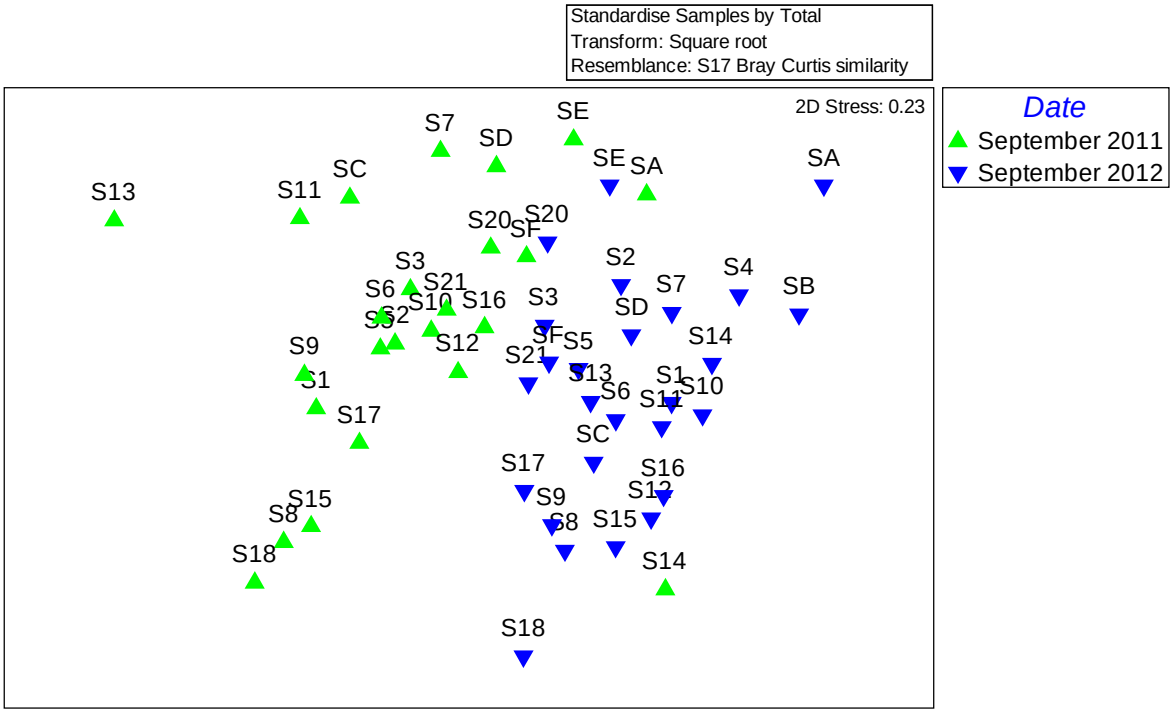


Figure 4. 7 Multi-dimensional scaling ordination (MDS) of bacterial composition in the River Wensum from September 2011 and 2012.

4.4.2 Bacterial diversity (Shannon index *H'* log)

4.4.2.1 Spatial and temporal variation of bacterial diversity

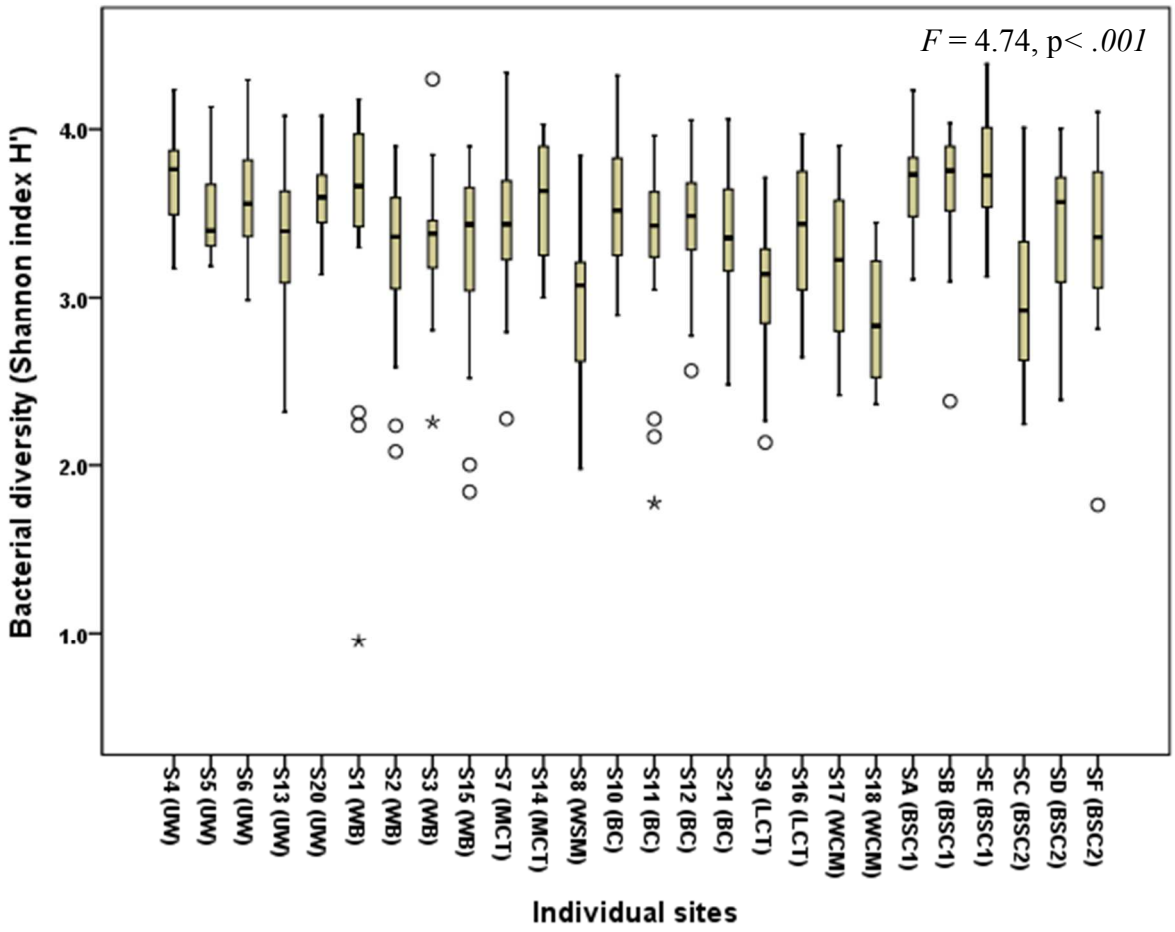
Bacterial diversity (*H'*) in the river water ranged from 1.0 at site S1 in August 2011, to 4.4 at site SE in May 2012, with an overall mean of 3.4.

Figure 4.8 and Table 4.1 show the spatial variation of bacterial diversity in the River Wensum from June 2011 to December 2012 and Figure 4.9 and Table 4.2 show the temporal variation.

Application of two-way ANOVA shows significant differences between sites (F = 4.74, P< 0.001) and times (F = 6.56, P< 0.001), with differences between sites explaining a larger proportion of the variance (18.3%) than differences between months (18.2%).

Generally, bacterial diversity is highest at upstream sites, with sites S4 and SE both having means of 3.6. Diversity decreases as the water moves downstream, and the lowest values are in the 4th order streams, with both S8 and S18 having means of 2.9 (Figure 4.8 and Table 4.1).

Diversity increased in wet and cold months with highest values recorded in February, May and December 2012, while it decreased during dry and warm months, with lowest values recorded in June, August and September 2011 (Figure 4.9 and Table 4.2).



Key symbols: UW = Upper Wensum, WB = Wensum Beck, MCT = Mid Catchment Tributaries, WCM = Wensum Swanton Morley, BC = Blackwater Catchment, LCT = Lower Catchment Tributaries, WCM: Wensum Costessey Mill, BSC1 = Blackwater subcatchment 1, BSC2 = Blackwater subcatchment 2.

Figure 4. 8 Box plot of spatial variation of bacterial diversity in the River Wensum (sites as individuals and groups) from June 2011 to December 2012.

Table 4. 1 Mean, standard error, minimum and maximum of bacterial diversity (Shannon index *H*') in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum (Shannon index *H'*)
S4	3.6 ± .033	3.2 - 4.2
S5	3.4 ± .029	3.2 - 4.1
S6	3.5 ± .029	3.0 - 4.3
S13	3.3 ± .027	2.3 - 4.1
S20	3.5 ± .029	3.1 - 4.1
S1	3.3 ± .029	1.0 - 4.2
S2	3.2 ± .028	2.1 - 3.9
S3	3.2 ± .029	2.3 - 4.3
S15	3.2 ± .029	1.8 - 3.9
S7	3.3 ± .029	2.3 - 4.3
S14	3.5 ± .029	3.0 - 4.0
S8	2.9 ± .027	2.0 - 3.8
S10	3.4 ± .029	2.9 - 4.3
S11	3.2 ± .029	1.8 - 4.0
S12	3.4 ± .027	2.6 - 4.1
S21	3.3 ± .030	2.5 - 4.1
S9	2.9 ± .030	2.1 - 3.7
S16	3.3 ± .029	2.6 - 4.0
S17	3.1 ± .029	2.4 - 3.9
S18	2.8 ± .029	2.4 - 3.4
SA	3.6 ± .028	3.1 - 4.2
SB	3.5 ± .029	2.4 - 4.0
SE	3.6 ± .029	3.1 - 4.4
SC	2.9 ± .029	2.2 - 4.0
SD	3.3 ± .029	2.4 - 4.0
SF	3.2 ± .030	1.8 - 4.1

Shannon index is a measure of bacterial diversity that takes into account both richness and evenness of bacterial species in a community. More bacterial diversity represented by high *H'* value, while less bacterial diversity represented by low *H'* value. If *H'* value equal to 0, then bacterial community represented by only one species in a given community (Hill *et al.* 2003).

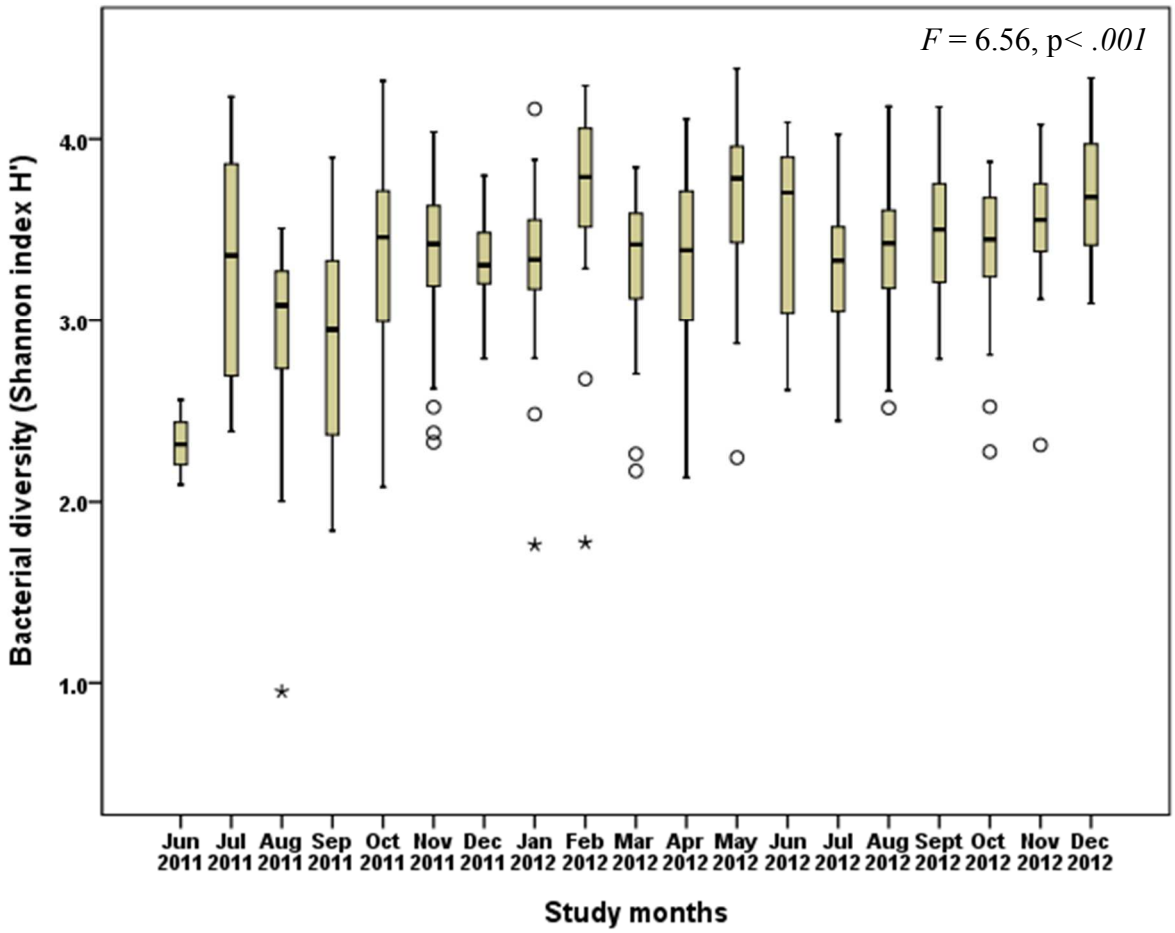


Figure 4. 9 Box plot of temporal variation of bacterial diversity (Shannon index *H'*) in the River Wensum (by month) from June 2011 to December 2012.

Table 4. 2 Mean, standard error, minimum and maximum of bacterial diversity (Shannon index *H*') in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum (Shannon index *H'*)
2011	June	2.4 ± .071	2.1 - 2.6
	July	3.3 ± .031	2.4 - 4.2
	August	2.9 ± .028	1.0 - 3.5
	September	2.9 ± .024	1.8 - 3.9
	October	3.3 ± .022	2.1 - 4.3
	November	3.3 ± .023	2.3 - 4.0
	December	3.3 ± .023	2.8 - 3.8
2012	January	3.3 ± .023	1.8 - 4.2
	February	3.7 ± .023	1.8 - 4.3
	March	3.3 ± .023	2.2 - 3.8
	April	3.3 ± .023	2.1 - 4.1
	May	3.6 ± .023	2.2 - 4.4
	June	3.5 ± .024	2.6 - 4.1
	July	3.3 ± .023	2.4 - 4.0
	August	3.4 ± .023	2.5 - 4.2
	September	3.5 ± .023	2.8 - 4.2
	October	3.4 ± .026	2.3 - 3.9
	November	3.6 ± .024	2.3 - 4.1
	December	3.7 ± .023	3.1 - 4.3

4.4.2.2 Environmental parameters and bacterial diversity (Shannon index *H*')

Mean, standard error, minimum and maximum of environmental parameters are all presented in the Appendices Tables A3-1 to A3-22.

Table 4.3 shows the correlations between environmental parameters and bacterial diversity in the River Wensum from June 2011 to December 2012 using Spearman's rank correlation and stepwise multiple regression analysis.

Spearman's rank correlation analysis revealed that bacterial diversity was positively related to TC, TN, arable land area, river flow rate, TOC and improved grassland, respectively, while it was negatively related to stream order, temperature, urban area and the presence of STWs, respectively.

However, among these significant parameters, stepwise multiple regression analysis revealed that bacterial diversity was positively related to TC and TN, while negatively related to

temperature and stream order. All these environmental parameters explain approximately 18 % of the variation of bacterial diversity in the river water.

Overall, bacterial diversity showed significant spatial and temporal differences, with values decreasing as the water moves downstream. Bacteria can enter the river from different sources: for example, drainage from agricultural land and input from sewage treatment works, especially during high rainfall. The bacterial diversity at upstream sites positively correlated with TC and TN and may simply reflect the transport of allochthonous organisms into upstream sites. As the water moves downstream, the common OTUs (702.09, 795.42, 591.79, 806.71, 817.79, 572.13 and 718) are able to multiply, reducing overall diversity. This effect is most marked when TC and TN are low and when water temperature is high. These variations in the abundance of common OTUs make the greatest contributions to dissimilarity of bacterial community composition between samples and so reducing the relative abundance of the rare OTUs.

Table 4. 3 Relationships between environmental parameters and bacterial diversity (Shannon index *H'*) in the River Wensum from June 2011 to December 2012 using Spearman's rank correlation and stepwise multiple regression analysis.

Environmental parameter	Spearman's rank correlation	Stepwise multiple regression
Temperature (˚C)	- .199 , p< 0.001	-.197, p< 0.001
pH	N.S.	-
Total nitrogen TN (mg/L)	.231, p< 0.001	.205, p< 0.001
Total phosphorus TP (µg/L)	N.S.	-
Total carbon TC (mg/L)	.252, p< 0.001	.230, p< 0.001
Total organic carbon TOC (mg/L)	.207, p< 0.001	-
Total suspended solid TSS (mg/L)	N.S.	-
Arable land (%)	.221, p< 0.001	-
Improved grassland (%)	.152, p< 0.01	-
Other grassland (%)	N.S.	-
Urban area (%)	- .141, p< 0.01	-
Rainfall (mm)	N.S.	-
Sewage treatment works (n)	-.111, p< 0.01	-
Stream order (n)	-.232, p< 0.001	-.164, p< 0.001
River flow rate (m^3/s)	.218, p< 0.001	-

4.4.3 Common DNA fragment sizes and their abundance

Table 4.4 and Figure 4.7 show the 20 commonest OTUs and their abundance in the river Wensum from June 2011 to December 2012.

As can be seen in Table 4.4, averaged across all the samples analysed, each of the 20 commonest OTUs make up only a relatively small proportion of the total, but some of these OTUs can be very common in individual samples. The commonest four OTUs, for example, have mean abundances of between 2.7 and 7.4%, but have maximum abundances in individual samples ranging from 27.1% at site SD to 81.9% at site SC.

Figure 4.11 shows a cumulative dominance curve of the abundance of the 20 commonest OTUs in the River Wensum from June 2011 and December 2012. The abundance of these 20 OTUs accounted for approximately (40.4 %) of total bacterial abundance in the river water.

Table 4. 4 Mean of relative abundance, cumulative abundance, % of variance between sites and months and maximum abundance of the commonest OTUs in the River Wensum from June 2011 to December 2012.

Common OTUs (ARISA)	F value (site)	F value (month)	% variance between sites	% variance between months	Mean abundance (%)	Cumulative abundance (%)	Max. abundance	Sites of max. abundance
702.09	25.95	9.04	52.2	13.1	7.4	7.4	81.9	SC
795.42	11.07	6.65	33.8	14.6	4.7	12.1	54.3	S2
591.79	6.91	5.27	25.6	14.0	2.7	14.8	27.1	SD
806.71	8.47	3.62	30.9	9.5	3.2	17.9	67.1	S11
705	2.72	12.94	9.6	33.0	1.8	19.7	29.5	SB
817.97	6.63	2.41	26.9	7.1	1.0	20.6	18.3	S17
572.13	7.69	4.42	28.5	11.8	2.8	23.5	41.9	SD
683.31	2.36	3.63	11.2	12.4	1.2	24.7	29.5	S21
756.76	6.56	16.12	19.0	33.7	3.6	28.2	22.6	S3
718.52	7.60	3.73	28.7	10.1	4.6	32.8	17.2	S7
776.61	1.81	3.88	8.7	13.4	0.6	33.4	9.1	S7
773.36	2.99	1.52	14.8	5.4	0.9	34.4	15.6	S17
694.57	3.53	1.87	16.7	6.4	1.3	35.7	14.1	S17
753.56	2.42	4.84	11.0	15.8	0.9	36.6	8.9	S3
678.11	3.25	2.81	14.8	9.2	1.0	37.7	7.47	SE
565.24	4.97	2.98	21.1	9.1	1.5	39.2	15.2	S7
843.5	1.90	3.28	9.3	11.6	0.2	39.4	3.0	SE
415.76	1.91	1.38	10.2	5.2	0.2	39.5	7.1	S20
596.69	3.19	2.37	15.0	8.0	0.7	40.2	11.3	SB
798.26	1.09	2.73	5.7	10.2	0.3	40.4	12.2	S8

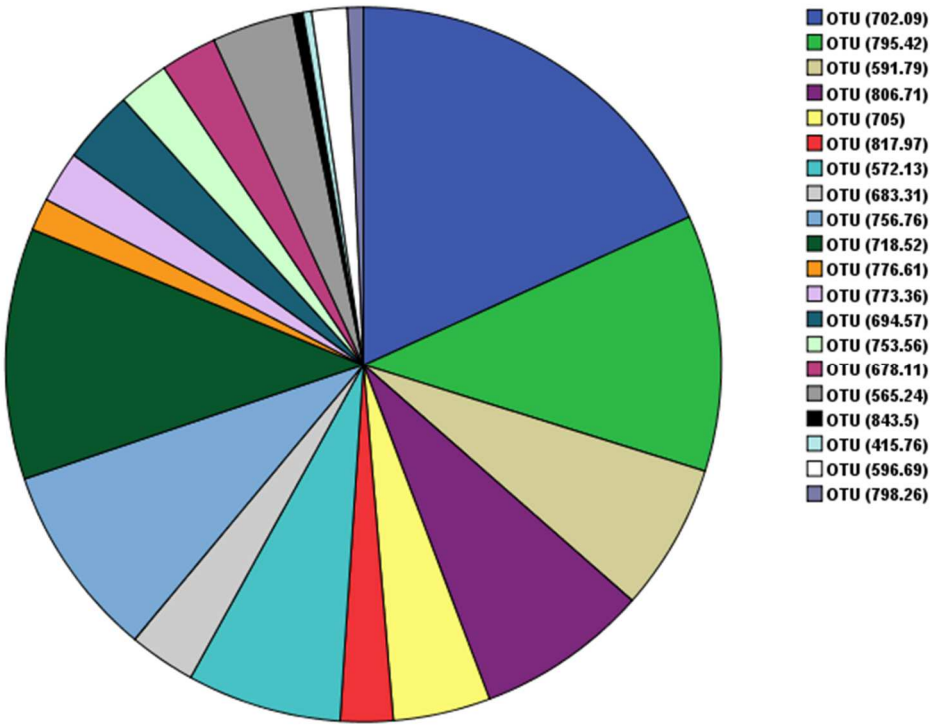


Figure 4. 10 Abundance of common DNA fragment sizes of bacterial communities in the River Wensum from June 2011 to December 2012.

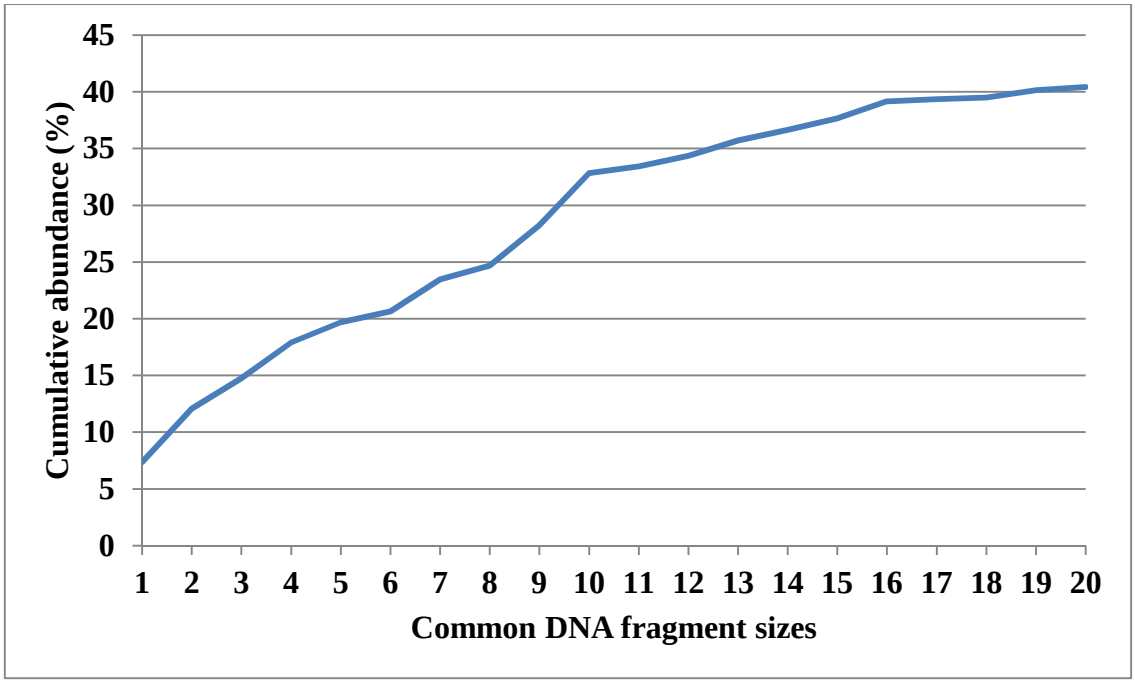


Figure 4. 11 Cumulative dominance curve of the abundance of commonest DNA fragment sizes in the River Wensum from June 2011 and December 2012.

4.4.3.1 Spatial and temporal variation of common DNA fragment sizes

Two-way ANOVA shows significant differences between sites for all 20 commonest OTUs, except OUT 798.26, and times, except OTUs 773.36 and 415.76 (Table 4.4).

Differences between sites explain larger proportions of the variances of the majority of common OTUs than differences between months (702.09, 795.42, 591.79, 806.71, 817.97, 572.13, 718.52, 773.36, 694.57, 678.11, 565.24, 415.76, and 596.69). However, differences between months explain larger proportions of the variances of seven common OTUs than differences between sites (705, 683.31, 756.76, 776.61, 753.56, 843.5, and 798.26) (Table 4.4).

Principal component analysis (PCA) (Table 4.5) reveals the overall patterns in the abundance of the 20 commonest OTUs across the Wensum catchment. Factor 1 explained 18.1% of the variation of OTUs, while factor 2 explained 8.5% of the variance. Factors 3 and 4 each explained a further 6 % of the variance.

PC 1 is essentially upstream-downstream and PC 2 represents OTUs that are varied between times. PC 1 is positively correlated with abundances of 702, 795, 591, 806 and 817.9, and negatively correlated with 756.76 and 718.52 (Table 4.5). Hierarchical partitioning shows that the strongest relationships with PC1 are flow, then temperature. PC2 is positively correlated with 705, and negatively correlated with 756.76 and 753.56 (Table 4.5). Hierarchical partitioning shows that this factor is most strongly related to percentage of arable, with weaker effects from the percentages of urban, TN, STWs and pH.

Canonical analysis of principal coordinates (CAP) (Figure 4.12) also displays the variations of OTUs between sites and times, and the correlations between environmental parameters and OTUs. Figure 4.13 shows the variations of OTUs between sites in the same axes of Figure 4.12. Also, Figure 4.14 shows a plot of the variations of the 20 commonest OTUs in the same axes of Figure 4.12.

Differences between sites explain a larger proportion (36.4%) of the variances of OTUs than differences between months (13.7%).

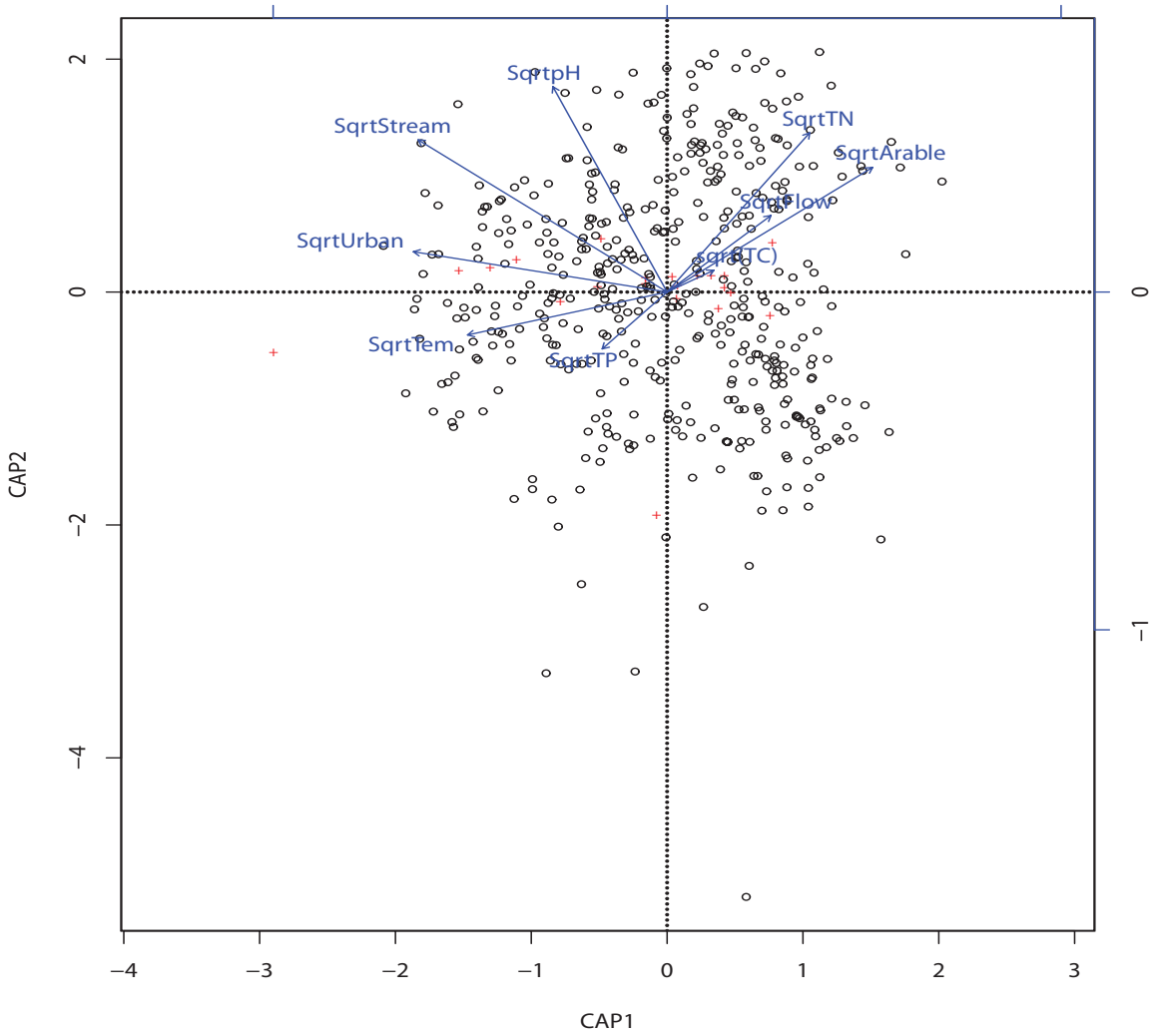
CAP 1 essentially corresponds to upstream-downstream position and CAP 2 reflects OTUs that vary between sampling times. OTUs become commoner as water moves downstream (3^{rd} and 4^{th} order streams) with the exception of site SC (2^{nd} order stream) that does not fit this pattern. These common OTUs grow faster than others and come to dominate the community as water moves downstream, resulting in the decreases of overall bacterial diversity. They are positively correlated with temperature, TP, pH and the presence of urban area. OTU (709.02) is at the end of axis 1 (downstream) and correlated positively with temperature, while OUT (565.24) and OTU (705) are at the other end and correlated positively with TC, TN, flow and arable land (Figure 4.12, Figure 4.13 and Figure 4.14). The permutation based ANOVA (using Adonis, see section 4.3.5) shows that there are significant but rather weak relationships with environmental variables: temperature (r^2 = 3.6%, p< .01), TP (r^2 = 0.95%, p< .05), urban area (r^2 = 4.3%, p< .01), stream order (r^2 = 2.1%, p< .01), pH (r^2 = 2.4%, p< .01), arable land (r^2 = 2.1%, p< .01),

TN (r^2 = 1.9%, p< .01), TC (r^2 = 0.99%, p< .01) and flow (r^2= 0.8%, p< .01). CAP 1 is positively correlated with TN, TC flow and arable land, while negatively correlated with temperature, TP, urban area, stream order and pH. CAP 2 is positively correlated with TN, TC, pH, urban area, stream order, flow and arable land, while negatively correlated with temperature and TP (Figure 4.12).

The overall pattern of community composition is that OTUs become commoner as the water moves downstream (3rd and 4th order streams). This presumably reflects the fact that these bacteria are actively growing in the river, and reducing the abundance of other taxa. The result of this is that the community becomes less diverse downstream. But there is one site (SC) that does not fit this pattern. A few OTUs exhibited greater and often increased abundance during storm events of high rainfall and flow rates, suggesting that these OTUs are of terrestrial origin that are flushed into upstream sites (OUT 705, 565.24, 756.76 and 753.56) (Table 4.5, Figures 4.12 and 4.13, 4.14 and Appendix Tables A 4.1-A 4.18, Figures A 4.1-A 4.18).

Figure 4.14 and Table 4.6, and Figure 4.15 and Table 4.7 give examples for the spatial and temporal variation of the first common OTU (702.09). The abundance of OTU 702.09 increases as water moves to downstream sites of the Wensum (3rd -4th order streams), with highest values recorded at 4th order streams (S8, S15 and S18) and at one upstream location, site SC. The abundance of this OTU increased during warm months (high water temperature), while it decreased during cold months (low water temperature), with highest values recorded in June 2011, August 2011 and July 2012.

Other figures and tables about spatial and temporal variation of the commonest OTUs are presented in the Appendix Tables A 4.1- A 4.18, Figures A 4.1-A 4.18.



Key symbols: Sqrt= square root transformation, Tem= temperature, TC= total carbon, TN= total nitrogen, TP= total phosphorus.

Figure 4. 12 Canonical analysis of principal coordinates (CAP) of the variations of ARISA OTUs between sites and times, and the correlations between OTUs and environmental parameters in the River Wensum from June 2011 to December 2012.

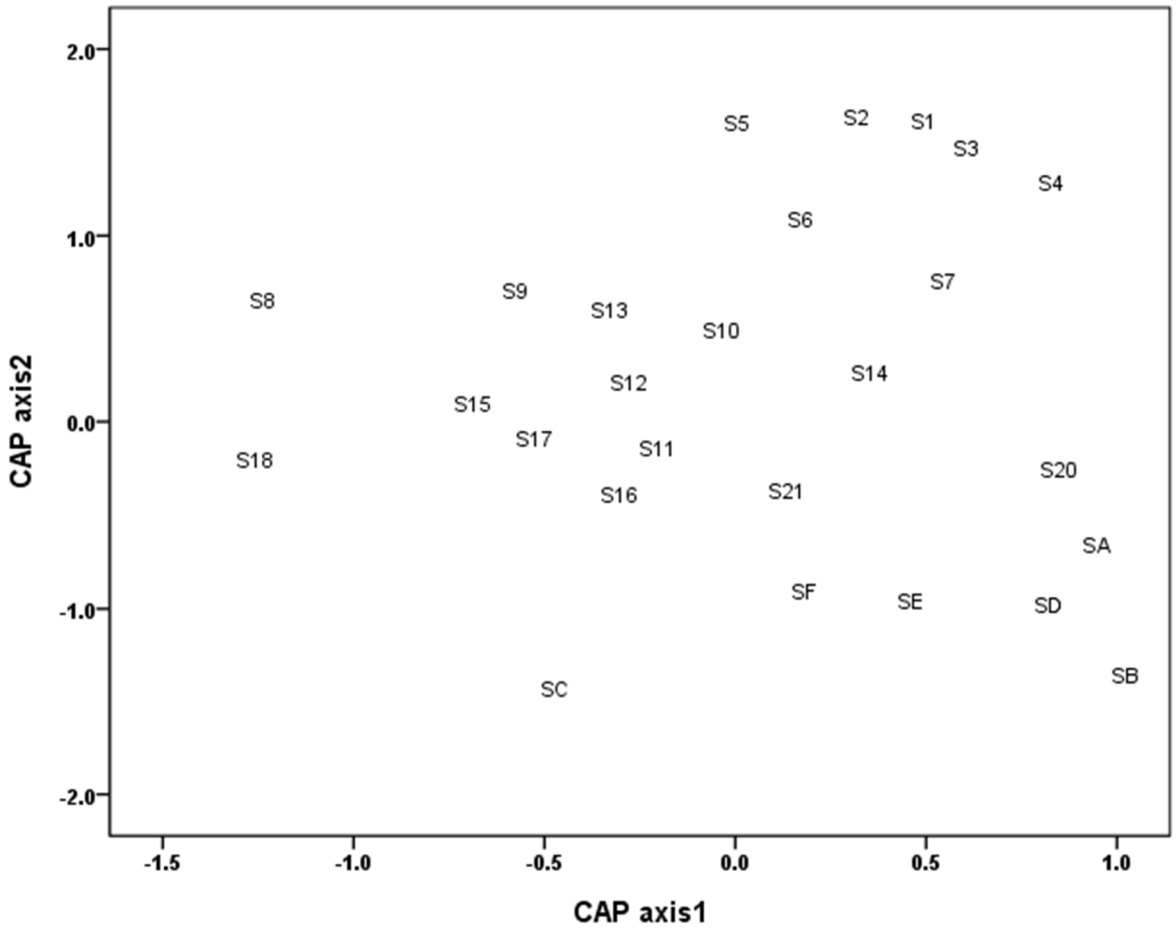


Figure 4. 13 Site centroids for canonical analysis of principal coordinates (CAP) of the variations of ARISA OTUs of the River Wensum from June 2011 to December 2012.

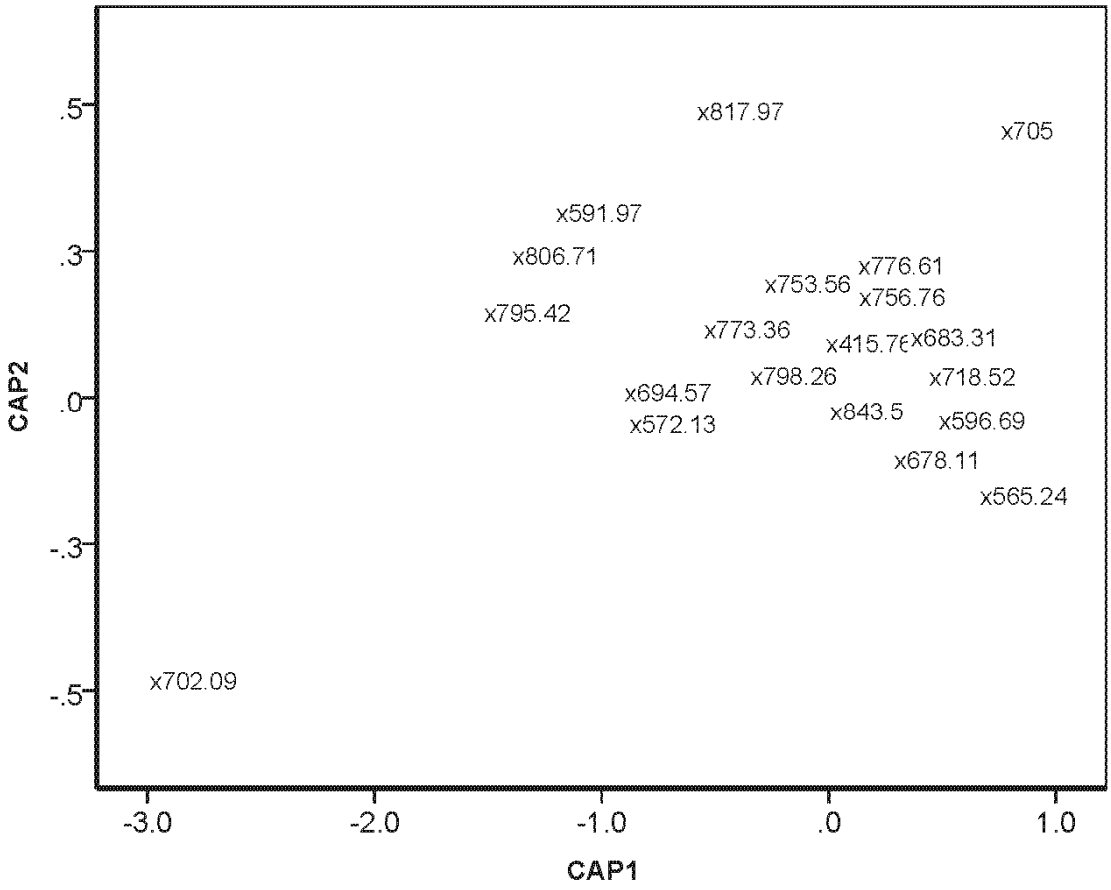
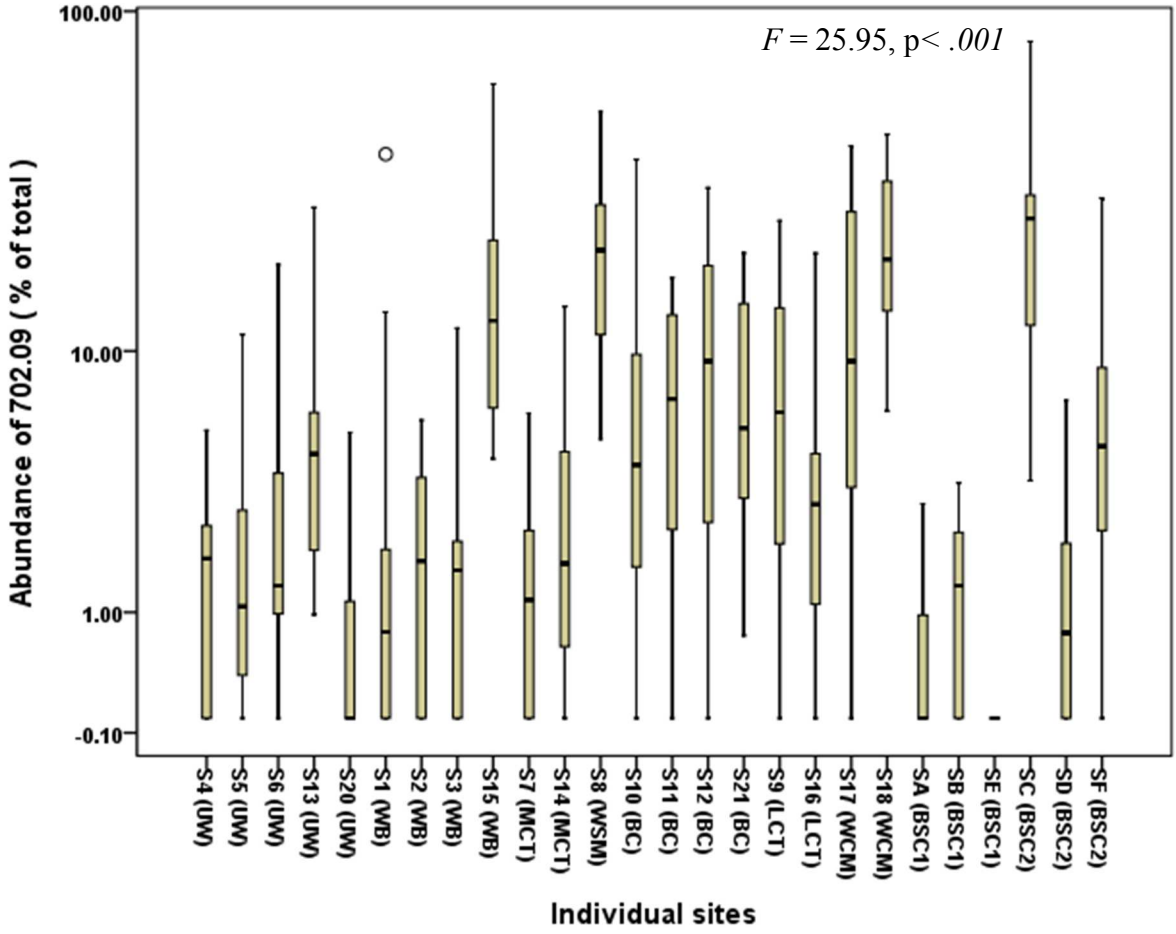


Figure 4. 14 Canonical analysis of principal coordinates (CAP) of the variations of the 20 commonest OTUs of the River Wensum from June 2011 to December 2012.

Table 4. 5 Principal component loadings of the 20 commonest OTUs in all sites of the River Wensum from June 2011 to December 2012.

OTU	Principal components	
	1	**2**
702.09	.679	-.292
795.42	.660	-.093
591.79	.535	.071
806.71	.700	.152
705	-.337	.529
817.97	.523	.211
572.13	.357	.298
683.31	-.274	.298
756.76	-.497	-.481
718.52	-.528	-.393
776.61	-.239	.397
773.36	.266	-.121
694.57	.249	-.047
753.56	-.159	-.535
678.11	-.416	.217
565.24	-.429	.176
843.5	-.111	.319
415.76	-.108	.152
596.69	-.424	.097
798.26	.232	.103
% variance explained	18.10%	8.50%



Key symbols: UW = Upper Wensum, WB = Wensum Beck, MCT = Mid Catchment Tributaries, WCM = Wensum Swanton Morley, BC = Blackwater Catchment, LCT = Lower Catchment Tributaries, WCM: Wensum Costessey Mill, BSC1 = Blackwater subcatchment 1, BSC2 = Blackwater subcatchment 2.

Figure 4. 15 Box plot of spatial variation of the common DNA fragment size (702.09) (sites as individuals and groups) from June 2011 to December 2012.

Table 4. 6 Mean, standard error, minimum and maximum (%) of the abundance of common DNA fragment size (702.09) in the River Wensum (by sites) from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	1.85 ± 0.299	0.00 - 5.56
S5	1.76 ± 0.261	0.00 - 11.23
S6	2.35 ± 0.262	0.00 - 18.43
S13	5.68 ± 0.244	0.97 - 27.21
S20	0.55 ± 0.261	0.00 - 5.47
S1	2.20 ± 0.261	0.00 - 38.85
S2	1.60 ± 0.253	0.00 - 6.01
S3	1.47 ± 0.261	0.00 - 11.77
S15	16.71 ± 0.261	4.45 - 61.89
S7	1.11 ± 0.261	0.00 - 6.33
S14	2.40 ± 0.261	0.00 - 13.81
S8	20.19 ± 0.244	5.20 - 51.50
S10	6.00 ± 0.261	0.00 - 37.45
S11	6.66 ± 0.261	0.00 - 16.83
S12	8.68 ± 0.238	0.00 - 31.04
S21	7.46 ± 0.269	0.72 - 19.94
S9	7.80 ± 0.269	0.00 - 24.86
S16	3.69 ± 0.261	0.00 - 19.91
S17	12.84 ± 0.261	0.00 - 41.05
S18	21.31 ± 0.261	6.45 - 44.29
SA	0.47 ± 0.248	0.00 - 3.06
SB	1.12 ± 0.262	0.00 - 3.65
SE	0.03 ± 0.254	0.00 – 0.03
SC	22.85 ± 0.254	3.72 - 81.94
SD	1.07 ± 0.254	0.00 - 6.97
SF	6.52 ± 0.270	0.00 - 28.94

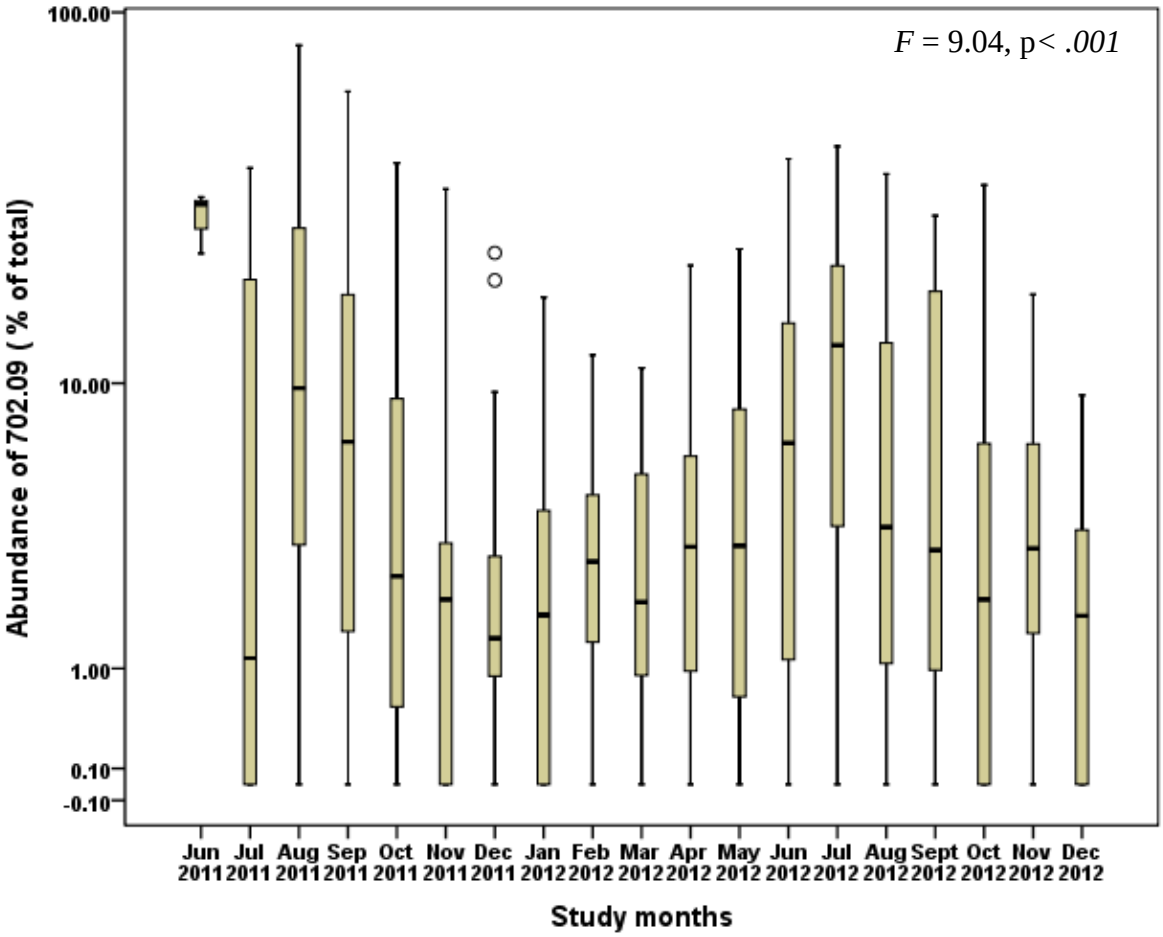


Figure 4. 16 Box plot of temporal variation of the common DNA fragment size (702.09) by months from June 2011 to December 2012.

Table 4. 7 Mean, standard error, minimum and maximum (%) of the abundance of the common DNA fragment size (702.09) in the River Wensum (by months) from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	18.03 ± .630	29.90 - 32.48
	July	5.46 ± .276	0.00 - 38.85
	August	10.84 ± .247	0.00 - 81.94
	September	8.44 ± .218	0.00 - 61.89
	October	4.17 ± .199	0.00 - 40.02
	November	2.69 ± .209	0.00 - 34.11
	December	1.99 ± .209	0.00 - 22.98
2012	January	1.76 ± .209	0.00 - 17.39
	February	2.61 ± .209	0.00 - 12.01
	March	2.20 ± .209	0.00 - 11.05
	April	3.05 ± .209	0.00 - 21.23
	May	3.15 ± .209	0.00 - 23.55
	June	6.51 ± .213	0.00 - 41.05
	July	11.15 ± .209	0.00 - 44.29
	August	5.55 ± .209	0.00 - 37.45
	September	5.56 ± .209	0.00 - 28.94
	October	3.10 ± .228	0.00 - 34.97
	November	3.46 ± .218	0.00 - 17.71
	December	1.36 ± .209	0.00 - 9.23

4.4.3.2 Environmental parameters and the abundance of common DNA fragment sizes

Mean, standard error, minimum and maximum of environmental parameters are all presented in the Appendix Tables A 3-1- A 3-22.

Table 4.8 and Table 4.9 show the correlations between environmental parameters and common OTUs using Spearman's rank correlation and stepwise multiple regression, and also proportions of those parameters explaining the variations of common OTUs.

As can be seen in Tables 4.8 and 4.9, the variation of the abundance of the majority of the commonest OTUs (702.09, 795.42, 591.79, 806.71, 705, 817.79, 572.13 and 683.31) was highly explained by stream order and temperature, while other parameters explained the variation of one to four common OTUs. Proportions of environmental parameters explaining the variations of common OTUs are varied, with values ranging from 0.4% to 45%.

For example, Spearman's rank correlation analysis (Table 4.8) showed that the abundance of first common OTU (702.09) was positively related to stream order, urban area, temperature, STWs and pH, while it was negatively related to arable land area, improved grassland area and

TN. However, among these significant parameters, stepwise multiple regression analysis (Table 4.8) showed that the most significant parameters that correlated negatively with the abundance of OTU (702.09) were arable land area (Figure 4.17), improved grassland area and TN, while the most significant parameters correlated positively with the abundance of this OTU were temperature (Figure 4.18), stream order, TP and urban area. All of these environmental parameters have contributed approximately (45%) of the variation in the abundance of this OTU.

Overall, OTU 702.09 enters the river from different sources, such as drainage from urban areas and discharges from sewage treatment works. The abundance of this OTU correlated negatively with TN at upstream sites. OTU 702.09 abundance increases as the water moves downstream (3^{rd}-4^{th} order streams) and during periods when water temperature is high. Its abundance at downstream sites correlated positively with TP.

Table 4. 8 Relationships between environmental parameters and common DNA fragment sizes in the Wensum River from June 2011 to December 2012.

Variable	OTU									
	702.09		795.42		591.79		806.71		705	
	1	2	1	2	1	2	1	2	1	2
Temperature (˚C)	.294**	.300	.165**	.135[a]	.260**	.301	.169**	.131	-.371**	- .190
pH	.203**	-	.258**	-	.141**	-	.245**	.115	N.S.	-
Total nitrogen TN (mg/L)	-.260**	- .172	N.S.	-	N.S.	-	- .111**	-	.272**	.122
Total phosphorus TP (µg/L)	.209**	.101	N.S.	-	N.S.	-	N.S.	-	-.162**	- .132
Total carbon TC (mg/L)	N.S.	-	-.090*	-	N.S.	-	N.S.	-	.103*	-
Total organic carbon TOC (mg/L)	N.S.	-	-.080*	-	N.S.	-	N.S.	-	.184**	-
Total suspended solid TSS (mg/L)	N.S.	-	-.147**	- .095	N.S.	-	N.S.	-	-.175**	-
Arable land (%)	-.436**	- .696	-.372**	-.403**	-.150**	-	- .277**	-	.159**	-
Improved grassland (%)	-.288**	- .640	-.298**	- .303	-.079*	-	- .196**	-	-.107*	-
Other grassland (%)	N.S.	-	N.S.	-	N.S.	-	-.099*	- .163	N.S.	-
Urban area (%)	.313**	.096	.262**	-	.252**	.110	.254**	-	N.S.	-
Rainfall (mm)	N.S.	-	N.S.	-	.101*	-	N.S.	-	N.S.	-
Sewage treatment works (n)	.287**	-	.270**	-	.150**	-	.255**	-	N.S.	-
Stream order (n)	.414**	.207	.415**	.256	.316**	.224	.368**	.323	-.136**	-.088
River flow (m^3/s)	N.S.	-	N.S.	-	N.S.	-	N.S.	-	.352**	.348
% variance explained	-	45%	-	20%	-	18 %	-	16%	-	30%

1= Spearman's rank correlation output, 2= regression analysis output, N.S. = not significant, (n) between brackets= numbers.

Table 4. 9 Relationships between environmental parameters and common DNA fragment sizes in the Wensum River from June 2011 to December 2012.

Variable	OTU									
	817.97		572.13		683.31		756.76		718.52	
	1	2	1	2	1	2	1	2	1	2
Temperature (˚C)	N.S.	-	N.S.	-	N.S.	-	-.177**	- .174	N	N
pH	.262**	.106	N.S.	-	N.S.	-	-.099*	-	N.S.	-
Total nitrogen TN (mg/L)	N.S.	-	-.123**	-	.099*	-	N.S.	-	N.S.	-
Total phosphorus TP (µg /L)	-.130*	-.202	.080*	-	N.S.	-	N.S.	-	-.104*	-
Total carbon TC (mg/L)	N.S.	-	N.S.	-	N.S.	-	N.S.	-	.162**	.148
Total organic carbon TOC (mg/L)	N.S.	-	.182**	-	N.S.	-	N.S.	-	N.S.	-
Total suspended solid TSS (mg/L)	-.212**	-	.160**	.125	.106*	-	N.S.	-	N.S.	-
Arable land (%)	-.111**	-	-.316**	-	.160**	-	.089*	-	.140**	-
Improved grassland (%)	.109*	-	.180**	-	-.136**	-	N.S.	-	N.S.	-
Other grassland (%)	N.S.	-	.124**	-	N.S.	-	N.S.	-	.089*	.121
Urban area (%)	.234**	-	.151**	-	N.S.	-	-.103*	- .099	-.285**	- .230
Rainfall (mm)	N.S.	-	.097*	.125	.112*	-	N.S.	-	.094*	-
Sewage treatment works (n)	.265**	.136	.102*	-	-.095*	-	-.083*	-	-.126**	-
Stream order (n)	.300**	.205	.362**	.328	-.154**	-.140	-.093*	-	-.091*	-
River flow (m^3/s)	N.S.	-	.115**	-	.078*	.202	-.080*	-	N.S.	-
% variance explained	-	14%	-	13%	-	0.6%	-	0.4%	-	1%

1= Spearman's rank correlation output, 2= regression analysis output, N.S. = not significant, (n) between brackets= numbers.

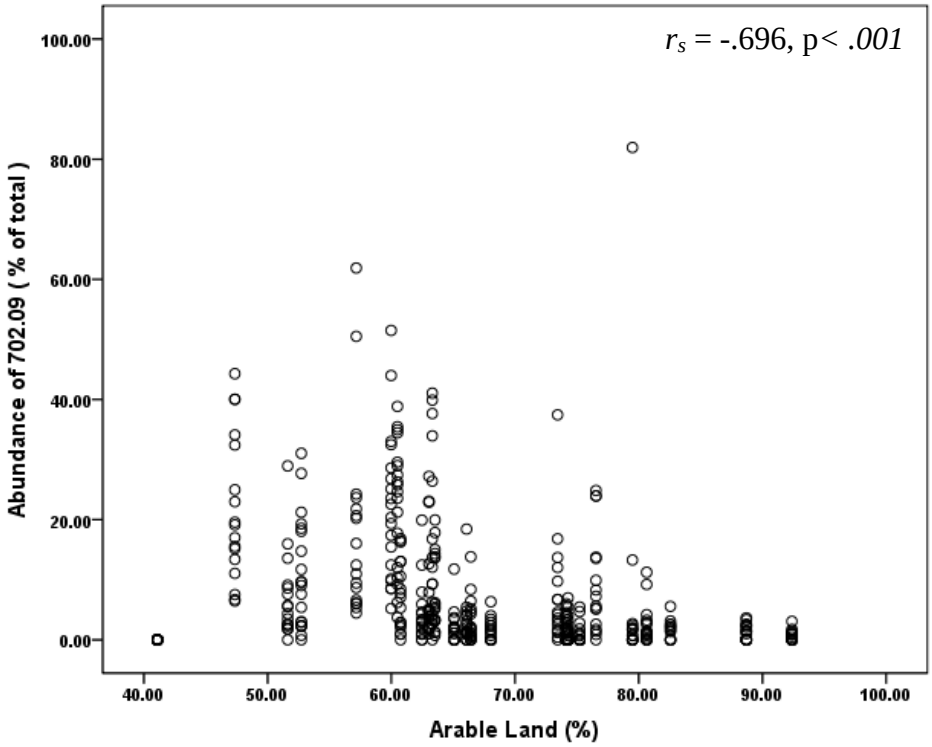


Figure 4. 17 Correlation between the abundance of the common OTU (702.09) and arable land from June 2011 to December 2012.

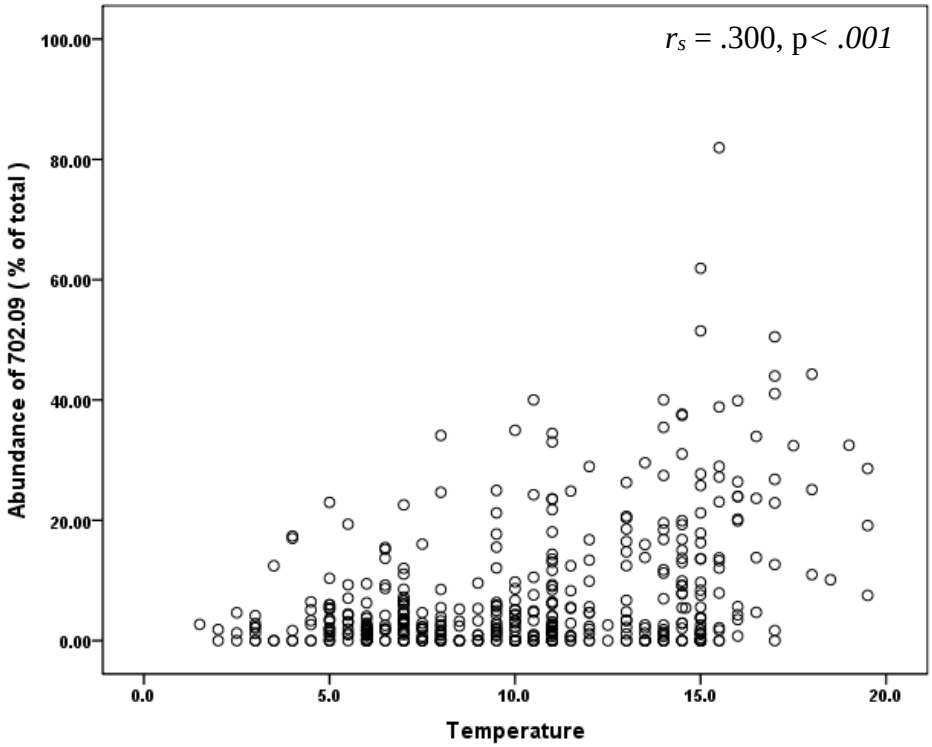


Figure 4. 18 Correlation between the abundance of the common OTU (702.09) and temperature from June 2011 to December 2012.

4.5 Discussion

4.5.1 Overall bacterial community composition

The results of this study indicated that the bacterial community composition in the River Wensum varied significantly both between sites and between months from June 2011 to December 2012. Bacterial diversity was higher at upstream sites during periods of high rainfall and river flow rate and also during cold months. Diversity decreased as water moves downstream, particularly during warm and dry months. The abundance of several common OTUs increases at downstream (3^{rd}-4^{th} order) sites, particularly in summer, presumably reflecting growth of common organisms, with consequent reduction in the overall diversity, as water moves downstream.

Site SC (2^{nd} order), however, has common bacteria similar in the pattern of common OTUs at downstream sites. The sources of this common bacteria could be septic waste as this site is located in an area with septic tanks and discharges that can potentially deposit microbes and nutrients into waterways, such as rivers and lakes (Ahmed *et al.* 2005).

The greatest differences in bacterial community composition were found between two downstream sites (S8 and S18) and three upstream sites (SA, SB and SE) in the Blackwater subcatchment.

Lear and Lewis (2009) found that bacterial biofilm community composition is more similar along stream sections than between streams. Fischer *et al.* (2009) reported a similar pattern in Sweden, which they attributed to differences between catchments. Dorigo *et al.* (2010) found large differences in bacterial biofilms between upstream (non-polluted) and downstream sections (polluted with pesticides) of the Morcilla River in France. This was attributed to extensive agriculture processes at downstream sites. Levine and Crump (2002) found that land use type has substantial effects on bacterial community composition between upstream and downstream sites of the River Ipswich in Massachusetts, US. Levine and Crump (2002) have suggested that the first establishment of the bacterial population was at upstream sites was mostly in an urban area (78% of total catchment) and then maintained down the length of the river. Bai *et al.* (2013) found that the bacterial composition at upstream sites receiving treated wastewater was different from that in downstream sites of the River Haihe in China receiving untreated wastewater. Also, upstream sites showed high abundance of ammonium-oxidizing bacteria and this attributed to their highly presence in the treated wastewater.

Olapade *et al.* (2005) found that the abundance of different bacterial populations was varied among 2^{nd} to 3^{rd} order streams in the USA. For example, *Burkholderia cepacia* showed higher abundance at a downstream site and correlated positively with dissolved organic carbon and nitrite. Similarly, Findlay *et al.* (2002) found that bacterial abundance varied among nine 1^{st} to 3^{rd} – order streams, and attributed this pattern to the variation of organic matter in these streams.

Upstream sites (SA, SB and SE) are located in an area of intensive arable agriculture. Fertilizers are normally used in grasslands and arable lands to improve crop yields (Vistousek *et al.* 1997; Wei *et al.* 2013). Runoff from these areas into streams can change the chemistry of water and may lead to changes of bacterial community composition (Kirk *et al.* 2004; Dorigo *et al.* 2010; Findlay 2010).

Previous studies give to little attention the correlation between grassland types and bacterial community composition and abundance. Studies are normally focused only on soil microbial communities. For example, Grayston *et al.* (2001) demonstrated that variations in the structure of bacterial communities in soil were related to the types of grassland.

Sites S8 and S18 are located on 4^{th} order streams in the proximity of urban areas (S8 = 3.11% and S18 = 4.89% of the total sub-catchment) and have of 3 and 2 STWs upstream, respectively. STWs may discharge nutrients and bacteria (Carpenter *et al.* 1998; Drury *et al.* 2013). These two sites also receive organic matter from upstream sites and adjacent areas.

The bacterial community composition was found to change significantly between months. Fluctuations of temperature and flow rate can have potential effects on bacterial community composition and structure (Bucci *et al.* 2014). Abed et al. (2011) used ARISA to show substantial changes in steam bacterial community composition and function in Oman after extreme flood events, with approximately three-quarters of the community being replaced. Zhang *et al.* (2012) found substantial seasonal variation in bacterial community composition and structure in three rivers, with temperature as the major responsible factor. Mueller-Spitz *et al.* (2009) found that bacterial composition in Lake Michigan was related more strongly to sampling date than to depth of water, showing a strong relationship to water temperature. Also, Crump and Hobbie (2005) found that bacterial composition in two temperate rivers was influenced by variation of temperature and flow rate. This study also revealed that many phylotypes were taxa related to soil and sediment bacteria, indicating the role of allochthonous bacteria in increasing diversity.

Fisher *et al.* (2000) attributed variation in the bacterial abundance (about 84%) along the the River Hull river to be due to water temperature. Lindstrom *et al.* (2005) found that the abundance of bacterial taxa in different lakes was strongly influenced by water temperature. For example, the *R. pickettii* was present in high abundance at low water temperature, while the Bacteroidetes were present in high frequency at high water temperature.

Saha *et al.* (2003) found that bacterial abundance and distribution in some canals of Kolkata were highly related to rainfall and flow rate, while other parameters, such as pH and total suspended solids had no effect. Jordaan and Bezuidenhout (2013) found that changes of abundance and diversity of Actinobacteria and Acidobacteria in the River Vaal, South Africa were positively related to rainfall. Also, Kumari *et al.* (2011) found that the abundance of ammonium-oxidizing bacteria in some freshwater environments was positively related to ammonia, nitrate and rainfall.

In Vaal River in South Africa, Jordaan and Bezuidenhout (2013) found that the abundance of some bacterial groups, such as Gammaproteobacteria and Deltaproteobacteria was positively

related to flow rate. However, Stepanauskas *et al.* (2003) reported different results for the relative abundance of some phylotypes of bacterioplankton in the delta of the Sacramento-San Joaquin River in California. They found that the relative abundance of members, such as *Microthrix* belonging to Actinobacteria, were high during summer and autumn seasons and were negatively correlated with river flow.

Phosphorus and nitrogen are two nutrients that can enter streams and cause eutrophication (Smith 2003). This phenomenon can affect bacterial community composition and abundance in freshwater environments (de Figueiredo *et al.* 2010; Kent and Bayne 2010). Wu and Hahn (2006) found that the abundance of *Polynucleobacter* (PnecB) in the Mondsee Lake were positively related to water temperature and TP. Also, Sorichetti *et al.* (2013) found that Cyanobacteria in 25 oligotrophic lakes were predominant at all levels of total phosphorus. Xiong *et al.* (2012) found that TC was negatively correlated with the relative abundance of Bacteroidetes and Acidobacteria in the sediments of an alkaline lake, while they were positively related to pH. Liu *et al.* (2013) found that the spatial and temporal distributions of bacterial communities in the Jiulong river in China were highly attributed to the variation of TN, TC, water temperature and phosphorus. Sun *et al.* (2013) found that approximately 91% of the variations of ammonium-oxidizing bacteria in the River Dongjiang in China were explained by the variation of total nitrogen and total carbon.

De Figueiredo *et al.* (2012) found that the abundance of Betaproteobacteria along the River Cértima in Portugal were correlated positively with TSS. At the discharge point of the Oloshi River in Nigeria, which receives high amounts of effluents, Nwaugo *et al.* (2007) recorded high abundance of some bacterial genus like *Klebsiella* spp. and *Corynebacterium* spp., which were related positively to total suspended solids and total dissolved solids.

In 23 streams in Hubbard Brook in the US, Fierer *et al.* (2007) found that pH showed strong effects on bacterial community composition and abundance of some dominant bacterial groups, such as Proteobacteria which were correlated positively with pH. In 17 acidic and natural streams in South Island, New Zealand, Lear *et al.* (2009) found that types and proportions of some bacterial groups were varied and strongly correlated with pH. For example, 38% of Alphaproteobacteria dominated natural streams (pH, 6.7-8.3) but this proportion was 21% in acidic streams (pH, 3.9-7.5). Also, members of Betaproteobacteria (29%) were dominant in acidic streams.

4.5.1.1 Bacterial diversity

There were significant changes in bacterial diversity between sites and months in the River Wensum. The increases in abundance of some OTUs may proportionately reduce the importance of other OTUs, especially affecting rare OTUs. Bacterial diversity was found to decrease at downstream sites of the river (during warm and dry months), whereas decreases in the abundance of OTUs during wet and cold months resulted in an increase in bacterial diversity.

Similar results are reported by Sekiguchi *et al.* (2002) who found that bacterial community diversity decreased downstream in the River Changjiang in China, and attributed this pattern to physical and chemical properties of the river water, such as water temperature, nutrient concentrations and flow rate. Similarly, Lemian *et al.* (2012) also found that bacterioplankton diversity decreased downstream in the River Xiamen Houxi in China and attributed this decrease to higher values of pH and total nitrogen (TN) at downstream sites, explaining about 48% of the total variation of bacterial community composition. However, other studies have found different trends of bacterial diversity. For example, Bushaw-Newton *et al.* (2012) found that bacterial diversity in sediments of the River Anacostia in the US increased moving downstream, attributing this to high concentrations of TC and TN at downstream sites.

Bacterial diversity was also negatively related to water temperature in the Wensum and this in agreement with Henne *et al.* (2013) who found that bacterial diversity in different types of freshwater was higher when water temperature is low. Dong *et al.* (2010) found that bacterial abundance in Tibetan Plateau lakes in China were positively related to water temperature and dry periods, while diversity was positively related to wet periods and water temperature.

The evolution of the community as water moves down a river (as mentioned in Chapter 1, section 1.6.1 and Chapter 3, section 3.5.1.1) depends on inputs of exogenous bacteria and on the relative growth rates of the OTUs present. Bacterial diversity increases at upstream sites and during the wet (high river flow) and cold (low water temperature) conditions, and is positively related to TN and TC. Then diversity declines as water moves downstream. Presumably, there are differences between OTUs in terms of their population growth rates in response to water temperature and pH. Then, these OTUs grow faster and come to make up a greater proportion of the bacteria present than other OTUs that are resources limited or are unable to grow in river water. These processes are especially important during periods of high water temperature or when residence time is long. Then, these fast growing OTUs come to dominate the community as water moves downstream, resulting in the decreases of overall bacterial diversity, an effect that is especially marked in periods of higher water temperatures.

The data obtained here show that there are significant, but rather weak, differences between sites in terms of bacterial composition and abundance. This is perhaps because water in the river environment is homogeneous, in contrast to environments, such as soil (which are more heterogeneous), and where we can find large differences in the bacterial community at fine scales (Ranjard *et al.* 2001; Kang and Mills 2006). So, this study suggests that there is not competition between bacterial OTUs. There are significant correlations between bacterial community composition and abundance and environmental parameters, but these correlations are not strong and thus do not clearly distinguish between sites that are more or less impacted by human activities. Consequently, bacteria do not appear to be good indicators of the ecological status of the river water, so will be of limited value for assessing compliance with the European Water Framework Directive. However, bacterial communities may be of use as an indicator for ecological health in more nutrient poor waters and the trophic state needs more research using different molecular techniques (Lear *et al.* 2009), such as Illumina sequencing and may require the development of new statistical methods to deal with this homogeneous environment.

4.6 Summary

In the research reported in this chapter, bacterial community composition was demonstrated to be significantly different between sites and sampling dates. Bacterial diversity decreases as water moves downstream, while bacterial abundance increases as water moves downstream, with only some fragment sizes able to grow and multiply at downstream sites. The dominant OTUs may control the substrates affecting other rarer OTUs and so reducing their abundance. Spatially, the large difference of bacterial community composition was between upstream and downstream sites. Temporarily, the large differences were between December 2011 and 2012 and also between September 2011 and 2012. Bacterial diversity and abundance showed significant relationships with some environmental parameters. However, there may be other chemical, physical and biological parameters beyond the current study that have potential effects on bacterial community composition in the River Wensum. For example, the effects of biological factors such as viruses and flagellates and chemical factors such as chlorophyll a. Also, the effect of heavy metals on bacterial community composition such as chromium, lead, cadmium, nickel and zinc.

Chapter Five

454 pyrosequencing technique for the analysis of bacterial communities in the River Wensum

5.1 Introduction

Microbial sequence data have played a fundamental role in classifying organisms. It is more informative than phenotypic information, can be readily interpreted (Woese 1987) and organisms can be taxonomically classified based on sequence differences (Wolska and Szweda 2012).

Metagenomics, the sequencing of genes from mixed communities of microorganisms and directly from environmental samples, reveals extensive microbial diversity overlooked by culture based methods (Hugenholtz *et al.* 1998; Petrosino *et al.* 2009). A widely used approach involves the amplification of regions of 16S rRNA, which has been widely used to construct bacterial phylogenies (Petrosino *et al.* 2009; Dall'Agnol *et al.* 2012).

Since its introduction in 2005, pyrosequencing has been shown to be an effective tool in assembling genomes of bacteria retrieved from short reads, which can be used to identify bacteria at genus and species level (Margulies *et al.* 2006). It has been used to study bacterial communities in different environments, including a deep underground mine in the US (Edwards *et al.* 2006), foods (Humblot and Guyot 2009), the Amazon River (Bai *et al.* 2010) and sea water (Thompson *et al.* 2011).

5.2 Aims

As introduced in Section 1.7, the aims of the research presented in this chapter are to use 454 pyrosequencing to characterise bacterial communities in the River Wensum including (i) spatial and temporal variations and associations with environmental factors, (ii) to determine the dominant bacterial phyla, (iii) to determine the commonest bacterial OTUs between sites and in time (December 2012), (iv) to describe the trend of the shift of abundance of the commonest bacterial OTUs when water moves to downstream sites, and (v) to identify the

taxonomic affinities of the commonest OTUs, based upon the most similar 16S sequences from cultured strains and the most similar environmental 16S sequences. This is to seek to infer their potential functional significance based on the characteristics of their nearest relatives.

5.3 Methods and materials

5.3.1 The selection of DNA templates

36 DNA templates were chosen for 454 pyrosequencing, with single samples from each site (S1-S18, S20-21 and SA-SF) on the Wensum from February 2012, and sites S1, S8, S18 S20 and SA-SF from December 2012. February 2012 was chosen as the most common ARISA fragments were present in that month, and December 2012 was a period of high flow rate (monthly average; 9.6 m^3/s measured at Costessey Mill).

DNA templates were quantified using a Nanodrop 1000 spectrophotometer (Thermo scientific, UK).

5.3.2 Oligonucleotide primers, PCR amplification and 454 pyrosequencing

Hypervariable regions V1 and V2 of the 16S rRNA gene were amplified using modified versions of the primers 27F and 338R (Fierer *et al.* 2008; Hamady *et al.* 2008) that have previously been used in the phylogenetic analysis of bacteria using pyrosequencing (Liu *et al.* 2007). The forward primer (5'-GCCTTGCCAGCCCGCTCAGTCAGAGTTTGATCCTGGCTCAG-3') contains 454 Life science primer B and 27F primer sequences joined by a linker sequence (TC) (underlined). The reverse primer (5'-GCCTCCCTCGCGCCATCAGNNNNNNNNNNNNCATGCTGCCTCCCGTAGGAGT-3'), contains the 454 Life Science primer A, 12 barcode bases, 338R sequences and a linker sequence (CA) between the barcode and 338R sequences (underlined).The primers were synthesised by Applied Biosystems. Appendix Table A 5.1 shows error-correcting barcodes used to tag each one of the 36 PCR products.

PCR was carried out in 25 µl of a mixture containing 12.5 µl of ACCUZYME™ Mix (Bioline, UK), 1 µl of forward primer and 1 µl of reverse primer, 1 µl of DNA (5ng DNA) and 9.5 µl of MilliQ water using the StepOne™ Real-Time PCR System (Applied Biosystems, UK). The PCR conditions were: 94 ˚C for 4 minutes, 30 cycles of 94 ˚C for 30 seconds, 50˚C for 1 minute and 72˚C for 1.30 minutes, final extension of 72 ˚C for 10 minutes. The PCR product was then held at 4 ˚C. Amplification was checked on 1% agarose gel diluted with 1% of Tris-Borate EDTA buffer (TBE, pH = 8.0) and stained with ethidium bromide (10 mg/ml) (Sigma, UK).

PCR products were purified using the Fermentas GeneJet purification kit (Thermo Scientific, UK) following the manufacture's protocol with small modifications; 22µl of binding buffer was added to 22 µl of PCR product and mixed thoroughly, and an optimal pH for DNA binding was shown by the appearance of a yellow colour. 22µl of isopropanol was added to this and was mixed thoroughly. The solution was transferred to the GeneJet purification column and centrifuged for 1 minute at 12000 rpm. 700µl of wash buffer was added and centrifuged for 1 minute at 12000 rpm. The centrifugation was repeated to ensure residual wash buffer was completely removed and DNA eluted using 20 µl of elution buffer (10 mM Tris-HCl, pH 8.5) and centrifugation at 12000 rpm for 1 minute. Purified DNA was stored at - 20˚C.

Purified DNAs were quantified using a Nanodrop 1000 spectrophotometer (Thermo scientific, UK). 10 ng of each of the purified DNAs were pooled for 454 pyrosequencing. 3 µl of the mixture was electrophoresed to check the purity on 2% agarose TBE gel and stained with ethidium bromide (10 mg/ml) (Sigma, UK). 10 µl of this mixture was sequenced from the 338R primer using 454 Titanium chemistry on a 454 GS FLX using a quarter of plate by the Centre for Genomic Research at the University of Liverpool.

5.3.3 Analysis of sequences

Sequences were processed using Mothur software version 1.32.0, available at the website www.mothur.org (Schloss 2009; Schloss *et al.* 2009) following the recommended 454 standard operating procedure (SOP) (downloaded 21.10.13) with some modifications. Mothur is a comprehensive, flexible and simple tool used for the analysis of sequencing data.

454 pyrosequencing reads were first assigned to individual samples using barcodes, allowing 1 base mismatch, and the forward primer and barcode sequences were removed. Then, sequences were aligned against the SILVA reference alignment (Pruesse *et al.* 2007), and chimeras identified using the Uchime tool (Edgar *et al.* 2011). Sequences were discarded if there were more than 2 mismatches in the forward primer sequence. The number of bases beyond the forward primer with good quality scores was less than 230 or they could not be satisfactorily aligned against the Silva reference alignment. Truncated sequences were eliminated by removing the shortest 2% as defined by the alignment of the 3' end of the read with the reference alignment. Sequences were assigned to the Silva reference taxonomy, chloroplast sequences were removed, and then grouped into OTUs based on 95% similarity.

The taxonomic assignments for the 40 commonest OTUs were first performed by Mothur. Then, the file containing a representative sequences for each of the commonest 40 OTUs was also used to find out the taxonomic affinities in more details using the basic local alignment search tool (BLAST) of the National Centre for Biotechnology Information (NCBI) on the website http/blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul *et al.* 1997).

A neighbour joining tree of all 40 commonest OTUs was generated using Mothur.

An Excel file of the abundance of the 40 commonest OTUs produced by Mothur was imported into SPSS for statistical analysis using principal component analysis (PCA). All graphs were produced using SPSS and Microsoft Excel (2010). I have not carried out the canonical analysis of principal coordinates (CAP) for the 454 data as the samples were collected on only two occasions.

5.4 Results

The ¼ plate of 454 pyrosequencing yielded 198,285 reads, with the majority of these in the range 350-380 bp.

After quality filtering and removal of short sequences using Mothur, 143,168 high quality reads were obtained with > 230 base pairs beyond the PCR primer.

The taxonomic assignments for these are shown in Table 5.1 and Figure 5.1.

All reads are classed as bacteria and 23 bacterial phyla were identified. The majority of them belong to the phyla proteobacteria (39.19%), Bacteroidetes (21.55%), Cyanobacteria (16.02%), Actinobacteria (2.92%) and Firmicutes (1.61%). Other bacterial phyla were found at lower frequency and 16.7% were unclassified at phylum level (Table 5.1). Aravindraja *et al.* (2013) found that the proportion of unclassified bacteria at phylum level ranged between 15% in marine sediments to about 37% in seawater samples (of total Illumina reads). In different water samples (aquifers) in the southern Algerian Sahara, Lenchi *et al.* (2013) found that 38% of total 454 pyrosequencing reads were unclassified bacteria at phylum level. Hence, the proportions obtained in this research were less than those published for seawater samples, but similar to the populations for marine sediments.

Unclassified bacteria at phylum level could be an artefact of the way that Mothur performs the classification. Bowman *et al.* (2012) could not classify the domain level of bacteria in seawater using the reference taxonomy (Mothur software) but could do so using Blast search. The results in this research showed that OTU1 and OTU9 were unclassified bacteria using Mother (Appendix Table A 5.2). However, a Blast search against 16S rRNA sequences (Bacteria and Archaea) obtained the full taxonomic affinities for the unclassified bacteria (Table 5.2).

Also, by running a Blast search against the NR full database found that OTU1 was an uncultured bacterium, clone SWB29 (similarity 96%, accession number AB294340). This clone was previously isolated from a deep coal aquifer in Japan (Shimizu *et al.* 2007). OTU9 was uncultured bacterium, clone ANTLV9_G05 (97% similarity, accession number DQ521564). This clone was previously isolated from the perennial ice cover of Lake Vida, Antarctica (Mosier *et al.* 2007).

Table 5. 1 Bacterial phyla, their numbers of reads and percentages of the total (143168) reads in the River Wensum from February 2012 (26 samples) and December 2012 (10 samples). Produced by Mothur programme, with the majority of reads in the range 350-380 base pair (bp).

Common bacterial phyla	454 pyrosequencing reads	(% of the total 454 reads)
Proteobacteria	56005	39.19
Bacteroidetes	30850	21.55
Unclassified bacteria	23877	16.68
Cyanobacteria	22942	16.02
Actinobacteria	4176	2.92
Firmicutes	2301	1.61
Candidate division TM7	1073	0.75
Fusobacteria	637	0.44
Acidobacteria	509	0.36
Nitrospirae	196	0.14
Verrucomicrobia	167	0.12
Gemmatimonadetes	147	0.10
Chloroflexi	118	0.082
Spirochaetes	115	0.080
Deferribacteres	13	0.009
Deinococcus-Thermus	11	0.008
Candidate division OP9	6	0.0042
Candidate division OP10	5	0.0040
Planctomycetes	5	0.0040
Lentisphaerae	4	0.0030
Thermotogae	4	0.0030
Candidate division OP11	3	0.0021
Chlorobi	2	0.0014
Synergistetes	2	0.0014

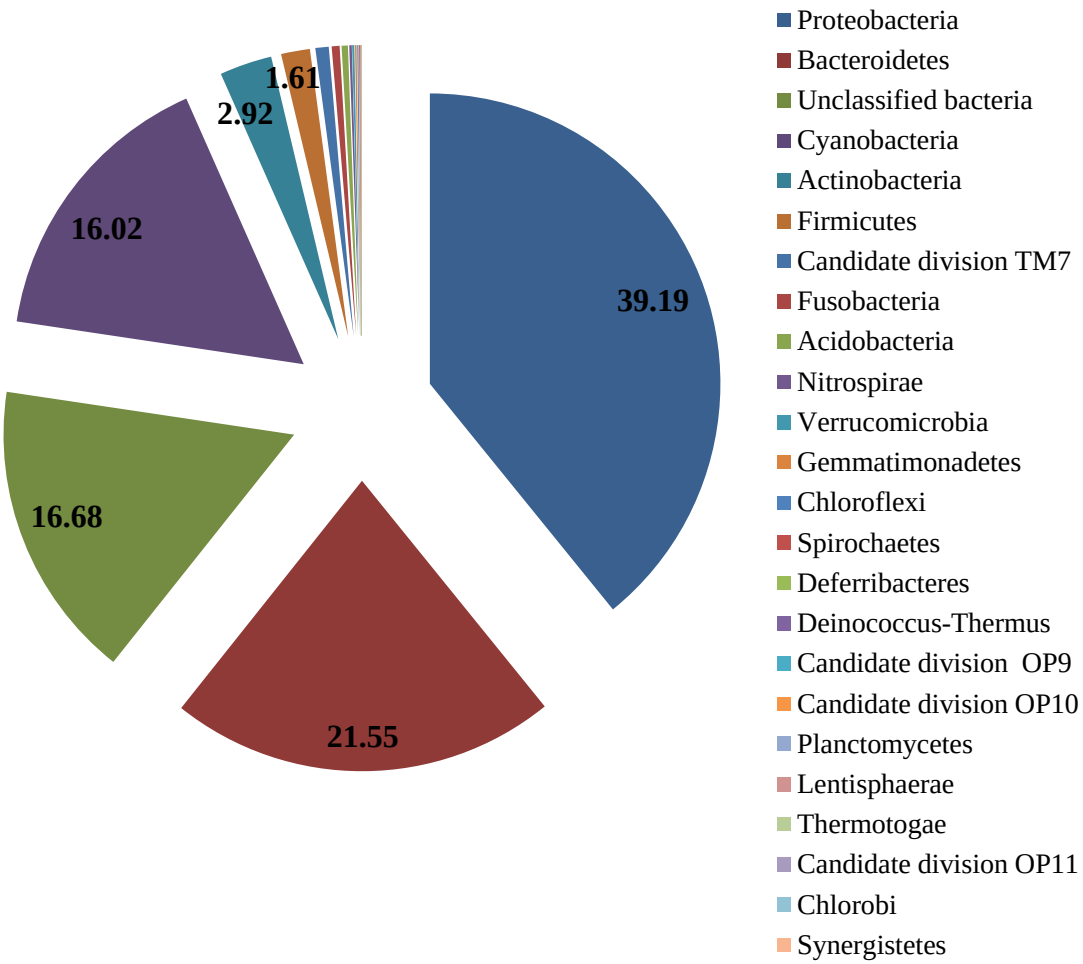


Figure 5. 1 Bacterial phyla in the River Wensum from February 2012 (26 samples) and December 2012 (10 samples). Produced by Mothur programme, with the majority of reads in the range 350-380 base pair (bp).

5.4.1 Commonest bacterial OTUs in all sites of the River Wensum

Figure 5.2 shows a neighbouring-joining tree for the 40 commonest bacterial OTUs in the River Wensum from February 2012 (26 samples) and December 2012 (10 samples).

Table 5.2 gives more detailed information on these 40 commonest bacterial OTUs and their taxonomic affinities including the most similar sequence retrieved using BLAST.

As can be seen in Figure 5.2 and Table 5.2, five major clusters were obtained in which members of each dominant phylum were clustered together. The majority of commonest bacteria OTUs were Proteobacteria accounting for 20.69% (classes of Beta (17.15%), Epsilon (1.67%), Gamma (0.9 %), Alpha (0.54%) and Delta (0.43%)) followed by Bacteroidetes accounting for 12.54 % (classes of Flavobacteria (9.95%), Sphingobacteria (1.60%) and Cytophagia (0.99%)), Cyanobacteria (6.84 %) and Actinobacteria (1.80 %).

In the neighbour joining tree (Figure 5.2), the cluster of Proteobacteria includes Betaproteobacteria (OTUs 2, 4, 5, 6, 11, 13, 16, 17, 22, 28, 31, 34, 37 and 40) and Gammaproteobacteria (OTUs 27 and 32). Epsilonproteobacteria includes OTUs 15, 36 and 38. Members of Actinobacteria (OTUs 20, 21 and 25) also were clustered together. Another cluster represents Bacteroidetes, including nine of Flavobacteria (OTUs 3, 8, 10, 12, 14, 18, 19, 23, 24 and 33) and Cytophagia (OTU14). The cluster of Cyanobacteria includes OTUs 1, 7, 30, 35 and 39.

The first common phylum was Cyanobacteria (mean abundance = 3.41% of total) and was represented by *Cyanothece* sp. (84% similarity to the nearest match in GenBank), followed by Betaproteobacteria (3.69%) represented by *Rhodoferax ferrireducens* (96% similarity) and Bacteroidetes (2.76%) represented by *Flavobacterium segetis* (95% similarity) Tables 5.2 and 5.3.

As bacteria reproduce asexually, the biological species concept of a population of interbreeding individuals (Mayr 1985; Mayr 1988) cannot be used. The erection of boundaries between species (and genera) are therefore essentially arbitrary choices, and traditional species and genus names may not consistently reflect phylogenetic distances. The “gold standard” used in culture based microbiology to assess whether two strains represent different species is DNA-DNA hybridisation, with strains being classed as con-specific when they have greater than 70% sequence similarity (Goris *et al.* 2007). The correlation between this overall level of DNA sequence similarity and similarity of 16S DNA sequences is imperfect. A similarity between 16S sequences of 97% is often taken as representing the boundary between species, but this figure is based on the comparison of full length 16S sequences (Chen *et al.* 2013; Tikhonov *et al.* 2014). Fox reports two strains which are different species by other criteria, but have 99.5% sequence similarity (Fox *et al.* 1992).

The conventionally used definition that “species” have 97% similarity of 16S sequences is based on comparison of full length 16S sequences. The mapping of sequence similarity thresholds for the full 16S onto the corresponding thresholds that should be used for a particular

hypervariable region is not clear. These thresholds will almost certainly be different from 97%, and probably lower.

For the 454 data, the OTU based approach is, in effect, assuming that organisms that share that level of sequence similarity (including variations due to sequencing errors) have substantial ecological similarity and behave in functionally similar ways. This study has identified the most similar 16S sequences from cultured strains and the most similar environmental 16S sequences to seek to characterise the taxonomic affinities of the commonest OTUs, and to infer the potential functional significance based on the characteristics of their nearest relatives. In some cases (the group of closely related OTUs) we can be reasonably confident about the taxonomic affinities of the OTUs. In other cases, such as the Cyanobacteria, the most similar sequence is only 85%. Here we can say that the sequence is likely to be a Cyanobacterium, but can say very little more.

Most crucially, we are not attempting to say that OTU1 is definitely to be identified with the cultured strain known as species *Cyanothece* sp. Even the literature using Sanger sequencing of full length 16S rarely attempts to be as precise as this.

Neither is this study seeking to define phylogenetic relationships between our OTUs. These need to be based on a near full length 16S sequence as a minimum. Bootstrap values were introduced by Felsenstein as a way of placing "confidence limits on phylogenies" (Felsenstein 1985), and assessing the extent to which they are likely to represent the "true" phylogeny of the organisms. The aim of this study was simply to identify the most similar DNA sequences in the databases, and then to use the much more robust phylogenies derived from these much longer sequences to infer the taxonomic affinities of the OTUs. The majority of bootstrap values are very low, reflecting that we are working with a relatively short and hypervariable sequence. We have reported bootstrap values greater than 50% on the neighbouring joining tree containing the reference sequences.

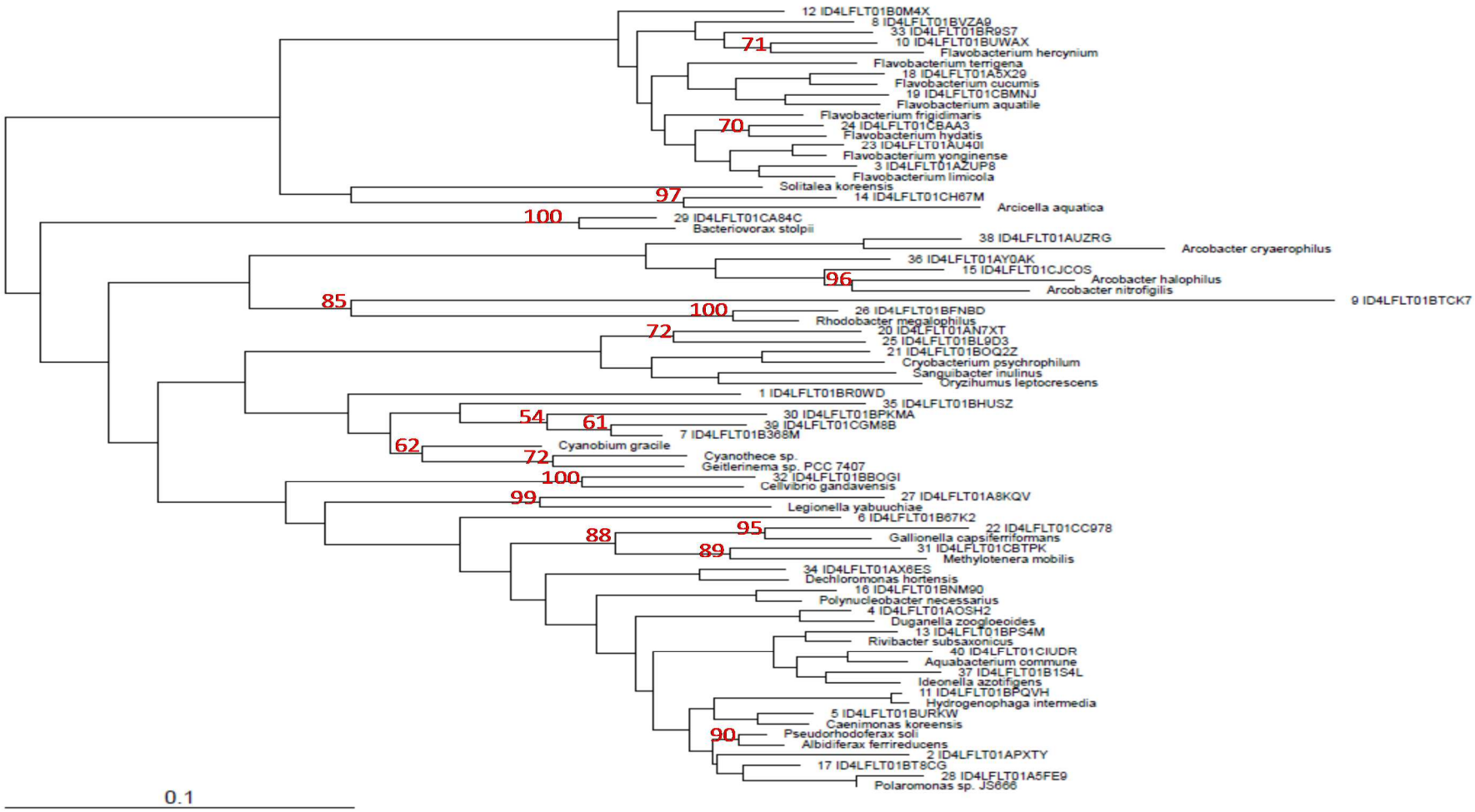


Figure 5. 2 Neighbouring-joining tree for the 40 commonest bacterial OTUs from the River Wensum and the closest relatives identified in Table 5.2, scale bar = 0.1. Numbers in red indicate bootstrap values greater than 50%.

Table 5. 2 The commonest bacterial OTUs in the River Wensum from February and December 2012, their phylogenetic and taxonomic affinities, identified using the BLAST searches of 16S rRNA gene sequences against GenBank.

OTU number	Phylum - class and subclass	Order	Family	Closest relative	Accession number	Similarity (%)
1	Cyanobacteria - Oscillatoriophycideae (*)	Chroococcales	Unclassified	*Cyanothece* sp. ATCC 51142 strain	NR 074316	84 %
2	Proteobacteria, Beta	Burkholderiales	Comamonadaceae	*Albidiverax ferrireducens* T118 strain DSM 15236	NR 074760	96 %
3	Bacteroidetes - Flavobacteria (**)	Flavobacteriales	Flavobacteriaceae	*Flavobacterium yonginense* strain HMD1001	NR 108535	96 %
4	Proteobacteria – Beta (**)	Burkholderiales	Oxalobacteraceae	*Duganella zoogloeoides* strain IAM 12670	NR 025833	96 %
5	Proteobacteria – Beta (**)	Burkholderiales	Comamonadaceae	*Caenimonas koreensis* strain EMB320	NR 043748	97 %
6	Proteobacteria – Beta (**) ;	Burkholderiales	Comamonadaceae	*Rhodoferax ferrireducens* T118 strain DSM 15236	NR 074760	88 %
7	Cyanobacteria - Oscillatoriophycideae (*)	Chroococcales	Unclassified	*Cyanobium gracile* PCC 6307 strain PCC 6307	NR 102447	88 %
8	Bacteroidetes – Flavobacteria (**) ;	Flavobacteriales	Flavobacteriaceae	*Flavobacterium frigidimaris* strain KUC-1 16S	NR 041057	95 %
9	Bacteroidetes - Sphingobacteria (**)	Sphingobacteriales	Sphingobacteriaceae	*Solitalea koreensis* strain R2A36-4	NR 044568	84 %
10	Bacteroidetes – Flavobacteria (**)	Flavobacteriales	Flavobacteriaceae	*Flavobacterium terrigena* strain DS-20	NR 044006	96 %
11	Proteobacteria – Beta (**)	Burkholderiales	Comamonadaceae	*Hydrogenophaga intermedia* strain S1	NR 024856	99 %
12	Bacteroidetes – Flavobacteria (**)	Flavobacteriales	Flavobacteriaceae	*Flavobacterium hercynium* strain : WB 4.2-33	NR 042520	97 %
13	Proteobacteria – Beta (**)	Burkholderiales	Unclassified	*Rivibacter subsaxonicum* strain : BF49	NR 042651	95 %

*= subclass, **= class, ***=suborder. Names of phylum, class, subclass, order, family and closest relative are based on the taxonomic affinity of the closest sequence in the BLAST search.

Table 5.2 (continued). The commonest bacterial phyla and their taxonomic affinities in the River Wensum from February and December 2012, using the BLAST tool and based on 454 pyrosequencing of 16S rRNA gene.

OTU number	Phylum – class and subclass	Order-suborder	Family	Closest relative	Accession number	Similarity (%)
14	Bacteroidetes – Cytophagia (**)	Cytophagales	Cytophagaceae	*Arcicella aquatica* strain NO-502	NR 029000	90 %
15	Proteobacteria - Epsilonproteobacteria (**)	Campylobacterales	Campylobacteraceae	*Arcobacter nitrofigilis* DSM 7299	NR 102873	90 %
16	Proteobacteria - Beta (**)	Burkholderiales	Burkholderiaceae	*Polynucleobacter necessarius* subsp. a*symbioticus* QLW-P1DMWA-1	NR 074689	96 %
17	Proteobacteria - Beta (**)	Burkholderiales	Comamonadaceae	*Pseudorhodoferax* soli strain TBEA3	NR 044574	97 %
18	Bacteroidetes - Flavobacteria (**)	Flavobacteriales	Flavobacteriaceae	*Flavobacterium cucumis* strain R2A45-3	NR 044107	96 %
19	Bacteroidetes – Flavobacteria (**)	Flavobacteriales	Flavobacteriaceae	*Flavobacterium aquatile* strain : DSM 1132	NR 042495	96 %
20	Actinobacteria – Actinobacteridae (*)	Actinomycetales - Micrococcineae (***)	Sanguibacteraceae	*Sanguibacter inulinus* strain ST50	NR 029277	92 %
21	Actinobacteria; Actinobacteridae (*)	Actinomycetales, Micrococcineae (***)	Microbacteriaceae	*Cryobacterium psychrophilum* strain : DSM 4854	NR 042170	95 %
22	Proteobacteria – Beta (**)	Gallionellales	Gallionellaceae	*Gallionella capsiferriformans* ES-2 strain ES-2	NR 074658	91 %
23	Bacteroidetes- Flavobacteria (**)	Flavobacteriales	Flavobacteriaceae	*Flavobacterium frigidimaris* strain KUC-1	NR 041057	96 %

*= subclass, **= class, ***=suborder. Names of phylum, class, subclass, order, family and closest relative are based on the taxonomic affinity of the closest sequence in the BLAST search.

Table 5.2 (continued). The commonest bacterial phyla and their taxonomic affinities in the River Wensum from February and December 2012, using the BLAST tool and based on 454 pyrosequencing of 16S rRNA gene.

OTU number	Phylum - class and subclass	Order	Family	Closest relative	Accession number	Similarity (%)
24	Bacteroidetes - Flavobacteria (**)	Flavobacteriales	Flavobacteriaceae	*Flavobacterium hydatis* strain ATCC 29551	NR 044695	99 %
25	Actinobacteria - Actinobacteridae (*)	Actinomycetales - Micrococcineae (***)	Intrasporangiaceae	*Oryzihumus leptocrescens* strain KV-628	NR 041253	90 %
26	Proteobacteria – Alpha (**)	Rhodobacterales	Rhodobacteraceae	*Rhodobacter megalophilus* strain : JA194	NR 042585	95 %
27	Proteobacteria - Gamma (**)	Legionellales	Legionellaceae	*Legionella yabuuchiae* strain OA1-2	NR 041322	88 %
28	Proteobacteria; Beta (**)	Burkholderiales	Comamonadaceae	*Polaromonas* sp. strain JS666	NR 074725	96 %
29	Proteobacteria – Delta (**)	Bdellovibrionales	Bacteriovoracaceae	*Bacteriovorax stolpii* strain DSM 12778	NR 042023	95 %
30	Cyanobacteria Oscillatoriophycideae (*)	Oscillatoriales	Unclassified	*Geitlerinema* sp. PCC 7407 strain PCC 7407	NR 102448	87 %
31	Proteobacteria – Beta (**)	Methylophilales	Methylophilaceae	*Methylotenera mobilis* strain JLW8	NR 102842	95 %
32	Proteobacteria- Gamma (**)	Pseudomonadales	Pseudomonadaceae	*Cellvibrio gandavensis* strain R-4069	NR 025419	96 %
33	Bacteroidetes - Flavobacteria (**)	Flavobacteriales	Flavobacteriaceae	*Flavobacterium limicola* strain ST-82	NR 024787	95 %
34	Proteobacteria - Beta (**)	Rhodocyclales	Rhodocyclaceae	*Dechloromonas hortensis* strain MA-1	NR 042819	96 %

*= subclass, **= class, ***=suborder. Names of phylum, class, subclass, order, family and closest relative are based on the taxonomic affinity of the closest sequence in the BLAST search.

Table 5.2 (continued). The commonest bacterial phyla and their taxonomic affinities in the River Wensum from February and December 2012, using the BLAST tool and based on 454 pyrosequencing of 16S rRNA gene.

OTU number	Phylum – class and subclass	Order-suborder	Family	Closest relative	Accession number	Similarity (%)
35	Cyanobacteria Oscillatoriophycideae (*)	Oscillatoriales	Unclassified	*Geitlerinema* sp. PCC 7407 strain	NR 102448	86 %
36	Proteobacteria - Epsilonproteobacteria (**)	Campylobacterales	Campylobacteraceae	*Arcobacter halophilus* strain LA31B	NR 041918	90 %
37	Proteobacteria – Beta (**)	Burkholderiales	Comamonadaceae	*Ideonella azotifigens* strain 1a22	NR 044521	95 %
38	Proteobacteria - Epsilonproteobacteria (**)	Campylobacterales	Campylobacteraceae	*Arcobacter cryaerophilus* strain A 169/B	NR 025905	90 %
39	Cyanobacteria; Oscillatoriophycideae (*)	Oscillatoriales	Unclassified	*Geitlerinema* sp. PCC 7407 strain	NR 102448	88 %
40	Proteobacteria - Beta (**)	Burkholderiales	Comamonadaceae	*Aquabacterium commune* strain B8	NR 024875	95 %

*= subclass, **= class, ***=suborder. Names of phylum, class, subclass, order, family and closest relative are based on the taxonomic affinity of the closest sequence in the BLAST search.

5.4.2 The abundance of the 40 commonest bacterial taxa (OTUs)

Figure 5.3 and Table 5.3 show the mean abundance, cumulative abundance, maximum abundance and sites of the maximum abundance of the 40 commonest OTUs in the River Wensum from February and December 2012.

The abundance of the all these 40 commonest OTUs accounted for about 42 % of the total bacterial abundance in the river water. This percentage is similar to that obtained by the ARISA tool of the abundance of the 20 commonest fragment sizes (40%) from the total abundance. However, the 40 commonest ARISA fragment sizes contributed about (55 %) of the total abundance. This means that ARISA contributed higher abundance but lower diversity than that obtained by 454 pyrosequencing.

As can be seen in Table 5.3, averaged across all the samples analysed, each of the 40 commonest OTUs make up only a relatively small proportion of the total, but some of these OTUs can be very common in individual samples. The commonest three OTUs, for example, have mean abundances of between 2.8 and 3.7%, but have maximum abundances in individual samples ranging from 14.7% at site SBD to 41.2% at site SC.

Results presented in Chapter 4 found that sites SC and S18 had the highest abundance of the first common OTU (702.09).

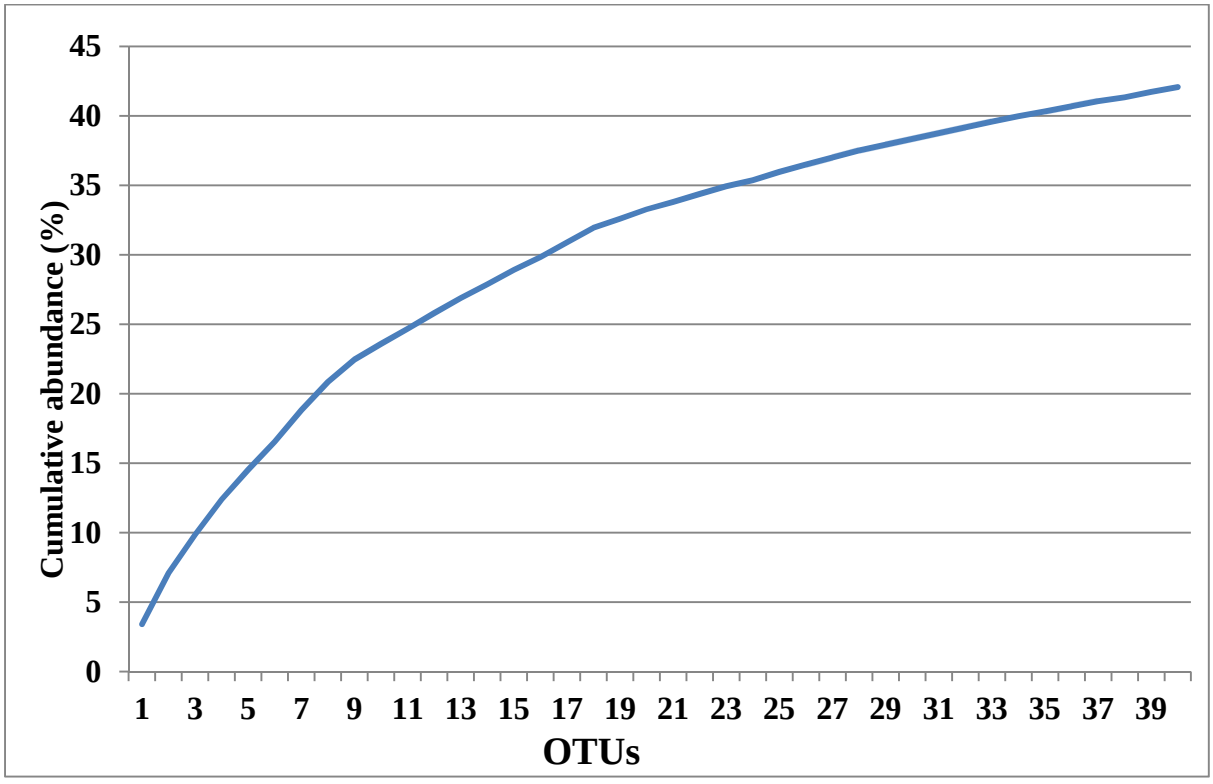


Figure 5. 3 Cumulative dominance curve of the abundance of the 40 commonest OTUs in the River Wensum from February and December 2012.

Table 5. 3 Mean of relative abundance, cumulative abundance, maximum abundance (% of the total) and sites of maximum abundance of the 40 commonest OTUs in the River Wensum from February 2012 (26 samples) and December 2012 (10 samples).

Commonest OTU	Mean abundance (%)	Cumulative abundance	Max. abundance	Sites of max. abundance
1	3.41	3.41	41.18	SC
2	3.69	7.10	14.65	SBD
3	2.76	9.86	25.47	S18
4	2.55	12.41	15.55	S20D
5	2.14	14.55	20.07	S9
6	2.00	16.55	25.52	S17
7	2.28	18.83	22.93	SDD
8	2.03	20.86	7.34	SC
9	1.60	22.46	5.47	SA
10	1.13	23.59	6.79	SC
11	1.10	24.69	16.87	S11
12	1.12	25.81	3.14	S20
13	1.09	26.90	2.27	SF
14	0.99	27.89	8.60	S9
15	1.02	28.91	6.31	S18
16	0.94	29.85	4.41	SCD
17	1.04	30.89	6.65	SBD
18	0.83	31.93	4.53	SC
19	0.66	32.59	8.55	S9
20	0.69	33.28	3.78	S16
21	0.53	33.81	1.95	S18
22	0.57	34.38	1.54	SD
23	0.54	34.92	1.68	SAD
24	0.46	35.38	4.56	S20
25	0.58	35.96	3.08	SDD
26	0.54	36.50	2.13	S9
27	0.50	37.00	3.57	S15
28	0.50	37.50	1.78	SBD
29	0.43	37.93	1.10	SE
30	0.41	38.34	2.59	S2
31	0.42	38.76	1.15	S9
32	0.40	39.16	1.55	SA
33	0.42	39.58	1.98	S16
34	0.39	39.97	3.93	SAD
35	0.34	40.31	6.37	S5
36	0.37	40.68	4.10	S18D
37	0.37	41.05	1.74	SD
38	0.28	41.33	6.28	SCD
39	0.40	41.73	2.24	S6
40	0.35	42.08	1.59	SAD

5.4.2.1 Spatial distribution of the commonest OTUs

Spatial distribution of the abundance of the all 40 commonest OTUs is presented in the Appendix (Table A 5.3).

Principal component analysis (PCA) (Table 5.4 and Figure 5.4) shows the overall patterns in the abundance of these common OTUs across the Wensum catchment. Factor 1 explained 19.8% of the variation of OTUs, while factor 2 explained 11.4% of the variance. Factors 3 and 4 each explained a further 8% of the variance.

Axis 1 is essentially upstream-downstream and axis 2 separates the December samples. Axis 1 is positively correlated with OTUs 1, 3, 5, 10, 14, 19, 20, 21, 25 and 26, and negatively correlated with OTUs 2, 4, 9, 12, 23 and 29. Axis 2 is positively correlated with OTUs 2, 17, 26, 34 and 40.

The overall patterns of community composition is that the most common OTUs are positively correlated with axis 1, so they are becoming commoner as the water moves downstream (3rd and 4th order). This presumably reflects the fact that these bacteria are actively growing in the river, and reducing the abundance of other taxa. The result of this is that the community becomes less diverse as it moves downstream. However, there are one or two sites, such as site SC that do not fit this pattern (Tables 5.2 and 5.4, Figure 5.4 and Appendix Table A5.3). .

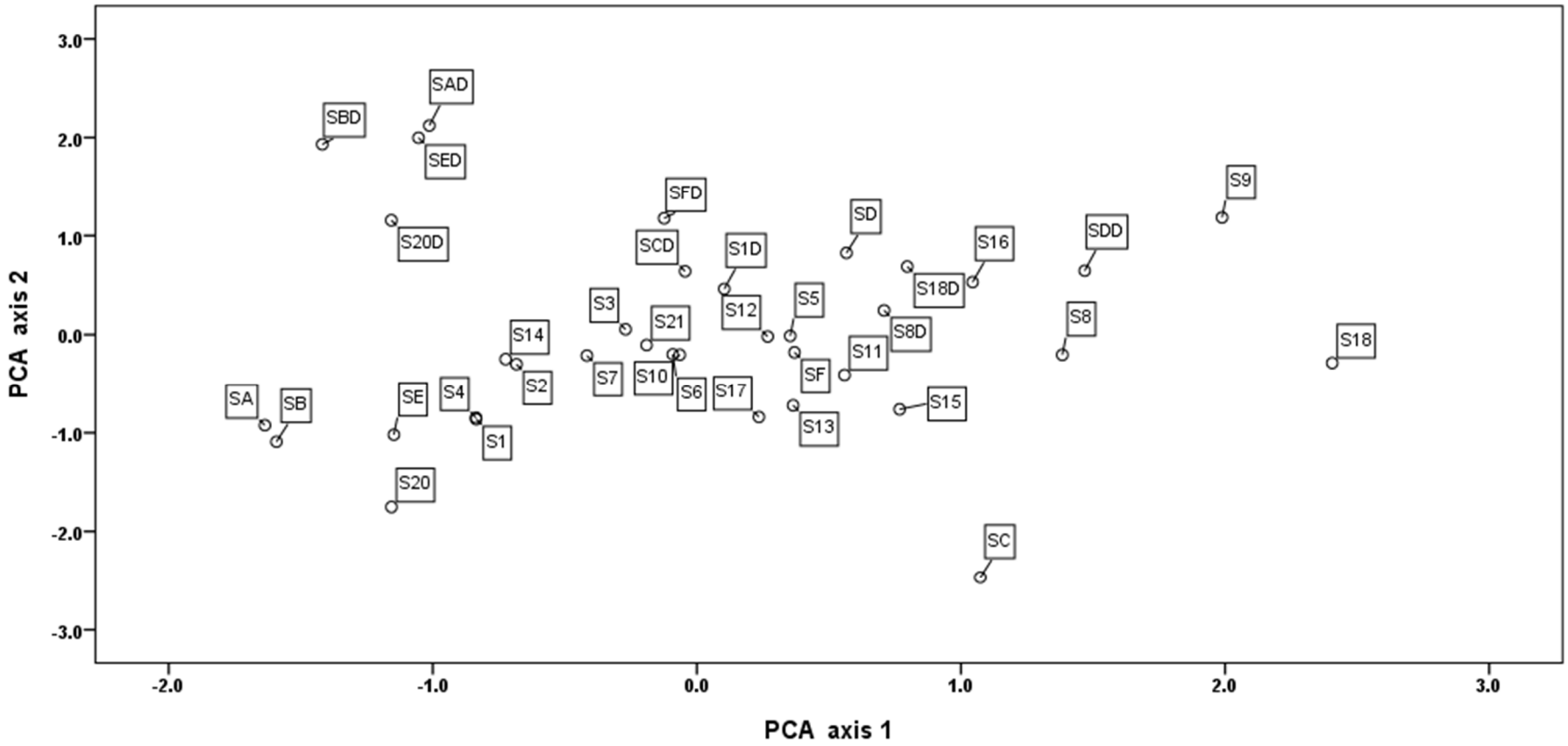


Figure 5. 4 Principal component analysis of the commonest OTUs (equal weight given to all OTUs) for all sites of the River Wensum from February and December 2011.

Table 5. 4 Principal component loadings of the commonest OTUs (equal weight given to all OTUs) for all sites of the River Wensum from February and December 2011.

OTU	Principal components	
	1	2
OTU1	0.254	-0.455
OTU2	-0.521	0.505
OTU3	0.591	-0.081
OTU4	-0.622	0.176
OTU5	0.644	0.087
OTU6	-0.03	-0.218
OTU7	0.413	0.037
OTU8	-0.267	-0.395
OTU9	-0.701	-0.198
OTU10	0.619	-0.358
OTU11	0.024	0.097
OTU12	-0.626	0.023
OTU13	-0.304	0.278
OTU14	0.693	0.252
OTU15	0.128	0.07
OTU16	0.194	0.181
OTU17	-0.43	0.58
OTU18	0.389	-0.399
OTU19	0.599	0.118
OTU20	0.731	0.242
OTU21	0.602	0.12
OTU22	-0.053	0.383
OTU23	-0.741	0.354
OTU24	-0.445	-0.446
OTU25	0.625	0.166
OTU26	0.530	0.59
OTU27	-0.055	-0.317
OTU28	-0.254	0.464
OTU29	-0.546	-0.402
OTU30	-0.014	-0.439
OTU31	0.133	0.348
OTU32	-0.606	-0.417
OTU33	-0.290	-0.017
OTU34	-0.232	0.621
OTU35	0.187	0.023
OTU36	0.147	0.264
OTU37	-0.062	0.312
OTU38	0.001	0.12
OTU39	0.464	0.043
OTU40	-0.363	0.724
% variance explained	19.80%	11.40%

1- Commonest OTUs at upstream sites.

The commonest OTUs at the upstream sites were Betaproteobacteria (OUTs 2 and 4), Deltaproteobacteria (OTU29), Gammaproteobacteria (OTU32), Sphingobacteria (OUT9) and Flavobacteria (OTUs 12 and 23) (Figure 5.4 and Table 5.4).

The variation of each one of these commonest bacteria was related to some environmental parameters as revealed by Spearman's rank correlation (see Appendix Table A 5.4).

The closest relative of OTU2 is *Albidiverax ferrireducens* T118 strain DSM 15236 (similarity 96%) which was proposed as a novel facultative anaerobic species by Finneran *et al.* (2003), and can support aerobic growth through maintaining energy from dissimilatory Fe(III)-reduction. It was isolated with other Fe (III)- reducing microbes from Oyster Bay sediments, US (Finneran *et al.* 2003). OTU2 can enter upstream sites from terrestrial areas, especially during high rainfall and river flow. So its abundance in the upstream sites positively correlates with TC and TN. OTU2 decreases as the water moves to downstream sites.

The closest relative of OTU4 is *Duganella zoogloeoides* strain IAM 12670 (96% similarity). This taxon was isolated from waste water environments by Hiraishi *et al.* (1997) and was suggested to play an important role in these environments. The *Duganella zoogloeoides* is able to degrade various pollutants and are present on activated sludge. It metabolizes dissolved organic matter and produces mucopolysaccharides (Schweitzer *et al.* 2000). OTU4 can enter upstream with water from terrestrial areas (arable lands) and groundwater into upstream sites. So, the abundance of this common OTU4 in upstream sites positively correlated with TC and TN. OTU4 decreases as the water moves to downstream sections of the river (Tables 5.2 and 5.4, Figure 5.4 and Appendix Table A 5.4).

Betaproteobacteria (OTU2) was the commonest bacteria in December 2012 after flooding events, indicating the role of flooding in flushing these bacteria from soil into upstream sites of the river.

The closest relative of OTU29 is the *Bacteriovorax stolpii* strain DSM 12778 (95% similarity) which is known as the terrestrial Bdellovibrio species (Baer *et al.* 2000). The species of *stolpii,* like other species of the genus *Bacteriovorax,* is known to be an obligatory predatory bacterium that preys on the other gram-negative bacteria in marine and freshwater environments (Pineiro *et al.* 2008). OTU29 can enter upstream sites from terrestrial areas (arable lands). Its abundance negatively correlated with TOC and can be diluted by high rainfall and river flow (Tables 5.2 and 5.4, Figure 5.4 and Appendix 5.4).

The closest relative of OTU32 is *Cellvibrio gandavensis* strain R-4069 (96% similarity). This strain is described as cellulolytic bacteria and was isolated from agricultural soil. It is highly able, like other strains belonging to the genus of *Cellvibrio,* to degrade cellulosic plant fibres (Lednicka *et al.* 2000; Mergaert *et al.* 2003). OTU32 can enter the upstream aquatic environment from terrestrial areas (arable lands). OUT 32 decreases as water moves to

downstream sites. Its abundance negatively correlated with TP and TOC at these sites (Tables 5.2 and 5.4, Figure 5.4 and Appendix Table A 5.4).

The closest relative of OTU9 is *Solitalea koreensis* strain R2A36-4 (84% similarity) which was isolated from greenhouse soils samples in Korea and found to be a food-associated taxon (Weon *et al.* 2009; Corry *et al.* 2011). OTU9 can enter upstream sites from terrestrial areas (arable lands). So its abundance in the upstream sites positively correlated with TC but negatively with TSS. OTU9 decreases as water moves to downstream sites (Tables 5.2 and 5.4, Figure 5.4 and Appendix 5.4).

The closest relative of OTU12 is the *Flavobacterium hercynium* strain : WB 4.2-33 (97% similarity) which was isolated from freshwater samples (hard water) in Germany by Cousin *et al.* (2007). OTU12 can come with water from terrestrial areas (arable lands) into upstream sites. Its abundance in the upstream sites positively correlated with TC. It decreases as water moves to downstream sections of the river and towards urban areas, and its abundance at these sites correlated negatively with TP (Tables 5.2 and 5.4, Figure 5.4 and Appendix 5.4).

The closest relative of OTU23 is the *Flavobacterium frigidimaris* strain KUC-1 16S (95% similarity) which was isolated from seawater in Antarctica and proposed to be a novel species by Nogi *et al.* (2005). Authors have described this species as an aerobic psychrotolerant bacterium that is highly able to catalyse organic materials under cold environments. OUT 23 can come from terrestrial area (arable lands), especially during high rainfall and river flow. Its abundance in the upstream sites positively correlated with TC and TN. OTU23 decreases as water moves to downstream sites. Its abundance in these sites negatively correlated with pH (Tables 5.2 and 5.4, Figure 5.4 and Appendix 5.4).

Overall, the commonest OTUs at the upstream sites are associated with water from terrestrial areas (most of them from soil) entering the river. The majority of the bacteria can multiply and increase in these sites and their abundance is correlated positively with TC, TN and arable areas, while they decrease as the water moves to downstream sites.

Most of the commonest OTUs at the upstream sites are soil bacteria (OTUs 2, 29, 32 and 9) and could be flushed from terrestrial areas into streams. Two of OTUs (12 and 23) are freshwater bacteria. OTU4 is a sewage bacterium and was found to be high at site S20 (about 16% of total abundance) and during December 2012 (high river flow) (Appendix Table A 5.3). Site S20 is dominated by groundwater and located in a rural area that has many septic tanks.

2- Commonest OTUs at downstream sites

PCA showed that the commonest OTUs at the downstream sites of the river were Cyanobacteria (OTU1), Flavobacteria (OTUs 3, 10 and 19), Cytophagia (OTU14), Actinobacteria (OTUs 20, 21 and 25) and Alphaproteobacteria (OTU26).

The correlations between environmental parameters and commonest OTUs at downstream sites as revealed by Spearman's rank correlation are presented in the Appendix (Table A 5.5).

The closest relative of OTU1 is *Cyanothece* sp. ATCC 51142 strain (84% similarity). This strain is unicellular diastrophic and was isolated from marine environments by Welsh *et al.* (2008) and has been found to play an important role in N_2-fixation. Also, Min and Sherman (2010) found that this strain was able to produce hydrogen (H_2) equal to the rate of nitrogen fixation. OTU1 can enter streams from different sources (especially from adjacent lakes) and increase as water moves to downstream sections. Bacteria can grow and multiply in these sites. In the Wensum, its abundance was positively correlated with TP but negatively correlated with TC, TOC and TN. Because the bacteria are growing at downstream of the river, they can be diluted and decrease in number after high rainfall and river flow. However, these bacteria were also found at one upstream site (SC) and may due to an adjacent lake that connect with the stream upstream of the sampling site. The other possibility for the source of these bacteria is septic sewages that are found in the vicinity of site SC (Tables 5.2, Table 5.4, Figure 5.4 and Appendix 5.5).

The closest relative of OTU3 is *Flavobacterium yonginense* strain HMD1001 (96% similarity) which was isolated first from mesotrophic lakes in Korea by Joung *et al.* (2012). OTU3 can enter to the river from different sources. Its abundance at upstream sites negatively correlated with TN. OTU3 increases as water moves downstream and its abundance in these sites correlated positively with TP (Tables 5.2 and 5.4, Figure 5.4 and Appendix Table A 5.5).

The closest relative of OTU10 is *Flavobacterium terrigena* strain DS-20 (96% similarity) which was first isolated from soil samples in Korea and described as a novel species by Yoon *et al.* (2007). OTU10 enter streams with soil water. Its abundance in the upstream sites negatively correlated with TN and TC and increases as water moves to downstream sites. The bacteria were found to be diluted by rainfall and high river flow (Tables 5.2 and 5.4, Figure 5.4 and Appendix Table A 5.5).

The closest relative of OTU14 is *Arcicella aquatica* strain NO-502 (90% similarity) and was isolated from a neuston biofilm in a freshwater lake in Russia (Nikitin *et al.* 2004). OTU14 can come with water from different sources and increase as water moves downstream and towards urban areas. The bacteria can grow and multiply at downstream sites with its abundance at these sites positively correlated with pH and TSS (Tables 5.2 and 5.4, Figure 5.4 and Appendix Table A 5.5).

The closest relative of OTU19 is *Flavobacterium aquatile* strain DSM 1132 (96% similarity) which was isolated first from water and soil samples by Frankland in 1889, and latterly was described as nitrate-reducing bacteria that are often found in freshwater and soil environments (Weeks 1954; Bernarder *et al.* 1996). OTU19 can come with water into the river from different sources and its abundance in the upstream sites negatively correlated with TC and TN. OTU19 increases as water moves to downstream sites and its abundance in these sites is positively correlated with TP and TSS (Tables 5.2 and 5.4, Figure 5.4 and Appendix Table A 5.5).

The closest relative of OTU20 is *Sanguibacter inulinus* strain ST50 (92% similarity). This species is known as a coryneform bacterium and was first isolated from cow's blood (Ventura *et al.* 2007). OTU20 can enter river water from different sources and increase as water moves downstream towards urban areas. Its abundance positively correlated with TSS (Tables 5.2 and 5.4, Figure 5.4 and Appendix Table A 5.5).

The closest relative of OTU25 is *Oryzihumus leptocrescens* strain KV-628 (90% similarity) which was first isolated from a paddy soil habitat in Japan (Kageyama *et al.* 2005). OTU25 can enter streams from terrestrial areas and its abundance in upstream sites is negatively correlated with TC. OTU25 increases as water moves downstream and its abundance at these sites is positively correlated with TP and TSS (Tables 5.2 and 5.4, Figure 5.4 and Appendix Table A 5.5).

The closest relative of OTU26 is *Rhodobacter megalophilus* strain JA194 (95% similarity) which was first isolated from soil samples in the Himalaya by Arunasri *et al.* (2008) and was proposed as a novel species of the genus *Rhodobacter*. It is often found in aquatic environments and can be phototrophic, photoheterotrophic and chemoheterotrophic aerobic bacteria (Arunasri *et al.* 2008). OTU26 can enter stream water from terrestrial areas (soil) and move downstream, especially after events of high rainfall followed by increased river flow. Suspended particles (TSS) can carry bacteria into streams. The abundance of OTU26 at downstream sites is positively correlated with TOC (Tables 5.2 and 5.4, Figure 5.4 and Appendix Table A 5.5).

Alphaproteobacteria (OTU26) was also the commonest bacteria in December 2012 after flooding. The bacteria could be flushed from soil to downstream sites (see Appendix Table 5.5). Alphaproteobacteria (OTU26) are often found in aquatic environments, indicating the suitability of freshwater environments for their growth and multiplication.

Overall, the commonest OTUs at downstream sites enter surface water from different sources and increase in abundance as water moves downstream. Most of the commonest bacteria in downstream sections are freshwater bacteria; Flavobacteria (OTUs 3, 10 and 19) and Cytophagia (OTU19). Alphaproteobacteria (OTU26) is often found in aquatic environments and also in soil. Two of the OTUs (10 and 20) are soil bacteria. The downstream bacterial OTUs showed a positive relationship with TP and the presence of urban areas.

5.4.2.2 Commonest OTUs in December 2012

The distribution in December 2012 of the abundance of the 40 commonest OTUs is presented in the Appendix (Table A 5.3). Principal component analysis (Figure 5.4 and Table 5.4) shows the overall patterns in the abundance of these common OTUs across the Wensum catchment. Principal component analysis showed that the commonest OTUs in December 2012 were Betaproteobacteria (OTUs 2, 17, 34 and 40) and Alphaproteobacteria (OTU26).

As mentioned in section 5.4.2.1, Betaproteobacteria (OTU2) is the commonest bacteria at the upstream sites of the river, while Alphaproteobacteria (OTU26) are the commonest bacteria at downstream sites. These are soil bacteria and can be flushed from terrestrial areas into streams and increase in number after rainfall and flooding events. Variations in these bacteria were related to some environmental parameters as revealed by Spearman's rank correlations (Appendix Table A 5.6).

The closest relative of OTU17 is *Pseudorhodoferax soli* strain TBEA3 (similarity 97%) which was isolated from soil and proposed to be a novel species. This strain is only able to utilize an organic sulfur compound (3,3'-thiodipropionic acid used as an antioxidant in food) as a source of carbon and energy, indicating its role in sulfur transformations (Bruland *et al.* 2009). OTU17 can enter stream water from terrestrial areas, especially during high rainfall and river flow. Its abundance positively correlated with TC (Tables 5.2 and 5.4, Figure 5.4 and Appendix 5.6).

The closest relative of OTU34 is *Dechloromonas hortensis* strain MA-1 (96% similarity). This strain was proposed by Wolterink *et al.* (2005) and was isolated from garden soil and also from sites polluted with chlorate (used in herbicides) and perchlorate compounds. It has found to play an important role in converting perchlorate to chlorate (perchlorate-reducing bacteria) and ultimately to chlorine plus oxygen, indicating its role in the field of bioremediation (Wolterink *et al.* 2005). OTU34 can enter stream water from soil, especially during high rainfall. Its abundance positively correlated with TN, TC and TOC (Table 5.2 and 5.4, Figure 5.4 and Appendix Table A 5.6).

The closest relative of OUT40 is *Aquabacterium commune* strain B8 (95% similarity) which was isolated from biofilms in drinking water distribution systems by Kalmbach *et al.* (1999) and proposed to be a novel species. Its growth behaviour is described as microaerophilic (it can grow at low concentrations of oxygen), conferring advantage on this bacterium in environments, such as sewage. This bacterium is often found in the source of drinking water (groundwater) at low level of oxygen concentration (Kalmbach *et al.* 1999). The species of *Aquabacterium commune* is known to be a sulfur-reducing bacterium (Bade *et al.* 2000). OTU40 was associated with groundwater at upstream sites, especially during high rainfall and river flow. Its abundance positively correlated with TN, TC and TOC but negatively correlated with pH. OTU40 was found to be abundant at sites SA and S20 which are dominated by groundwater (Table 5.2 and 5.4, Figure 5.4 and Appendix Table A 5.6)

Overall, in December 2012, the commonest OTUs are associated with water from terrestrial areas and groundwater entering streams, with high numbers after rainfall events followed by flooding. OTUs 2, 17, 34 and 40 increase at upstream sites and their abundance is positively correlated with TN and TC, while OTU26 multiplies at downstream sites, with its abundance positively correlated with TOC.

5.4.2.3 Other common OTUs

1- Betaproteobacteria

The closest relative of OTU6 is *Rhodoferax ferrireducens* T118 strain DSM 15236 (similarity 96%) which was proposed as a novel facultative anaerobic species by Finneran *et al.* (2003), and can support aerobic growth through maintaining energy from dissimilatory Fe(III)-reduction. It was isolated with other Fe (III)- reducing microbes from Oyster Bay sediments, US (Finneran *et al.* 2003).

The closest relative of OTU5 is *Caenimonas koreensis* strain EMB320 (similarity 97%) which was isolated from activated sludge by Ryu *et al.* (2008) and proposed to be a novel genus and species. This species is strictly aerobic and has been found to play an important role in removing phosphorus (Ryu *et al.* 2008).

The closest relative of OTU11 is *Hydrogenophaga intermedia* strain S1 (similarity 99%) which was reported the first time as a bacterial strain by Feigel and Knackmuss (1988) and found to have a high ability to degrade 4-aminobenzenesulfonic acid in aerobic conditions. As a consequences of this ability, it has been of great value in bioremediation studies has been gained (Gan *et al.* 2012).

The closest relative of OUT13 is *Rivibacter subsaxonicum* strain BF49, which was isolated first from hardwater biofilms by Stackebrandt *et al.* (2008). The genus of *Methylibium* is known to be an ammonium-oxidizing bacteria (Mao *et al.* 2011).

The closest relative of OTU28 is *Polaromonas* sp. strain JS666 (similarity 96%), which is known to be pollutant-reducing bacterium and resistant to metals. It plays an important role in bioremediation at environmental sites polluted with chlorinated solvent. It has a high ability to degrade hydrocarbon and xenobiotic compounds (Mattes *et al.* 2008).

The closest relative of OTU37 is *Ideonella azotifigens* strain 1a22 (similarity 95%), which was isolated from soil associated with the rhizosphere from agricultural areas in New York, by Noar and Buckley (2009) and was proposed to be a novel species of bacteria exhibiting great ability in N_2- fixation.

The closest relative of OTU16 is *Polynucleobacter necessarius* subsp. asymbioticus QLW-P1DMWA-1 (96% similarity), which was found to be the most dominant bacterium of free-living bacteria in freshwater environments. It contains sub-species which are cosmopolitan and ubiquitous in such environments (Meincke *et al.* 2012), and is known to live by utilising low molecular weight humic materials (Hahn *et al.* 2012).

The closest relative of OUT22 is *Gallionella capsiferriformans* strain ES-2 (91% similarity). Species belonging to this strain are proposed as a novel in freshwater. This strain plays an a crucial role in Fe-oxidation and has a high ability to tolerate heavy metals in freshwater

environments (Emerson *et al.* 2013). Generally, the genus of *Gallionella* is known to be Iron-oxidizing bacteria (Lear *et al.* 2009).

The closest relative of OTU31 is *Methylotenera mobilis* strain JLW8 (95% similarity), which was isolated from terrestrial and freshwater environments and found to play an important role in carbon cycling (Lapidus *et al.* 2011).

2- Epsilonproteobacteria

OTU15 is closely related to *Arcobacter nitrofigilis* DSM 7299 (90% similarity) and this strain is known to be non- pathogenic and described as a Nitrogen-fixing bacterium, and was first isolated from a cordgrass root in Canada (Pati *et al.* 2010).

The closest relative of OTU36 is *Arcobacter halophilus* strain LA31B and was first known as an obligate halophilic (salt-loving) bacteria of the *Arcobacter* genus, isolated from a hypersaline lagoon in Hawaii and proposed as a novel species (Donachie *et al.* 2005).

The closest relative of OTU38 is *Arcobacter cryaerophilus* strain A 169/B (90% similarity) which was isolated first from animals by Neill Campbell *et al.* (1985) and proposed to be a novel pathogenic species. The *Arcobacter cryaerophilus* is known to be pathogenic for animals and also humans and this was confirmed by the isolation of this species from many causes of diarrhoea and septicaemia in humans (Pejchalova *et al.* 2006).

3- Gammaproteobacteria

The closest relative of OTU27 is *Legionella yabuuchiae* strain OA1-2, and this contains species that were proposed to be novel to the gene *Legionella* after the first isolation from soil samples polluted with industrial wastes in Japan (Kuroki *et al.* 2007). Approximately 21 species of the genus *Legionella* have been found to cause diseases in humans and are found in different contaminated environments, such as wet soils, rivers and water distribution systems (Kuroki *et al.* 2007). As yet, the species of *yabuuchiae* of the genus *Legionella* has not been confirmed to be causative agents of human disease (Ayres *et al.* 2010; Tai *et al.* 2012).

4- Bacteroidetes (Flavobacteria)

The closest relative of OTU8 is *Flavobacterium frigidimaris* strain KUC-1 16S (95% similarity) and was isolated from seawater in Antarctica and proposed to be a novel species by Nogi *et al.*

(2005). Authors have described this species as an aerobic psychrotolerant bacterium that is very able to catalyse organic materials in cold environments.

The closest relative of OTU18 is *Flavobacterium cucumis* strain R2A45-3 (96% similarity) which was isolated first from soil samples in Korea used in cultivating different vegetables, such as lettuce and cucumber (Weon *et al.* 2007). This species of *Flavobacterium cucumis* is described as a food-associated bacterium (Corry *et al.* 2011).

The closest relative of OUT24 is *Flavobacterium hydatis* strain ATCC 29551 (99% similarity) which was isolated from salmon in Michigan, USA (Gherna and Woese 1992) and is known to be a causative agent in fish diseases (Welker *et al.* 2005; Dworkin and Falkow 2006).

The closest relative of OTU33 is *Flavobacterium limicola* strain ST-82 (95% similarity) which was first isolated from cold freshwater sediments in Japan and described as a psychrotolerant bacterium. This species has found to have a high ability in decomposing organic matters (Tamaki *et al.* 2003).

5- Cyanobacteria

The other closest relative of OTU7 is *Cyanobium gracile* strain PCC 6307 (88% similarity). This strain is an unicellular picocyanobacterium which was first isolated from a freshwater lake in the US by Gerloff, Fitzgerald *et al.* (1950) and found to play an important role in nitrogen cycles (Shih *et al.* 2013).

The other closest relatives of OTUs (30, 35 and 39) is the *Geitlerinema* sp. PCC 7407 strain. This species was isolated first from freshwater environments but the source of this strain is unknown (Rippka *et al.* 1979). This type of filamentous Cyanobacteria is known to produce bioactive exometabolites, such as 4,4' dihydroxybiphenyl and harmane (Caicedo *et al.* 2012).

6- Actinobacteria

The closest relative of OTU21 is *Cryobacterium psychrophilum* strain DSM 4854 (95% similarity). This species was isolated first from soil in Antarctic by Inoue and Komagata in 1976 and described as a psychrophilic bacterium (Suzuki *et al.* 1997).

In summary, members of bacterial phyla in the River Wensum are involved in nitrogen cycles (OTUs 1,6, 7, 13, 15,19 and 37) (Hahn 2006), the cycling of metals (Lisle *et al.* 2004) as shown in some members that are involved in Fe-reduction (OTUs 2 and 22), sulfur-reduction (OTU17), phosphorus removal (OTU5), sulfur-reduction (OTU17), carbon cycles (OTU31) and degradation of pollutants including dissolved organic matter (OTUs 4, 8, 16, 28, 33 and 40) and cellulose fibres (OTUs 32 and 9). The removal of some chemical compounds is apparent in some members (OTUs 11, 34, 26) indicating their role in bioremediation. Some members of

Cyanobacteria (OTUs 30, 35 and 39) are involved in producing bioactive exometabolites. Campylobacteraceae has one human pathogenic member (OTU38). Also, one species of the genus *Flavobacterium* is a causative agent in fish diseases (OTU24).

5.5 Discussion

Previous studies have shown that 454 pyrosequencing of the hypervariable region (V1-V2) of the 16S rRNA genes gives detailed information on the composition and abundance of bacterial communities (Edwards *et al.* 2006; Amend *et al.* 2010; Engelbrektson *et al.* 2010) and this approach has given a comprehensive picture in the study of the bacterial communities in the River Wensum in February 2012 (26 samples) and December 2012 (10 samples).

The results revealed that bacteria in the River Wensum are dominated by common freshwater groups: Proteobacteria, Bacteroidetes, Cyanobacteria and Actinobacteria. Comparable studies using high-throughput techniques to investigate the diversity of freshwater bacteria are rare. Jordaan and Bezuidenhout (2013) carried out a similar study in the River Vaal, South Africa and also found that Cyanobacteria, proteobacteria (Beta, Alpha and gamma), Bacteroidetes and Actinobacteria were the dominant bacterial groups found in river water, with other phyla such as Acidobacteria and Firmicutes found in low percentages. In a freshwater lake, Moller *et al.* (2013) found that Bacteroidetes, Actinobacteria and Verrucomicrobia were the dominant bacterial groups, whereas Proteobacteria and Cyanobacteria were less important. In the River Mississippi, Staley *et al.* (2013) found that approximately 93% of (Illumina) reads belonged to Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria and Verrucomicrobia. Using FISH combined with DAPI staining, Klammer *et al.* (2002) found that 60% DAPI stained cells in the Tarun and Traunsee Rivers in Austria were Betaproteobacteria, Alphaproteobacteria and Cytophaga-Flavobacterium, with Betaproteobacteria the most abundant of these groups in both lake and river water. Similar techniques were utilized by Kenzaka *et al.* (2001) to study bacterial community composition in two urban rivers in Malaysia and Thailand that are contaminated with untreated sewage. Kenzaka *et al.* (2001) found Betaproteobacteria and Gammaproteobacteria were dominant in both rivers, making up 5% to 39% and 4% to 41% of the total, respectively.

5.5.1 Proteobacteria

Proteobacteria are known to be of group of Gram-negative bacteria comprising a vast majority of organisms belong to 6 classes Alpha-Beta-Gamma-Delta-Epsilon and Zeta- Proteobacteria (Newton *et al.* 2011). The role of this phylum in freshwater can vary among these classes (Matcher *et al.* 2011). The phylum of Proteobacteria in the River Wensum was the most abundant with 21 of the commonest OTUs. This phylum was dominated by the class of Betaproteobacteria (OTUs 2, 4, 5, 6, 11, 13, 16, 17, 28, 31, 34, 37 and 40), followed by

Epsilonproteobacteria (OTUs 15, 36 and 38) Gammaproteobacteria (OTUs 27 and 32), Alphaproteobacteria (OUT26) and Deltaproteobacteria (OTU29).

Betaproteobacteria were found to be the most abundant class in the River Wensum. They are also known to be abundant in many different freshwater environments, representing sometimes about 70% of the total abundance (Newton *et al.* 2011). But this class is also found to be in low abundance in oceans compared with the class of Alphaproteobacteria. Betaproteobacteria can be co-cultured with *Cryptomonas sp*. (Algae) and can be associated with different particles and Cyanobacteria as well. Members of this class in freshwaters are fast growing and nutrient loving (Newton *et al.* 2011). Species belonging to this class are known to play an important role in the cycling of nitrogen. They oxidize ammonium (ammonium-oxidizing bacteria) to nitrate first and then support plant life with the fixed nitrogen (Newton and Madison 2008).

This study found that the order of Burkholderiales was the commonest order belonging to Betaproteobacteria and has six families, with the most abundant being the family of Comamonadaceae. Members of this family are aerobic and most of them have flagella to facilitate their movements (Garrity *et al.* 2005). This family is known to be found in high abundance in rivers (Crump *et al.* 2009; Bai *et al.* 2013). The other five families belong to the order Burkholderiales were Oxalobacteraceae, Burkholderiaceae, Gallionellaceae, Methylophilaceae and Rhodocyclaceae. The family Oxalobacteraceae has members that can strictly live in either aerobic or anaerobic conditions and most of them are able to fix nitrogen (Garrity *et al.* 2005). The family Burkholderiaceae is known to have some pathogenic members (Garrity *et al.* 2005). The family Gallionellaceae is known as iron bacteria (Emerson *et al.* 2013). The family Methylophilaceae contains members ubiquitous in natural environments, such as freshwaters and saline waters, indicating their important role in such environments. Members are fast growing and can tolerate a high levels of C_1 substrates (Vorobev *et al.* 2013). The family Rhodocyclaceae has members which are known as aerobic denitrifying bacteria and some of them play an important role in the field of bioremediation (Garrity *et al.* 2005).

Epsilonproteobacteria was the second most abundant class of the phylum proteobacteria and all their closest relatives belonged to the order Campylobacterales and the family Campylobacteraceae. Epsilonproteobacteria are distributed in marine, terrestrial and freshwater environments, performing important biogeochemical roles in their own environments and they are abundant in sulphuric environments. However, there is little information about their distribution as the class has few cultured representatives. However, the family Campylobacteraceae has been well studied due to the pathogenicity of most members (Rossmassaler *et al.* 2012). In this study, all three OTUs had relatives closest to the genus of *Arcobacter*. The genus *Arcobacter* was proposed by Vandamme *et al.* (1991) for species previously assigned to the genus *Campylobacter*. This genus includes pathogenic species like *Arcobacter cryaerophilus* and non-pathogenic species like *Arcobacter nitrofigilis*. Some species of *Arcobacter* are found in sea water, while others are associated with animals (Pati *et al.* 2010). Some *Arcobacter* play an important role in treating waters through reducing nitrate (Essahale *et al.* 2010).

This study also revealed that two of the commonest OTUs (27 and 32) belonged to the class Gammaproteobacteria. This class contains the most studied of all bacterial groups (Newton and Madison 2008) including the enteric bacteria, such as *Escherichia coli* and most numbers of pathogens belong to this class, such as *Vibrio*, *Salmonella* and *Pseudomonas*. (Williams *et al.* 2010). In contrast to Betaproteobacteria, the highest abundance of the class of Gammaproteobacteria is normally found in saline waters, such as sea waters and saline lakes rather than fresh water environments. Pathogenic members of this class can be associated with human and animal wastes (Newton and Madison 2008). At the lower taxonomic level, the results presented here found that the closest relatives belonged to two families: Legionellaceae and Pseudomonadaceae.

One common OTU (26) was found to belong to the class of Alphaproteobacteria. Members of this class are able to establish symbiotic relationships with root nodules of plants, and so can facilitate atmospheric nitrogen fixation by plants. In return, the plants can provide the bacteria with nutrients. Also, other members of this class are responsible for many zoonotic diseases. The class of Alphaproteobacteria are known to be more abundant in sea water than in freshwater environments (Newton and Madison 2008).

This study also found that one common OTU (29) belonged to the class of Deltaproteobacteria. Members of this class are aerobic or anaerobic and many of them play a major role in the cycling of elements, such as sulphur and iron (Garrity *et al.* 2005).

5.5.2 Bacteroidetes

This study revealed that the second dominant bacterial phylum in the River Wensum was Bacteroidetes with 11 common OTUs (3, 8, 9, 10, 12, 14, 18, 19, 23, 24 and 33). This phylum contains Gram-negative bacteria, commonly known as Cytophaga-Flavobacterium Bacteroidetes. Members of this phylum are obligatory aerobic or anaerobic and can be found in different environments, such as soils, freshwaters and marine waters. Members of this phylum are also known to be abundant in the faeces of animals and humans. Large percentages of particle-attached bacteria are comprised of Bacteroidetes which play an important role in degrading biopolymers (cellulose) (Newton and Madison 2008). Bacteroidetes is considered to be the second most abundant phylum after proteobacteria in aquatic environments and members of this phylum are known to be the major consumer of high molecular weight DOM (O'Sullivan *et al.* 2006). Bacteroidetes in freshwater environments are found in high abundance following the decline of Cyanobacteria blooms and members like *Flavobacterium* of this phylum are normally the dominant genus, indicating the importance of nutrients availability to these members (Eiler and Bertilsson 2004; Eiler and Bertilsson 2007). This research found that the most common OTUs belonged to the class Flavobacteria, the order Flavobacteriales and the family Flavobacteriaceae, and the genus *Flavobacterium* was found to be dominant. *Flavobacterium* comprises about fifty species that have been isolated from different environments, such as soil freshwaters, and marine waters. The highest abundance of these

species is found in cold fresh and marine waters, exhibiting a high ability to uptake and degrade organic matter (Miyashita *et al.* 2010). Most species belonging to this genus are also psychrotolerant or psychrophilic and they can be mesophilic or halophilic. Some species, however, are considered opportunistic pathogens and have been confirmed to be causative agents of fish disease, such as *Flavobacterium columnare* and *Flavobacterium hydatis* (Dworkin and Falkow 2006).

Two of the other common OTUs (9 and 14) belong to the families Sphingobacteriaceae and Cytophagaceae, respectively. The family Sphingobacteriaceae is known to have a high ability to degrade nutrients (Vishnivetskaya *et al.* 2011).

5.5.3 Cyanobacteria

This study found that Cyanobacteria are the third most dominant bacterial phylum in the River Wensum (OTUs 1, 7, 30, 35 and 39). Cyanobacteria are gram–negative bacteria distributed in many environmental habitats, such as fresh and marine waters and terrestrial habitats (Chlipala *et al.* 2011). All members of Cyanobacteria (filamentous and unicellular) are photosynthetic releasing oxygen to the atmosphere. Carbon dioxide can be fixed by all of the bacteria but only some strains are known as N_2-fixation bacteria (Min and Sherman 2010). Taxonomically, Cyanobacteria were mistakenly classed as belonging to algae because they contain photopigments (Chlorophyll a and Carotene β-carotene) that are necessary for photosynthesis. However, Cyanobacteria are the same as bacteria in terms of their cellular and organismal contents. The common name of Cyanobacteria is blue-green algae and this is because of some pigments that are produced by most members, giving cells a bluish colour which becomes apparent at high growth concentrations (Pope and Patel 2008; Whitton 2012). Nowadays, Cyanobacteria represent a new and important source of some pharmaceutical compounds and also many chemical metabolites. However, many toxins, such as hepatotoxins and neurotoxins can be produced by some members during their blooms. The presence of these toxins in drinking water resources is harmful to human health and the environment (Chlipala *et al.* 2011).

This research found that all the common members of Cyanobacteria present belonged to the order Oscillatoriales (OTUs 30, 35 and 39) and the order Chroococcales (OTUs 1 and 7). Ferrari *et al.* (2011) found that Cyanobacteria in the Uruguay River were mainly represented by members of the order of Chroococcales and Oscillatoriales.

5.5.4 Actinobacteria

Actinobacteria was the last dominant phylum in the River Wensum and had three common OTUs (20, 21 and 25). The phylum Actinobacteria are known as gram-positive bacteria with high Guanine and Cytosine (G+C) contents. Actinobacteria are one of the major and diverse phyla in the bacterial domain and compromise more than 35 families. Actinobacteria are able

to produce extracellular enzymes and secondary metabolites and this is due to their physiological diversity and metabolic capability. They are also considered to be a major source of antibiotic production. Actinobacteria are also known to form spores and can be found in different environments, such as terrestrial, fresh and marine water environments. Actinobacteria play a major role in decomposing and recycling organic matter (Ventura *et al.* 2007; Newton and Madison 2008). In the River Wensum, three common OTUs of the order of Actinomycetales were found represented by the families Sanguibacteraceae (OTU20), Microbacteriaceae (OTU21) and Intrasporangiaceae (OTU25).

5.5.5 The abundance and distribution of the commonest OTUs

The abundance of the 40 commonest OTUs in the River Wensum accounted for approximately 42% of the total abundance in the river water. This percentage is lower than that obtained by ARISA where the 40 commonest DNA fragment sizes accounted for about 55% of the total bacterial abundance, indicating that ARISA gave higher abundance but lower diversity than 454 pyrosequencing. Zhu *et al.* (2013) investigated bacterial community composition in sea sediments in China using pyrosequencing. In shallow sea sediments, 62 abundant OTUs made up 22% of sequences, while 62 common OTUs in deep sea sediments made up about 57% of total sequences. In seawater samples, Chow *et al.* (2013) found that the five most abundant OTUs from different depths (0 to 5 m) made up about 52% of the total bacterial sequences. Hence, the samples from the River Wensum are less diverse than shallow water sediments (Zhu *et al.* 2013) , but show similar diversity to deep sea sediments (Zhu *et al.* 2013) and also seawater samples (Chow *et al.* 2013).

The abundance of the 40 commonest OTUs increases as water moves downstream in the River Wensum and the highest abundance of all these commonest OTUs was found at sites SC and S18. The downstream site S18 is at the outflow of the study catchment, a 4th order stream located in an urban area. This location receives organic matter and bacteria from upstream sites and as runoff from urban areas and sewage treatment works. Site SC also shows a high abundance of all the commonest OTUs especially Cyanobacteria, despite being an upstream site. These organisms are growing at a site with presumably preferable environmental conditions. Increased Cyanobacteria at site SC may due to discharges from septic sewage (Ahmed *et al.* 2005) or an adjacent lake which is connected to the stream and can release these bacteria into it. In arctic tundra, Crump *et al.* (2007) found that the bacterial community composition in swage runoff was very similar to connected lakes, and was attributed to dispersal processes. Also Nelson *et al.* (2009) found matching results for different lakes and streams in California, USA.

The commonest bacteria at upstream sites were Proteobacteria (Beta, Delta and Gamma) and Bacteroidetes (Sphingobacteria and Flavobacteria). On the other hand, Cyanobacteria, Actinobacteria, Bacteroidetes (Cytophagia and Flavobacteria) and Alphaproteobacteria were the commonest bacteria at the downstream sites.

Sekiguchi *et al.* (2002) determined the succession of bacterial community structure along the River Changjiang in China. Upstream sites were dominated by Betaproteobacteria and Bacteroidetes. These decreased as water moved to downstream sites of the river with bacterial communities becoming more dominated by gram-positive bacteria, such as Actinobacteria. Sekiguchi *et al.* (2002) attributed the succession to changes of nutrients, water temperature, river flow and pH. In different streams in Spain, Simek *et al.* (2001) found that Betaproteobacteria and Cytophaga/Flavobacterium were the most common bacteria at upstream sites and were largely allochthonous in origin.

This study showed that the most common organisms at upstream sites are soil bacteria (OTUs 2, 29, 32 and 9), indicating the importance of allochthonous bacteria. These comprise freshwater bacteria (OTUs 12 and 23) and one sewage bacteria (OTU4). These organisms play different roles in these environments. For example, Betaproteobacteria (OTU2) have a role in Fe (III)-reduction (Finneran *et al.* 2003), Betaproteobacteria (OTU4) in the degradation of various pollutants and dissolved organic matter (Hiraishi *et al.* 1997), Bacteroidetes (OTU9) in the degradation of nutrients (Weon *et al.* 2009) and Betaproteobacteria (OTU17) in the reduction of organic sulfur (Bruland *et al.* 2009). Bacteroidetes (OTU23) catalyse organic matter in cold environments (Nogi *et al.* 2005), Deltaproteobacteria (OUT29) prey on the other gram-negative bacteria (Pineiro *et al.* 2008) and Gammaproteobacteria (OTU32) degrade cellulosic plant fibres (Lednicka *et al.* 2000).

The majority of the commonest bacteria in the downstream section of the River Wensum are freshwater bacteria (OTUs 1, 3, 10, 19 and 26). Two of the OTUs (10 and 20) are soil bacteria. These organisms play an important role in these environments. For example, Cyanobacteria (OTU1) in N_2-fixation (photosynthetic bacteria) (Welsh *et al.* 2008), Bacteroidetes (OTU19) in reducing nitrate (Weeks 1954) and Alphaproteobacteria (OTU26) in being phototrophic, photoheterotrophic and chemoheterotrophic (Arunasri *et al.* 2008).

In December 2012, during high river flow events (mean; 9.6 m^3/s at Costessey Mill) and rainfall (82 mm), the commonest bacteria found were Betaproteobacteria (OUT 2, 17, 34 and 40) followed by Alphaproteobacteria (OTU26). Chen *et al.* (2013) assessed the effects of dry and wet seasons on bacterial structure in different streams along the River Chongqing in China and found that bacterial diversity and abundance were greater in the wet season compared to the dry season with Betaproteobacteria more common in the wet season and Actinobacteria in the dry season. Changes in bacterial communities show most for Betaproteobacteria in the wet season (high rainfall events), which are not only attributed to environmental parameters, such as water temperature, but also to terrestrial source areas and tributaries that discharge into stream waters.

This study found that high rainfall and high flow events during December 2012 discharged many of the commonest OTUs from terrestrial source area and groundwater into upstream sites of the River Wensum (SA, SB, SC, SD, SE, SF and S20), increasing these commonest bacteria. At upstream sites on the Mississippi River, abundance and diversity of most common members of the dominant bacterial groups were also positively related to rainfall (Staley *et al.* 2013) as

a result of rainfall carrying bacteria from terrestrial source areas into streams, and so affecting the relative abundance of these bacterial communities rather than their presence or absence.

In the research carried out in this study, 454 pyrosequencing and ARISA (Chapter 4) were found to be adequate in assessing the bacterial community composition present of the River Wensum. Unlike ARISA, 454 pyrosequencing makes it possible to identify actual microorganisms. However, 454 pyrosequencing is relatively expensive and prohibits processing high numbers of samples.

For the future, Illumina sequencing, which can give potentially 10^9 500 bp reads offers the benefits of both 454 pyrosequencing and ARISA. It is possible to have barcodes for both the forward and reverse primers. Hence, with 96 of each, it is possible to multiplex $96 \times 96 = 9216$ samples (Harbers and Kahl 2012; Liu *et al.* 2012; Wong *et al.* 2013). Such that, for a very large study, the costs are competitive with ARISA.

5.6 Summary

In the research presented in this chapter, 454 pyrosequencing offered an insight into the bacterial community composition of the River Wensum. The dominant bacterial groups in the river water were found to be common in various freshwater environments and were Proteobacteria, Bacteroidetes, Cyanobacteria and Actinobacteria. The taxonomic affinities of these common bacteria provide important information about their relatives, the first environments from which they were isolated and also their environmental roles. For example, most members in the River Wensum belong to the commonest bacterial OTUs that are involved in the cycling of nitrogen and metals. The abundance of the majority of the commonest bacteria were found to increase as water moves downstream (3^{rd}-4^{th} stream order), with the highest abundance recorded at sites S18 and SC. The most common bacteria at upstream sites were Proteobacteria (Beta, Gamma and Delta) and Bacteroidetes (Sphingobacteria and Flavobacteria). The majority of Proteobacteria and Bacteroidetes at upstream sites are considered to be soil bacteria and these decreased in abundance as water moved downstream. The commonest bacteria at downstream sites in the River Wensum are Cyanobacteria that are involved in N_2-fixation, Bacteroidetes (Cytophagia and Flavobacteria) and Alphaproteobacteria. Most of these bacteria are considered to be freshwater bacteria and increased in abundance at downstream sites. The common bacteria in December 2012 were Betaproteobacteria and Alphaproteobacteria. These bacteria are soil bacteria that can be flushed from terrestrial source areas into streams after flood events. The relative abundance of the commonest OTUs changed in December 2012 compared to February 2012, suggesting the role of high rainfall and flow rate at 10 selected sites in shifting the relative abundance of bacterial communities between these two periods.

Chapter Six

Conclusions and future work recommendations

6.1 Conclusions

Determining bacterial community composition and dynamics is a fundamental task in microbial ecology because of their rapid response to natural and anthropogenic pressures, and the role that they play in nutrient and carbon cycles (Kirchman *et al.* 2003; Daims and Wagner 2007). Most studies have been focused on marine and soil environments with much less effort on freshwater systems, despite the widespread occurrence and importance to humans of these systems worldwide (Debroas *et al.* 2009). Most studies assessing bacteria in freshwater environments have been focused on indicator bacteria, but composition and dynamics of bacterial communities in these environments have had less attention (Sigua *et al.* 2010). The effects of spatial and temporal variations, abiotic and biotic factors on bacterial community composition, structure and dynamics are still poorly understood (Lawrence *et al.* 2004; Lindstrom *et al.* 2005). Molecular techniques based on DNA have revolutionized our understanding of bacterial community composition and abundance in natural environments (Muyzer *et al.* 1993). In addition, the use of metagenomic approaches and other applications of high throughput sequencing methods to investigate bacterial communities in freshwaters is still rare (Debroas *et al.* 2009).

This study set out to investigate bacterial community composition and abundance in the River Wensum from June 2011 to February 2013. It also aimed to determine the effects of spatial and temporal variation and environmental factors on bacterial community composition and abundance. The River Wensum has been subject to inputs of high amounts of nitrogen and phosphorus as a result of intensive agriculture practices (upstream) and also discharges from sewage treatment works (downstream), causing problems for the river ecology and particularly in altering bacterial community composition and abundance. The research presented in this thesis presents one of only few studies of the bacterial community composition and abundance in a lowland arable catchment. It is also one of very few studies to carry out a detailed investigation of the temporal characterisation of bacterial communities in a river system. In addition, the microbiological techniques used here were applied for the first time to samples

from the river Wensum. The main conclusions from the research presented in this thesis are detailed as follows.

Total bacterial numbers were assessed from June 2011 to February 2013 using standard methods, epifluorescence microscopy and DAPI staining. It is concluded that total bacterial numbers (Chapter 3) ranged from 0.21×10^6 cells/mL to 5.34×10^6 cells/mL (mean = 1.1×10^6 cells/mL). Total bacterial numbers varied both spatially and temporally with greater differences between times than sites. Bacterial numbers increased as water moves downstream with the highest numbers recorded in 4th order streams. Temporally, the highest total bacterial numbers were recorded in June and August 2011 (summer), while the lowest numbers were recorded in December 2012 and February 2013 (winter). The variations of total bacterial numbers showed some relationship with environmental parameters including water temperature, TP, TC, TN, stream order, river flow and the numbers of adjacent sewage treatment works. Approximately 52% of the differences in total bacterial abundance were related to these parameters.

In February 2013, total heterotrophic bacteria were determined using the traditional heterotrophic bacterial count method and then the proportions of total bacterial numbers were calculated. Heterotrophic plate counts (Chapter 3) showed significant variations between sites, but did not show significant relationships to any environmental parameters. However, the highest heterotrophic plate counts were of the downstream sites S14 and S8. These two sites are downstream of the three sewage treatment works, most likely indicating the role of treated sewage in increasing heterotrophic bacterial numbers, especially those responsible for the degradation of ammonium (ammonium-oxidizing bacteria) as shown in the 454 pyrosequencing data in Chapter 5. In addition, percentages of total bacteria that are culturable ranged from 0.48% to 7%, and were negatively related to total bacterial numbers.

Shifts of bacterial community composition were assessed using the automated ribosomal intergenic analysis (ARISA) technique from June 2011 to December 2012. ARISA fingerprints (Chapter 4) showed significant spatial and temporal shifts in the composition and abundance of the bacterial community. Bacterial diversity is highest at upstream sites, while it decreases as water moves downstream. On the other hand, bacterial abundance increases as water moves downstream. However, site SC, which is impacted by septic waste discharges, is upstream and does not fit this pattern. It is more like the downstream sites and presented a high abundance of a commonly identified OTU (702.09). Multidimentional scaling (MDS) displays differences of bacterial community composition between sites and times. There is a large shift between upstream sites of the sub-catchment areas (SA, SB and SE) and downstream sites (S8 and S18) of the river Wensum and this was attributed to catchment characteristics. Upstream sites are small streams located in intensive agricultural areas, and because they are the primary link between terrestrial areas and aquatic environments, these locations receive bacteria and nutrient runoff, especially during high rainfall and flooding events. Downstream sites, on the other hand, are large streams located near urban areas and are influenced by a number of sewage treatment works (STWs). The discharge of bacteria and nutrients from both urban areas and STWs affect bacterial communities in river water.

Temporally, there is a large shift of bacterial composition in the same months of different years, particularly between December 2011 and December 2012. The mean water temperature was the same in both, but water flow was very different (mean= 2.30 m^3/s and 9.60 m^3/s respectively). Comparing September 2011 and September 2012 (mean water temperatures were 14.2 ˚C and 11 ˚C and mean water flow was 1.88 m^3/s and 2.32 m^3/s, respectively). Hierarchical partitioning analysis showed that water flow and temperature were the strongest factors affected the temporal variations of bacterial community composition in the River Wensum. However, bacterial community composition was very similar between summer 2011 and summer 2012, and between autumn 2011 (October and November) and autumn 2012 (October and November). Temperature and river flow therefore play an important role on the temporal variation of bacterial composition.

To investigate the significant effects of spatial and temporal variations and environmental parameters on the abundance of common bacterial OTUs in the river, the abundance of the commonest 20 OTUs were analysed individually and showed significant differences between sites and months. Common OTUs made up 40.4% of the total community. The abundance of the majority of these OTUs showed variations, with greater abundance between sites than between months in the downstream sites of the river, especially at sites S8 and S18. This result indicates that these OTUs grow and multiply in the river and that changes in their abundance are based on the fluctuations of physical and chemical parameters of the river water. On the other hand, a few OTUs exhibited greater and often increased abundance during high rainfall and flood events, suggesting that these represent bacteria of terrestrial origin that are flushed into the lower streams.

The variations in abundance of the commonest OTUs were related to a number of environmental parameters, including stream order, temperature, TN, TP, TC, TSS, pH, arable land, improved grassland, other grassland, urban area, flow rate, STWs and rainfall. These parameters accounted for different proportions of the variations of the commonest OTUs, with highest values explained for the first five commonest OTUs (16% to 45%). Changes in bacterial diversity were related to fewer environmental parameters, including TC, TN, temperature and stream order. These parameters explained about 18% of the variation of bacterial diversity.

The structure of bacterial communities in the river Wensum water was determined using 454 pyrosequencing for February (26 samples) and December 2012 (10 samples). 454 pyrosequencing of 16S rRNA (Chapter 5) showed that bacterial communities in the river Wensum contained phyla that are found to be common in the other freshwater environments worldwide (Jordaan and Bezuidenhout 2013; Moller *et al.* 2013; Staley *et al.* 2013). The dominant bacterial phyla were Proteobacteria (the classes of Beta, Epsilon, Gamma, Delta and Alpha in decreasing order of importance), Bacteroidetes (the classes of Flavobacteria, Cytophagia and Sphingobacteria in decreasing order of importance), Cyanobacteria and Actinobacteria. Principal component analysis showed that the 40 commonest OTUs belonging to these dominant phyla made up 42% of individuals.

The commonest bacteria at upstream sites were Proteobacteria (OTUs 2 and 4), Deltaproteobacteria (OTU29), Gammaproteobacteria (OTU32), Sphingobacteria (OUT9) and

Flavobacteria (OTUs 12 and 23). Most of them (OTUs 2, 9, 17, 29 and 32) are soil bacteria, suggesting that these bacteria are terrestrial in origin and are flushed into the lower order streams. Most of them showed positive relationships with TN and TC and the presence of arable areas. On the other hand, the commonest bacteria at downstream sites were Cyanobacteria (OTU1), Flavobacteria (OTUs 3, 10 and 19), Cytophagia (OTU14), Actinobacteria (OTUs 20, 21 and 25) and Alphaproteobacteria (OTU26). Most of these bacteria are freshwater bacteria. These bacteria become more common as water moves downstream, but there is one site (SC) which is upstream that was an exception to this pattern. Bacteria are actively growing in the river, diluting other taxa and reducing the diversity as water moves downstream. Most of the bacterial OTUs showed a positive relationship with TP and the presence of urban areas. The highest abundance of all the commonest OTUs was recorded at site S18 (4^{th} order stream) and site SC (upstream site).

Commonest bacteria in December 2012 were Betaproteobacteria (OTUs 2, 17, 34 and 40) and Alphaproteobacteria (OTU26). These are soil bacteria and showed greater abundance during this time of high rainfall and flood events, suggesting that these represent bacteria of terrestrial origin that are flushed into the lower stream order.

The majority of the 40 commonest bacterial OTUs belonging to the dominant phyla were found to be responsible for recycling of nitrogen (OTUs 1, 6, 7, 13, 15, 19 and 37), confirming that the river Wensum is exposed to high concentration of nutrients, such as nitrogen as a result of agriculture practices and discharges from sewage treatment works. Some of the bacterial OTUs are involved in Fe-reduction (OTUs 2 and 22), sulphur-reduction (OTU17), phosphorus removal (OTU5), carbon cycling (OTU31), removal of some chemical compounds (OTUs 11, 34, 26), degradation of dissolved organic matter (OTUs 4, 8, 16, 28, 33 and 40), and degradation of cellulose fibres (OTUs 32 and 9). Three of the bacterial OTUs are able to produce bioactive exometabolites (OTUs 30, 35 and 39). Results also revealed two pathogenic members, one belonging to Epsilonproteobacteria which can cause diarrhoea and septicaemia in humans (OTU38), and the other belonging to *Flavobacterium* which is known to be a causative agent to fish diseases (OTU24).

The most common 454 OTUs and ARISA OTUs increase in abundance as water moves downstream (into 3^{rd} and 4^{th} order streams). A few bacterial OTUs are more common in 1^{st} order streams, apparently because they are terrestrial in origin, although site SC is different in respect of this trend given the influence of local septic waste discharges.

The 20 commonest ARISA OTUs make up about 40.4% of the total, while the 40 commonest 454 OTUs make up about 42% of all individuals, indicating that ARISA gives higher abundance but less diversity than 454 OTUs. So, either the same ARISA OTU can represent more than one 454 OTU or ARISA is overloading many OTUs because their abundance is below the limit of fluorescence (weight. off).

6.1.1 Comment on the techniques used in this research

Many studies have shown epifluorescence microscopy and DAPI staining to be reliable, successful and suitable direct methods for enumerating bacterial cells in freshwater (Hobbie *et al.* 1977; Porter and Feig 1980; Clarke and Joint 1986; Gasol *et al.* 1999; Garren and Azam 2010; Yamaguchi *et al.* 2011). Standard methods of determining heterotrophic plate counts using R2A medium and spread plates (Reasoner and Geldreich 1985) are efficient in recovering large numbers of bacteria from the targeted environments.

ARISA has been shown to provide reliable, robust and reproducible results of bacterial communities in the targeted environments (Fisher and Triplett 1999; Brown and Fuhrman 2005; Yannarell and Triplett 2005). It is considered to be a powerful technique that can be utilized to determine spatial and temporal shifts of bacterial community composition (Jones *et al.* 2007). Large samples can be processed by the ARISA tool and estimates of relative abundance of bacterial groups can be provided by it (Crump *et al.* 2003; Bending *et al.* 2007).

ITSF/ITSReub primer pair has been shown to be reproducible and can give a high number of peaks and wide spacer sizes (Cardinale *et al.* 2004).

Studies of bacterial communities have shown 454 pyrosequencing to be powerful tool that can give rapid characterization, better representation and large sequence depth of the bacterial community composition. It can be applied directly to environmental samples without the need for cloning (Edwards *et al.* 2006; Binladen *et al.* 2007; Bowers *et al.* 2009; Matcher *et al.* 2011). According to Engelbrektson (2010), amplicons of the hypervariable region (V1-V2) of the gene 16S rRNA was found to give higher estimates of the richness of bacterial species. In addition, because this study used error correcting barcodes, the assignment of sequences to samples was easy and successful (Parameswaran *et al.* 2007; Hamady *et al.* 2008). Also, according to Amend (2010), 454 pyrosequencing reads expressed the relative abundance of each taxon and can be used to compare between bacterial communities present in samples.

6.2 Future work recommendations

DNA for 454 pyrosequencing was selected from the same DNA that was used for ARISA in February and December 2012. These DNA were selected because of the presence of the most common DNA fragment sizes for these months in the ARISA results. It was found that using ARISA is a powerful tool to estimate the spatial and temporal variations of bacterial composition in river water. Then from these results and checking the times of the presence of the most common bacteria, DNA from these times can be selected to discover bacterial communities using 454 pyrosequencing. This is because 454 pyrosequencing is expensive and needs more effort than ARISA and could not be applied to the whole study period. Alternatively, Illumina sequencing offers the benefit of both 454 and ARISA. Illumina

sequencing is now giving long enough reads in sufficiently large numbers of samples, and at a competitive price.

Because of the presence of two common pathogenic bacteria *Arcobacter cryaerophilus*, which is responsible for diarrhoea and septicaemia in humans (the highest abundance of 0.46% was at site SC), and *Flavobacterium hydatis* which is a causative agent to fish diseases (the highest abundance of 0.28% was at site S20), more investigation in river water, especially at sites S20 and SC, is required.

The high frequency of the Betaproteobacteria class suggests that it is an important component of the bacterial community in the River Wensum. It is recommended that the use of specific molecular probes to study Betaproteobacteria in more detail across the river is required due to their widespread occurrence and responsibility for many biogeochemical processes, such as nitrogen cycles. The Bacteroidetes phylum was dominated by the genus Flavobacterium in the river water, so further investigation of this genus and its specific role in the river water is also required. .

Much research has been focused on total coliform bacteria as an indicator of water contamination. However, work focusing on the whole bacterial community as an indicator of water contamination is scarce. The data presented in this thesis revealed common OTUs across different tributaries of the River Wensum that are significantly correlated with different environmental parameters. For example, most common OTUs at upstream sites were correlated positively with TC and TN, and some common OTUs at downstream sites were correlated positively with TP. Although, results presented in this thesis do not show strong relationships with ecological status, there is a possibility in the future to use the whole bacterial community as an indicator of the changes of river health rather than just focusing on traditional indicators, once powerful alternative molecular techniques and sufficient physiochemical and biological date are provided at spatial and temporal scales.

Lear *et al.* (2009) used ARISA to assess the bacterial community in four streams exposed to human impacts, and found that the bacterial community was able to discriminate the most impacted streams from others. However, Lear *et al.* (2009) recommended the use of alternative techniques, such as high-throughput sequencing and statistical tools to improve sensitivity of the analyses of the whole bacterial community. This approach allows the use of bacterial community as a reliable ecological indicator of freshwater health.

The research presented in this thesis is for a lowland catchment and revealed that the dominant bacterial phyla were Proteobacteria, Bacteroidetes, Cyanobacteria and Actinobacteria. Crump and Hobbie (2005) found the same frequency of bacterial phyla in two lowland river catchments. However, Winter *et al.* (2007) found that the Danube river was highly dominated by Cytophaga-Flavobacterium-Bacteroidetes, followed by the less frequent Actinobacteria and Cyanobacteria. This study is one of few studies to provide detailed information about bacterial communities in a lowland river water catchment. Further investigations of the bacterial community composition especially at sites showing common bacterial species between the

upstream (agricultural activities) and downstream (urban areas) in this lowland river are required, and temporal factors should not be neglected.

Other types of chemical parameters such as DOC (Sabater *et al.* 1993) and chlorophyll *a* (Siam and Ghobrial 2000) and also biological parameters, such as grazing by viruses and flagellates were not measured in this research and may have potential effects on the differences of total bacterial numbers (Gasol *et al.* 1999; Riemann *et al.* 2000). Similarly, there are other environmental parameters beyond this research that may also play an important role in shifting the composition of bacterial communities, for example, the effects of biological factors, such as viruses and flagellates (Riemann *et al.* 2000; Simek *et al.* 2001; Hewson and Fuhrman 2007), and chemical factors, such as chlorophyll *a* (Winter *et al.* 2007). In addition, heavy metals may also influence bacterial community composition, for example, chromium and lead (Vilchez *et al.* 2011), cadmium, nickel and zinc (Fechner *et al.* 2011) and arsenite (Quemeneur *et al.* 2010). It is recommended that these parameters are included in future investigations.

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APPENDIX to Chapter Three

Table A 3.1 Mean, standard error, minimum and maximum of water temperature in degrees Celsius in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	9.8 ± .041	2.0 – 15.5
S5	10.1 ± .038	4.0 – 15.0
S6	9.9 ± .038	3.5 – 14.6
S13	10.2 ± .035	3.0 – 17.0
S20	10.7 ± .038	4.5 – 15.0
S1	10.1 ± .038	1.5- 17.0
S2	11.6 ± .035	4.5 – 17.5
S3	10.2 ± .038	3.5 – 15.0
S15	10.5 ± .038	3.5 – 18.0
S7	9.6 ± .038	4.0 – 14.5
S14	9.7 ± .038	2.5 – 16.5
S8	11.0 ± .035	4.0 – 19.5
S10	9.2 ± .038	2.0 – 15.5
S11	9.4 ± .038	3.0 – 15.0
S12	9.3 ± .035	3.0 – 15.0
S21	9.3 ± .038	2.5 – 15.0
S9	10.6 ± .038	4.5 – 16.0
S16	9.4 ± .038	2.5 – 16.0
S17	9.6 ± .038	3.0 – 17.0
S18	10.6 ± .038	4.0 – 19.5
SA	9.6 ± .038	3.0 – 15.0
SB	9.9 ± .038	4.5 – 15.0
SE	10.7 ± .038	3.0 – 15.0
SC	10.5 ± .038	5.0 – 15.5
SD	10.3 ± .038	4.0 – 15.5
SF	10.1 ± .038	3.0 – 15.0

Table A 3.2 Mean, standard error, minimum and maximum of water temperature degrees Celsius in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	16.6 ± .081	14.5 – 19.0
	Jul y	15.7 ± .051	15.0 - 19.5
	August	15.3 ± .037	13.5 - 17.5
	September	14.2 ± .032	13.0 - 15.5
	October	10.8 ± .032	10.0 – 12.0
	November	7.9 ± .031	6.5 - 9.5
	Dec ember	5.7 ± .031	5.0 – 6.0
2012	January	3.3 ± .031	1.5 – 5.0
	February	7.1 ± .031	6.0 – 8.0
	March	7.1 ± .031	5.0 – 11.0
	April	10.0 ± .031	8.0 – 12.0
	May	10.9 ± .031	9.5 – 13.0
	Jun e	14.3 ± .031	12.0 - 19.5
	July	15.1 ± .031	12.5 – 18.0
	August	15.4 ± .031	13.5 - 19.5
	September	11.6 ± .031	10.0 – 14.0
	October	10.3 ± .031	9.5 – 11.0
	November	7.0 ± .031	5.0 – 10.0
	Dec ember	5.7 ± .031	4.5 – 7.0
2013	February	4.9 ± .031	3.0 – 7.0

Table A 3.3 Mean, standard error, minimum and maximum of pH in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± Standard error	Minimum and maximum
S4	8.19 ± .005	7.99 - 8.37
S5	8.11 ± .004	7.97 - 8.33
S6	8.02 ± .004	7.75 - 8.34
S13	8.14 ± .004	7.67 - 8.45
S20	8.09 ± .004	7.81 - 8.44
S1	8.23 ± .004	8.01 - 8.46
S2	8.19 ± .004	8.03 - 8.40
S3	8.20 ± .004	8.06 - 8.41
S15	8.21 ± .004	7.76 - 8.43
S7	8.14 ± .004	7.86 - 8.46
S14	8.25 ± .004	7.75 - 8.49
S8	8.21 ± .004	7.92 - 8.47
S10	8.18 ± .004	7.72 - 8.29
S11	8.13 ± .004	7.66 - 8.46
S12	8.12 ± .004	7.67 - 8.43
S21	8.26 ± .004	7.83 - 8.46
S9	8.17 ± .004	7.76 - 8.29
S16	8.17 ± .004	7.69 - 8.46
S17	8.30 ± .004	7.82 - 8.49
S18	8.23 ± .004	7.82 - 8.48
SA	8.02 ± .004	7.59 - 8.27
SB	7.97 ± .004	7.45 - 8.29
SE	7.95 ± .004	7.43 - 8.31
SC	7.91 ± .004	7.37 - 8.22
SD	7.96 ± .004	7.30 - 8.21
SF	7.96 ± .004	7.45 - 8.28

Table A 3.4 Mean, standard error, minimum and maximum of pH in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	8.31 ± .009	8.23 - 8.45
	July	8.15 ± .006	7.84 - 8.34
	August	8.17 ± .004	8.06 - 8.39
	September	7.99 ± .004	7.59 - 8.14
	October	8.14 ± .004	7.94 - 8.33
	November	8.06 ± .004	7.88 - 8.25
	December	8.09 ± .004	7.86 - 8.30
2012	January	8.11 ± .004	7.88 - 8.35
	February	8.19 ± .004	7.96 - 8.49
	March	8.24 ± .004	8.09 - 8.46
	April	7.82 ± .004	7.37 - 8.27
	May	8.10 ± .004	7.30 - 8.40
	June	8.16 ± .004	7.95 - 8.46
	July	8.08 ± .004	7.92 - 8.18
	August	8.11 ± .004	7.92 - 8.30
	September	8.19 ± .004	8.00 - 8.36
	October	8.04 ± .004	7.86 - 8.23
	November	8.16 ± .004	7.95 - 8.36
	December	8.08 ± .004	7.79 - 8.27
2013	February	8.37 ± .004	8.21 - 8.49

Table A 3.5 Mean, standard error, minimum and maximum of total nitrogen (TN) in mg/L in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	11.5 ± .079	1.5 - 20.8
S5	10.6 ± .072	9.4 - 14.3
S6	9.1 ± .072	7.1 - 11.9
S13	9.4 ± .068	7.8 - 12.4
S20	9.7 ± .072	7.9 - 12.3
S1	7.1 ± .072	0.6 - 14.4
S2	10.3 ±.068	7.1 – 12.0
S3	5.8 ± .072	4.5 - 9.8
S15	7.7 ± .072	6.1 - 10.9
S7	5.9 ± .072	4.8 - 8.9
S14	7.0 ± .072	4.3 - 11.6
S8	7.2 ± .068	5.6 – 10.0
S10	3.5 ± .072	2.5 - 7.5
S11	5.4 ± .072	4.3 - 7.8
S12	5.3 ± .068	4.4 – 7.0
S21	3.8 ± .072	2.0 - 6.5
S9	5.2 ± .072	3.9 - 7.8
S16	6.0 ± .072	4.5 - 8.8
S17	6.1 ± .072	5.1 - 8.4
S18	6.1 ± .072	3.8 - 8.7
SA	6.3 ± .072	2.7 - 13.4
SB	8.7 ± .072	7.3 - 10.8
SE	6.4 ± .072	3.9 - 11
SC	6.1 ± .072	5.1 - 7.4
SD	7.2 ± .072	5.5 - 9.5
SF	5.8 ± .072	4.4 - 10.5

Table A 3.6 Mean, standard error, minimum and maximum of total nitrogen (TN) in mg/L in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	6.7 ± .009	5.6 - 10.3
	July	5. 5 ± .006	2.8 - 10.5
	August	6.2 ± .004	0.8 - 11.5
	September	5.7 ± .004	0.6 - 11.6
	October	6.0 ± .004	0.8 - 10.7
	November	5.7 ± .004	1.5 - 10.9
	December	7.2 ± .004	3.7 – 19.0
2012	January	7.3 ± .004	3.1 - 14.5
	February	6.9 ± .004	3.4 – 12.0
	March	7.6 ± .004	3.8 - 13.1
	April	7.1 ± .004	3.5 - 12.7
	May	6.9 ± .004	3.2 –11.0
	June	6.7 ± .004	2.5 - 12.5
	July	6.5 ± .004	2.7 - 13.4
	August	6.4 ± .004	2.6 - 10.5
	September	7.1 ± .004	3.0 – 12.0
	October	7.0 ± .004	3.7 - 20.8
	November	7.9 ± .004	5.0 - 13.1
	December	9.8 ± .004	5.8 - 16.2
2013	February	9.1 ± .004	4.8 -14.1

Table A 3.7 Mean, standard error, minimum and maximum of total phosphorus (TP) in µg/L in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	82 ± .537	30 - 319
S5	57 ± .489	34 - 91
S6	132 ± .489	90 - 211
S13	66 ± .460	43 - 113
S20	75 ± .489	30 - 166
S1	160 ± .489	83 - 393
S2	79 ±.460	57 - 112
S3	75 ± .489	40 - 136
S15	81 ± .489	55 - 110
S7	93 ± .489	63 - 130
S14	197 ± .489	68 - 497
S8	112 ± .460	78 - 151
S10	56 ± .489	23 - 126
S11	297 ± .489	72 - 538
S12	242 ± .460	63 - 455
S21	86 ± .489	46 - 167
S9	44 ± .489	24 - 62
S16	58 ± .489	37 - 86
S17	159 ± .489	94 - 228
S18	108 ± .489	54 - 173
SA	101 ± .489	29 - 214
SB	96 ± .489	37 - 160
SE	69 ± .489	28 - 120
SC	151 ± .489	81 - 406
SD	80 ± .489	50 - 216
SF	76 ± .489	42 - 118

Table A 3.8 Mean, standard error, minimum and maximum of total phosphorus (TP) in µg/L in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	minimum and maximum
2011	June	141 ± 1.052	63 - 455
	July	92 ± .662	37 - 328
	August	87 ± .476	31 - 344
	September	111 ± .412	32 - 400
	October	105 ± .412	29 - 400
	November	104 ± .404	37 - 388
	December	98 ± .404	24 - 339
2012	January	112 ± .404	26 - 393
	February	95 ± .404	34 - 327
	March	92 ± .404	41 - 367
	April	81 ± .404	23 - 351
	May	78 ± .404	28 - 310
	June	102 ± .404	50 - 383
	July	120 ± .404	50 - 297
	August	143 ± .404	59 - 538
	September	120 ± .404	41 - 528
	October	126 ± .404	49 - 377
	November	91 ± .404	37 - 222
	December	101 ± .404	51 - 161
2013	February	71 ± .404	37 - 112

Table A 3.9 Mean, standard error, minimum and maximum of total carbon (TC) in mg/L in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	minimum and maximum
S4	73.83 ± .046	60.06 - 94.20
S5	71.28 ± .042	56.48 - 88.36
S6	63.99 ± .042	57.36 - 75.05
S13	67.56 ± .040	58.74 - 82.48
S20	71.87 ± .042	64.07 - 83.10
S1	77.94 ± .042	65.05 - 99.51
S2	65.64 ± .040	57.73 - 79.65
S3	70.06 ± .042	59.18 - 89.52
S15	67.58 ± .042	57.62 - 85.75
S7	72.53 ± .042	62.05 - 88.43
S14	71.55 ± .042	60.40 - 90.32
S8	68.87 ± .040	61.56 - 85.66
S10	77.92 ± .042	63.62 - 99.87
S11	72.71 ± .042	63.35 - 87.01
S12	74.95 ± .040	64.31 - 89.71
S21	75.63 ± .042	59.91 - 87.89
S9	71.65 ± .042	58.29 - 90.78
S16	65.58 ± .042	53.33 - 75.06
S17	76.20 ± .042	65.77 - 91.68
S18	67.73 ± .042	59.99 - 82.07
SA	77.10 ± .042	52.11 - 95.88
SB	70.01 ± .042	62.87 - 81.85
SE	74.75 ± .042	64.54 - 92.45
SC	71.19 ± .042	61.37 - 85.01
SD	61.00 ± .042	51.39 - 71.08
SF	71.86 ± .042	61.74 - 86.75

Table A 3.10 Mean, standard error, minimum and maximum of total carbon (TC) in mg/L in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	65.28 ± .090	57.73 - 72.15
	July	65.29 ± .057	54.50 - 71.60
	August	64.97 ± .041	56.22 - 78.16
	September	60.74 ± .035	51.39 - 66.20
	October	66.11 ± .035	58.05 - 77.36
	November	69.60 ± .035	57.13 - 81.23
	December	64.69 ± .035	58.29 - 78.99
2012	January	66.67 ± .035	57.30 - 77.01
	February	70.86 ± .035	61.78 - 84.77
	March	70.77 ± .035	63.50 - 78.11
	April	71.71 ± .035	56.50 - 82.18
	May	75.01 ± .035	57.10 - 84.01
	June	73.44 ± .035	63.62 - 83.98
	July	86.49 ± .035	70.21 - 99.87
	August	76.34 ± .035	62.73 - 88.60
	September	74.08 ± .035	63.23 - 86.97
	October	76.72 ± .035	69.35 - 84.60
	November	82.19 ± .035	69.53 - 95.80
	December	73.48 ± .035	63.31 - 80.98
2013	February	70.71 ± .035	54.88 - 78.69

Table A 3.11 Mean, standard error, minimum and maximum of total organic carbon (TOC) in mg/L in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	16.13 ± .066	6.30 - 37.50
S5	11.40 ± .060	1.95 - 22.60
S6	11.31 ± .060	3.95 - 20.91
S13	10.57 ± .056	2.02 - 20.58
S20	10.87 ± .060	4.99 - 21.90
S1	15.21 ± .060	8 .00 - 27.50
S2	11.94 ± .056	3.90 - 23.50
S3	12.82 ± .060	4.22 - 24.50
S15	12.89 ± .060	4.66 - 22.67
S7	13.02 ± .060	5.22 - 24.60
S14	14.15 ± .060	5.05 - 25.90
S8	12.68 ± .056	5.50 - 22.45
S10	14.50 ± .060	5.73 - 25.70
S11	14.95 ± .060	5.47 - 25.90
S12	14.14 ± .056	4.59 - 24.90
S21	12.61 ± .060	3.68 - 24.80
S9	11.42 ± .060	2.14 - 21.80
S16	13.09 ± .060	6.05 - 21.90
S17	13.67 ± .060	3.25 - 24.15
S18	12.72 ± .060	4.99 - 22.10
SA	12.65 ± .060	1.20 - 23.80
SB	12.67 ± .060	2.92 - 21.80
SE	13.36 ± .060	4.16 - 24.90
SC	15.16 ± .060	7.54 - 24.90
SD	15.85 ± .060	8.76 - 25.80
SF	14.37 ± .060	6.99 - 24.80

Table A 3.12 Mean, standard error, minimum and maximum of total organic carbon (TOC) in mg/L in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	7.01 ± .129	3.86 - 7.50
	July	12.81 ± .081	7.32 - 17.05
	August	9.82 ± .058	7.6 0 - 13.51
	September	7.18 ± .050	3.74 - 10.84
	October	6.74 ± .050	4.46 - 9.21
	November	7.99 ± .049	5.34 - 10.92
	December	10.01 ± .049	6.90 - 14.52
2012	January	4.60 ± .049	1.20 - 8.88
	February	11.20 ± .049	9.04 - 15.97
	March	14.31 ± .049	11.04 - 24.18
	April	17.28 ± .049	14.14 - 20.18
	May	20.74 ± .049	8.59 - 24.40
	June	9.55 ± .049	6.91 - 12.86
	July	20.27 ± .049	16.24 - 23.33
	August	11.75 ± .049	7.36 - 16.41
	September	12.95 ± .049	9.35 - 18.38
	October	22.75 ± .049	18.78 - 36.27
	November	23.55 ± .049	19.20 - 37.10
	December	23.98 ± .049	19.50 - 37.50
2013	February	23.98 ± .049	19.50 - 37.50

Table A 3.13 Mean, standard error, minimum and maximum of total suspended solids (TSS) in mg/L in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	4.27 ± .236	0.30 - 122.30
S5	2.67 ± .215	1.10 - 6.80
S6	1.87 ± .215	0.60 - 4.90
S13	1.80 ± .202	0.20 - 8.00
S20	2.15 ± .215	0.30 -7.80
S1	4.50 ± .215	0.30 - 41.70
S2	5.41 ± .202	1.72 - 15.50
S3	5.64 ± .215	1.58 - 26.90
S15	2.35 ± .215	0.80 - 7.80
S7	4.36 ± .215	1.10 - 20.10
S14	3.50 ± .215	1.18 - 11.10
S8	2.44 ± .202	1.10 – 11.00
S10	9.64 ± .215	1.72 - 49.80
S11	7.48 ± .215	3.26 - 15.66
S12	5.29 ± .202	2.5 - 9.20
S21	4.43 ± .215	1.54 - 17.20
S9	6.11 ± .215	3.20 - 13.90
S16	3.82 ± .215	1.74 - 13.40
S17	3.43 ± .215	1.00 - 20.10
S18	4.65 ± .215	1.20 - 24.20
SA	7.37 ± .215	1.10 – 37.00
SB	10.73 ± .215	1.90 - 50.36
SE	5.04 ± .215	0.70 - 39.56
SC	8.66 ± .215	4.30 - 23.90
SD	8.18 ± .215	3.90 - 38.60
SF	6.14 ± .215	1.42 - 14.20

Table A 3.14 Mean, standard error, minimum and maximum of total suspended solids (TSS) in mg/L in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	5.54 ± .463	1.66 - 7.88
	July	3.53 ± .291	1.14 - 9.14
	August	6.54 ± .209	1.12 - 16.04
	September	4.06 ± .181	0.60 - 39.56
	October	4.03 ± .181	1.48 - 50.36
	November	2.85 ± .178	0.74 - 12.94
	December	2.86 ± .178	0.86 - 10.44
2012	January	3.86 ± .178	0.66 - 17.06
	February	3.70 ± .178	0.30 - 34.80
	March	4.59 ± .178	1.00 - 38.60
	April	3.68 ± .178	0.80 - 14.90
	May	3.65 ± .178	1.20 – 22.0
	June	2.89 ± .178	0.3 - 8.70
	July	5.52 ± .178	0.8 - 26.90
	August	4.92 ± .178	0.2 - 24.90
	September	4.41 ± .178	0.9 - 19.90
	October	11.05 ± .178	1.5 - 122.30
	November	4.70 ± .178	0.9 - 37.00
	December	7.57 ± .178	4.1 - 17.00
2013	February	9.89 ± .178	3.1 - 24.20

Table A 3.15 Percentages of arable land in sub-catchment areas of the River Wensum. Data from Land Cover Map 2007 (LCM2007).

Individual site	Sub-catchment area (ha)	% of catchment in arable land
S4	1528.32	82.56
S5	1108.56	80.64
S6	5681.00	66.08
S13	2125.25	63.04
S20	1818.06	75.22
S1	1177.69	79.49
S2	712.94	74.14
S3	1811.94	65.07
S15	3282.19	57.16
S7	957.94	68.04
S14	1552.38	66.42
S8	5123.13	59.98
S10	10.12.69	73.44
S11	694.69	60.75
S12	527.38	52.72
S21	682.00	63.53
S9	1072.19	76.54
S16	1729.69	62.47
S17	4419.63	63.31
S18	2973.50	47.35
SA	496.75	92.38
SB	118.75	88.70
SE	17.44	41.09
SC	212.88	60.49
SD	491.81	74.28
SF	124.31	51.62

A 3.16 Percentages of improved grassland in sub-catchment area of the River Wensum. Data from Land Cover Map 2007 (LCM2007).

Individual site	Sub-catchment area (ha)	% of catchment in improved grassland
S4	176.88	9.55
S5	102.31	7.44
S6	1556.63	18.11
S13	772.50	22.92
S20	403.44	16.69
S1	178.00	12.01
S2	130.88	13.61
S3	558.13	20.04
S15	1147.56	19.98
S7	291.63	20.71
S14	475.19	20.33
S8	1724.25	20.40
S10	256.44	18.60
S11	218.63	19.12
S12	370.06	37.00
S21	163.31	15.21
S9	204.00	14.56
S16	445.63	16.10
S17	1334.25	19.11
S18	1564.69	24.92
SA	15.38	2.86
SB	2.19	1.63
SE	21.19	49.93
SC	65.94	18.74
SD	56.00	8.46
SF	71.13	29.61

A 3.17 Percentages of other grassland in sub-catchment area of the River Wensum. Data from Land Cover Map 2007 (LCM2007).

Individual site	Sub-catchment area (ha)	% of catchment in other grassland
S4	76.75	4.15
S5	20.81	1.51
S6	318.69	3.71
S13	31.25	0.93
S20	38.94	1.61
S1	52.81	3.56
S2	59.56	6.19
S3	252.44	9.07
S15	282.06	4.91
S7	80.25	5.70
S14	166.13	7.11
S8	552.81	6.47
S10	29.13	2.11
S11	66.13	5.78
S12	32.88	3.29
S21	84.94	7.91
S9	33.38	2.38
S16	157.50	5.69
S17	256.94	3.68
S18	300.13	4.78
SA	10.75	2.00
SB	7.81	5.84
SE	2.25	5.30
SC	0.63	0.18
SD	14.00	2.11
SF	8.44	3.50

A 3.18 Percentages of urban areas in sub-catchment areas of the River Wensum. Data from Land Cover Map 2007 (LCM2007).

Individual site	Sub-catchment area (ha)	% of catchment in urban area
S4	20.56	1.11
S5	9.94	0.72
S6	190.50	2.22
S13	195.94	5.81
S20	45.56	1.89
S1	21.94	1.48
S2	5.31	0.55
S3	13.31	0.48
S15	549.88	9.58
S7	11.44	0.81
S14	28.00	1.20
S8	265.63	3.11
S10	10.56	0.77
S11	76.00	6.65
S12	3.69	0.37
S21	13.44	1.25
S9	23.00	1.64
S16	40.94	1.48
S17	322.44	4.62
S18	307.25	4.89
SA	3.94	0.73
SB	1.25	0.93
SE	0.00	0.00
SC	1.13	0.32
SD	7.38	1.11
SF	2.56	1.06

Table A 3.19 Monthly mean rainfall for north Norfolk. Data obtained from Wensum Alliance. Location of gauging station NGR TG 108 257.

Year	Month	(mm)
2011	June	55.8
	July	47.6
	August	63.6
	September	25.6
	October	22.0
	November	23.8
	December	62.4
2012	January	39.0
	February	13.2
	March	52.2
	April	125.2
	May	42.4
	June	95.2
	July	66.8
	August	80.4
	September	48.8
	October	83.8
	November	78.4
	December	82.0
2013	February	41.4

Table A 3.20 Monthly mean flow rate for the River Wensum at Costessey Mill gauging station. Data obtained from the Environment Agency. Location of the gauging station NGR TG 176 127.

Year	Month	(m^3/s)
2011	June	1.82
	July	1.76
	August	1.85
	September	1.88
	October	1.65
	November	1.94
	December	2.30
2012	January	2.74
	February	2.48
	March	3.74
	April	4.48
	May	3.80
	June	2.90
	July	2.98
	August	2.53
	September	2.32
	October	3.60
	November	6.47
	December	9.60
2013	February	7.07

Table A 3.21 Numbers of sewage treatment works (STWs) and stream order for sub-catchment areas draining to individual sampling sites in the River Wensum.

Individual sites	Number of sewage treatment works	Stream order at sampling site
S4	1	2
S5	1	3
S6	2	2
S13	1	3
S20	0	2
S1	2	2
S2	0	3
S3	2	3
S15	3	4
S7	1	3
S14	3	3
S8	3	4
S10	0	3
S11	1	3
S12	1	4
S21	1	1
S9	0	3
S16	0	3
S17	3	3
S18	2	4
SA	0	1
SB	0	1
SE	0	2
SC	0	2
SD	0	2
SF	0	3

A 3.22 F-statistic values and p-values of all physical and chemical parameters and total bacterial abundance from June 2011 to February 2013.

Variable	Sites and dates	*F*-Statistic	*P*-value
Temp.	Individual sites	4.57	p< 0.001
	Months	403.36	p< 0.001
pH	Individual sites	19.62	p< 0.001
	Months	27.44	p< 0.001
Total nitrogen TN	Individual sites	28.07	p< 0.001
	Months	9.98	p< 0.001
Total phosphorus TP	Individual sites	28.96	p< 0.001
	Months	4.48	p< 0.001
Total carbon TC	Individual sites	39.29	p< 0.001
	Months	101.71	p< 0.001
Total organic carbon TOC	Individual sites	12.21	p< 0.001
	Months	292.39	p< 0.001
Total suspended solid TSS	Individual sites	6.36	p< 0.001
	Months	6.28	p< 0.001
Total bacterial abundance	Individual sites	11.174	p< 0.001
	Months	39.930	p< 0.001

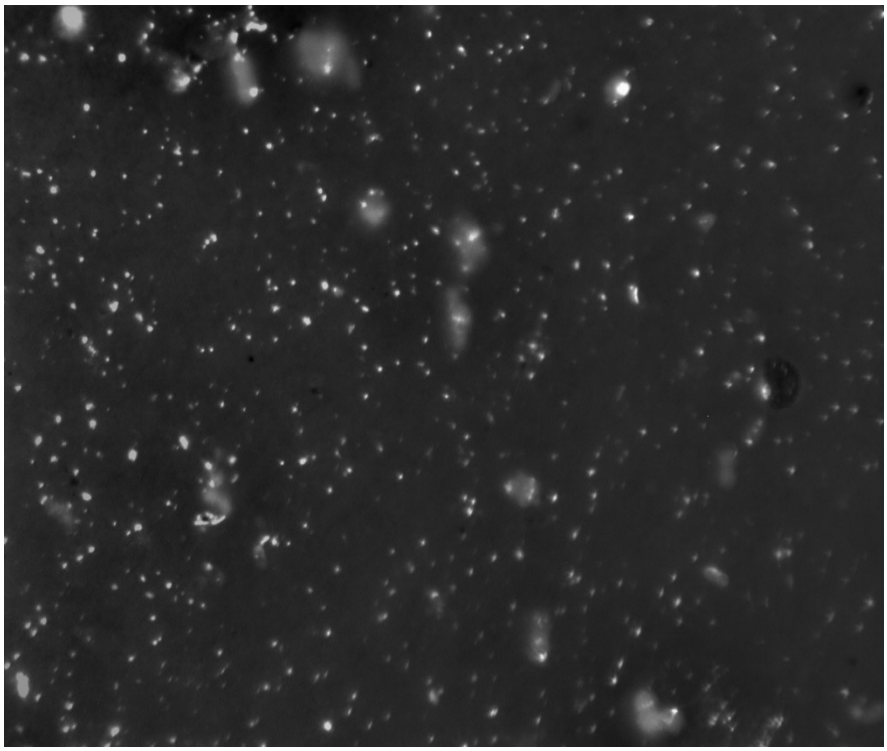

Figure A 3.23 Photograph of bacterial cells in sample 8 in the River Wensum from June 2011, using epifluorescence microscopy.

Table A 3.24 National Grid Reference (NGR), latitude and longitude, sampling date and times for all sites in the River Wensum from June 2011 to February 2012.

Site	NGR	Latitude	Longitude	Year 2011							Year 2012	
				June	July	August	September	October	November	December	January	February
				Date and time	Date and time	Date and time	Date and time	Date and time	Date and time	Date and time	Date and time	Date and time
S1	TF 93270 12707	52° 40.631'	00° 51.551'	-	-	16/08 13.20	20/09 1.10	24/10 14.05	22/11 13.25	9/12 12.30	16/01 12.19	22/02 12.02
S2	TF 96668 16215	52° 42.448'	00° 54.690'	15/06 15.20	11/07 14.20	16/08 13.40	20/09 1.30	24/10 14.25	22/11 13.45	9/12 13.50	16/01 12.38	22/02 12.20
S3	TF 98293 19110	52° 43.972'	00° 56.236'	-	-	16/08 14.00	20/09 1.47	24/10 14.45	22/11 14.05	9/12 14.00	16/01 12.54	22/02 12.35
S4	TF 89845 23780	52° 46.670'	00° 48.900'	Dry	Dry	Dry	Dry	Dry	22/11 13.00	9/12 13.05	16/01 11.47	22/02 11.40
S5	TF 87251 26799	52° 48.351'	00° 46.698'	-		16/08 12.35	20/09 12.20	24/10 13.05	22/11 12.30	9/12 12.50	16/01 11.08	22/02 11.20
S6	TF 86699 27970	52° 48.993'	00° 46.248'	-	-	16/08 12.20	20/09 12.13	24/10 12.5	22/11 12.15	9/12 12.45	16/01 11.21	22/02 11.13
S7	TF 96132 29217	52° 49.465'	00° 54.682'	-	11/07 13.00	16/08 11.35	20/09 11.15	24/10 11.45	22/11 11.40	9/12 12.55	16/01 10.38	22/02 10.42
S8	TG 02032 18450	52° 43.533'	00° 59.530'	15/06 16.30	11/07 15.20	16/08 14.30	20/09 2.20	24/10 15.15	22/11 14.35	9/12 15.25	16/01 13.24	22/02 12.55
S9	TG 03525 16928	52° 42.680'	01° 00.798'	-	-	16/08 14.45	20/09 2.45	24/10 15.45	22/11 14.50	9/12 14.30	16/01 13.43	22/02 13.05
S10	TG 09000 21665	52° 45.107'	01° 05.835'	-	-	16/08 10.10	20/09 10.25	24/10 10.45	22/11 10.50	9/12 11.40	16/01 9.48	22/02 10.07
S11	TG 09502 21274	52° 44.884'	01° 06.266'	-	-	16/08 10.25	20/09 10.15	24/10 10.35	22/11 10.40	9/12 9.48	16/01 10.02	22/02 9.58
S12	TG 10745 18781	52° 43.512'	01° 07.272'	15/06 10.40	11/07 10.50	16/08 10.55	20/09 4.55	24/10 10.15	22/11 10.25	9/12 9.37	16/01 9.20	22/02 9.45
S13	TF 91896 29315	52° 49.609'	00° 50.917'	15/06 13.05	11/07 12.25	16/08 11.50	20/09 11.13	24/10 12.05	22/11 11.55	9/12 12.30	16/01 10.55	22/02 10.55
S14	TG 01653 24531	52° 46.818'	00° 59.417'	-	-	16/08 11.15	20/09 11.00	24/10 11.30	22/11 11.25	9/12 11.55	16/01 10.23	22/02 10.32
S15	TF 99845 20065	52° 44.452'	00° 57.648'	-	11/07 15.00	16/08 14.20	20/09 2.07	24/10 15.00	22/11 14.20	9/12 14.20	16/01 13.09	22/02 12.43
S16	TG 12681 18593	52° 43.366'	01° 08.983'	-	11/07 11.00	16/08 9.50	20/09 9.45	24/10 10.05	22/11 10.10	9/12 9.30	16/01 9.05	22/02 9.40
S17	TG 18477 11188	52° 39.239'	01° 13.829'	-	-	16/08 9.00	20/09 9.05	24/10 9.12	22/11 9.20	9/12 9.03	16/01 14.30	22/02 9.15
S18	TG 17661 12729	52° 40.089'	01° 13.167'	-	11/07 9.25	16/08 9.20	20/09 9.13	24/10 9.35	22/11 9.45	9/12 9.12	16/01 14.40	22/02 9.20
S20	TF 87881 24059	52° 46.862'	00° 47.164'	15/06 14.10	-	16/08 12.50	20/09 1.35	24/10 13.20	22/11 12.45	9/12 13.00	16/01 11.37	22/02 11.30
S21	TG 08277 20230	52° 44.350'	01° 05.138'	-	-	16/08 10.40	20/09 10.55	24/10 11.05	22/11 11.05	9/12 11.42	16/01 9.35	22/02 10.16
SA	TG 11072 25682	52° 47.222'	01° 07.829'	16/07 9.45	12/07 9.30	-	21/09 13.15	25/10 9.15	23/11 9.40	9/12 10.01	17/01 13.00	21/02 9.50
SB	TG 11090 25691	52° 47.226'	01° 07.846'		12/07 9.35	-	21/09 13.25	25/10 9.17	23/11 9.44	9/12 10.01	17/01 13.05	21/02 9.55
SC	TG 12270 26381	52° 47.570'	01° 08.921'	16/07 10.35	12/07 10.00	-	21/09 14.00	25/10 9.55	23/11 11.30	9/12 11.45	17/01 10.40	21/02 10.35
SD	TG 12740 25397	52° 47.029'	01° 09.300'		12/07 10.10	-	21/09 14.25	25/10 10.05	23/11 12.30	9/12 10.30	17/01 10.25	21/02 10.45
SE	TG 11647 25689	52° 47.213'	01° 08.340'	16/07 9.57	12/07 9.40	-	21/09 14.45	25/10 9.35	23/11 10.32	9/12 10.17	17/01 12.45	21/02 10.15
SF	TG 12520 24604	52° 46.607'	01° 09.073'	16/07 11.05	12/07 11.05	-	21/09 14.55	25/10 10.4	23/11 13.10	9/12 10.52	17/01 9.38	21/02 11.30

Table A 3.25 National Grid Reference (NGR), latitude and longitude, sampling date and times for all sites in the River Wensum from March 2012 to February 2013.

Site	NGR	Latitude	Longitude	Year 2012										Year 2013
				March	April	May	June	July	August	September	October	November	December	February
				Date and time	Date and time	Date and time	Date and time	Date and time	Date and time	Date and time	Date and time	Date and time	Date and time	Date and time
S1	TF 93270 12707	52° 40.631'	00° 51.551'	19/03 13.08	23/04 12.20	17/05 12.10	20/06 11.55	18/07 11.38	22/08 11.55	19/09 10.52	17/10 11.40	20/11 11.55	18/12 13.08	18/12 12.00
S2	TF 96668 16215	52° 42.448'	00° 54.690'	19/03 13.26	23/04 12.30	17/05 12.15	20/06 12.15	18/07 12.11	22/08 12.13	19/09 11.08	17/10 12.13	20/11 10.12	18/12 13.22	18/12 12.10
S3	TF 98293 19110	52° 43.972'	00° 56.236'	19/03 13.41	23/04 12.40	17/05 12.30	20/06 12.25	18/07 12.25	22/08 12.30	19/09 11.25	17/10 12.30	20/11 12.25	18/12 13.35	18/12 12.40
S4	TF 89845 23780	52° 46.670'	00° 48.900'	19/03 12.41	23/04 11.45	17/05 11.40	20/06 11.25	18/07 11.11	22/08 11.30	19/09 10.25	17/10 11.30	20/11 11.30	18/12 12.40	18/12 11.30
S5	TF 87251 26799	52° 48.351'	00° 46.698'	19/03 12.13	23/04 11.15	17/05 11.15	20/06 11.05	18/07 10.49	22/08 10.56	19/09 10.02	17/10 11.05	20/11 11.10	18/12 12.20	18/12 11.10
S6	TF 86699 27970	52° 48.993'	00° 46.248'	19/03 11.58	23/04 11.00	17/05 11.05	20/06 10.55	18/07 10.39	22/08 10.50	19/09 9.25	17/10 10.55	20/11 11.00	18/12 12.05	18/12 11.00
S7	TF 96132 29217	52° 49.465'	00° 54.682'	19/03 11.22	23/04 10.30	17/05 10.40	20/06 10.20	18/07 10.09	22/08 9.55	19/09 9.20	17/10 10.23	20/11 10.35	18/12 11.35	18/12 11.00
S8	TG 02032 18450	52° 43.533'	00° 59.530'	19/03 14.19	23/04 13.05	17/05 12.50	20/06 13.00	18/07 12.57	22/08 12.58	19/09 11.52	17/10 12.56	20/11 12.55	18/12 14.05	18/12 12.35
S9	TG 03525 16928	52° 42.680'	01° 00.798'	19/03 14.40	23/04 13.50	17/05 13.00	20/06 13.10	18/07 13.10	22/08 1.10	19/09 12.10	17/10 1.10	20/11 1.05	18/12 14.17	18/12 13.00
S10	TG 09000 21665	52° 45.107'	01° 05.835'	19/03 10.38	23/04 9.40	17/05 10.05	20/06 9.30	18/07 9.04	22/08 9.20	19/09 8.40	17/10 9.40	20/11 10.00	18/12 11.00	18/12 10.10
S11	TG 09502 21274	52° 44.884'	01° 06.266'	19/03 10.27	23/04 9.50	17/05 10.00	20/06 8.50	18/07 9.28	22/08 9.10	19/09 8.50	17/10 9.15	20/11 9.50	18/12 10.50	18/12 9.48
S12	TG 10745 18781	52° 43.512'	01° 07.272'	19/03 10.05	23/04 9.25	17/05 9.45	20/06 8.30	18/07 8.55	22/08 8.55	19/09 8.20	17/10 9.00	20/11 9.40	18/12 10.35	18/12 9.35
S13	TF 91896 29315	52° 49.609'	00° 50.917'	19/03 11.4	23/04 10.50	17/05 10.50	20/06 10.35	18/07 10.22	22/08 10.25	19/09 9.32	17/10 10.40	20/11 10.50	18/12 11.50	18/12 10.55
S14	TG 01653 24531	52° 46.818'	00° 59.417'	19/03 11.07	23/04 10.15	17/05 10.30	20/06 10.05	18/07 9.57	22/08 10.10	19/09 9.08	17/10 10.09	20/11 10.25	18/12 11.23	18/12 10.32
S15	TF 99845 20065	52° 44.452'	00° 57.648'	19/03 14.07	23/04 12.50	17/05 12.40	20/06 12.50	18/07 12.47	22/08 12.45	19/09 11.4	17/10 12.45	20/11 12.40	18/12 13.52	18/12 12.30
S16	TG 12681 18593	52° 43.366'	01° 08.983'	19/03 9.55	23/04 9.15	17/05 9.40	20/06 8.20	18/07 8.46	22/08 8.47	19/09 8.10	17/10 10.55	20/11 9.30	18/12 10.25	18/12 9.44
S17	TG 18477 11188	52° 39.239'	01° 13.829'	19/03 9.10	23/04 8.35	17/05 9.30	20/06 14.10	18/07 8.10	22/08 8.09	19/09 12.52	17/10 8.10	20/11 1.55	18/12 9.35	18/12 9.10
S18	TG 17661 12729	52° 40.089'	01° 13.167'	19/03 9.27	23/04 8.50	17/05 9.10	20/06 13.45	18/07 8.18	22/08 8.24	19/09 12.35	17/10 8.25	20/11 1.40	18/12 9.45	18/12 9.15
S20	TF 87881 24059	52° 46.862'	00° 47.164'	19/03 12.25	23/04 11.30	17/05 11.30	20/06 11.20	18/07 11.02	22/08 9.31	19/09 10.20	17/10 11.20	20/11 11.15	18/12 12.30	18/12 11.35
S21	TG 08277 20230	52° 44.350'	01° 05.138'	19/03 10.5	23/04 10.00	15/05 10.10	19/06 9.45	18/07 9.38	22/08 11.13	19/09 8.30	17/10 9.54	20/11 10.10	18/12 11.07	18/12 10.20
SA	TG 11072 25682	52° 47.222'	01° 07.829'	19/03 9.50	24/04 9.10	15/05 9.12	19/06 9.12	16/07 8.30	21/08 8.35	18/09 8.29	16/10 8.40	19/11 10.35	17/12 9.05	18/12 9.50
SB	TG 11090 25691	52° 47.226'	01° 07.846'	19/03 9.55	24/04 9.15	15/05 9.18	19/06 9.15	16/07 8.35	21/08 8.40	18/09 8.40	16/10 8.45	19/11 10.30	17/12 10.15	18/12 9.55
SC	TG 12270 26381	52° 47.570'	01° 08.921'	19/03 11.15	24/04 9.40	15/05 9.50	19/06 9.50	16/07 10.35	21/08 9.25	18/09 9.27	16/10 9.40	19/11 11.00	17/12 10.55	18/12 10.30
SD	TG 12740 25397	52° 47.029'	01° 09.300'	19/03 12.30	24/04 9.55	15/05 10.00	19/06 10.00	16/07 9.35	21/08 9.35	18/09 9.50	16/10 9.50	19/11 11.15	17/12 11.10	18/12 10.43
SE	TG 11647 25689	52° 47.213'	01° 08.340'	19/03 10.15	24/04 9.30	15/05 8.51	19/06 8.51	16/07 9.05	21/08 9.10	18/09 9.11	16/10 9.25	19/11 9.35	17/12 10.35	18/12 10.10
SF	TG 12520 24604	52° 46.607'	01° 09.073'	19/03 1.12	24/04 10.50	15/05 10.40	19/06 10.40	16/07 10.15	21/08 10.05	18/09 10.2	16/10 10.20	19/11 11.45	17/12 11.33	18/12 11.45

APPENDIX to Chapter Four

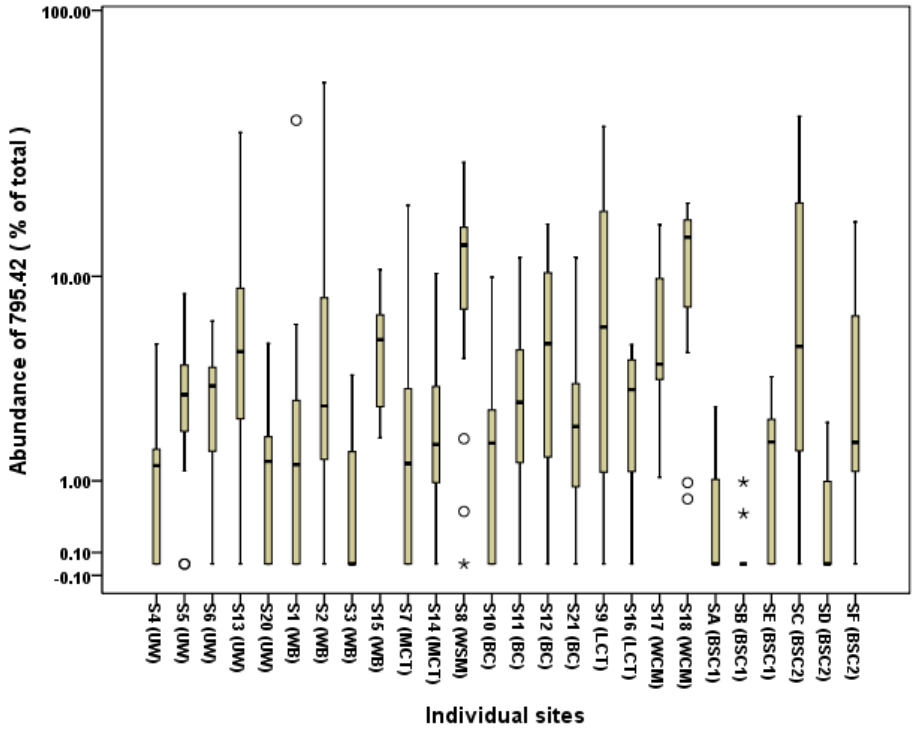

Figure A 4.1 Spatial variation of the common OTU (795.42) by sites from June 2011 to December 2012.

Table A 4.1 Mean, standard error, minimum and maximum of the abundance (%) of the common OUT (795.42) in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	0.96 ± .295	0.00 - 5.26
S5	3.46 ± .258	0.00 - 8.51
S6	3.11 ± .258	0.00 - 6.58
S13	5.60 ± .241	0.00 - 35.47
S20	1.31 ± .258	0.00 - 5.29
S1	1.96 ± .258	0.00 - 39.41
S2	6.23 ± .250	0.00 - 54.34
S3	0.65 ± .258	0.00 - 3.83
S15	5.88 ± .258	1.86 - 10.66
S7	1.65 ± .258	0. 00 - 18.88
S14	2.31 ± .258	0.00 - 10.27
S8	10.14 ± .241	0.00 - 27.43
S10	1.87 ± .258	0.00 - 9.91
S11	3.21 ± .258	0.00 - 11.88
S12	4.88 ± .235	0.00 – 16.00
S21	2.41 ± .266	0.00 - 11.89
S9	7.98 ± .266	0.00 - 37.38
S16	2.9 ± .258	0.00 - 5.22
S17	6.64 ± .258	1.06 - 15.91
S18	11.98 ± .258	0.72 - 19.25
SA	0.45 ± .244	0.00 - 2.70
SB	0.06 ± .259	0.00 - 0.98
SE	1.14 ± .251	0.00 - 3.76
SC	8.62 ± .251	0.00 - 40.78
SD	0.26 ± .251	0.00 - 2.25
SF	3.32 ± .267	0.00 - 16.32

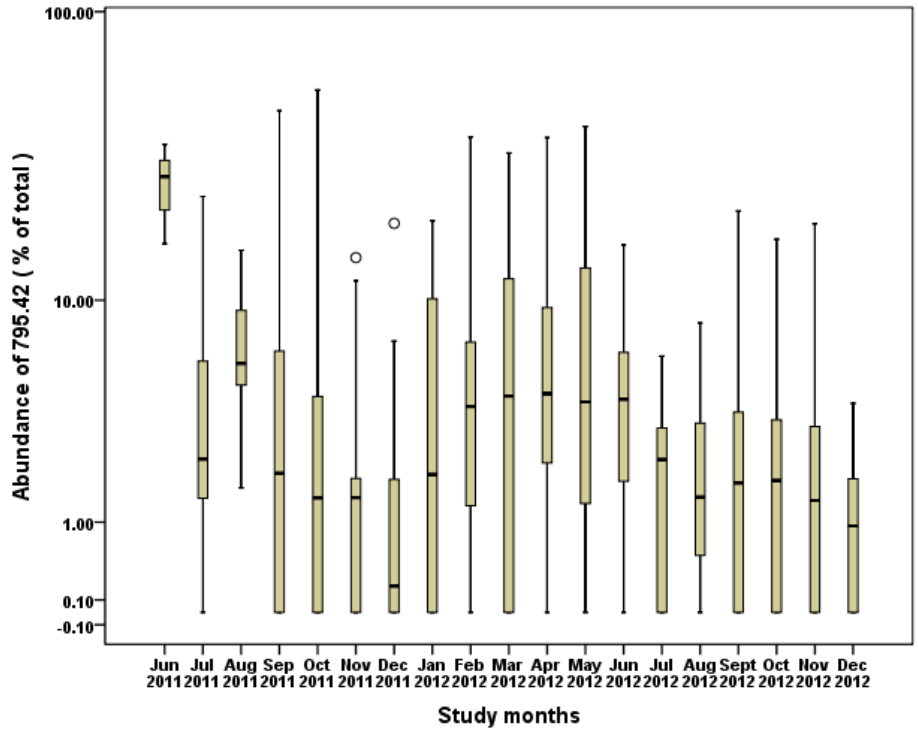

Figure A 4.2 Temporal variation of the common OTU (795.42) by months from June 2011 to December 2012.

Table A 4.2 Mean, standard error, minimum and maximum of the abundance (%) of the common OUT (795.42) in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	18.02 ± .622	16.0 - 35.47
	July	4.69 ± .272	1.60 - 23.43
	August	5.48 ± .243	0.00 - 15.15
	September	2.58 ± .215	0.00 - 46.17
	October	2.51 ± .196	0.00 - 54.34
	November	1.10 ± .206	0.00 - 14.27
	December	0.69 ± .206	0.00 - 18.87
2012	January	2.65 ± .206	0.00 - 19.28
	February	4.69 ± .206	0.00 - 37.56
	March	4.41 ± .206	0.00 - 33.11
	April	5.57 ± .206	0.00 - 37.38
	May	5.35 ± .206	0.00 - 40.78
	June	3.55 ± .211	0.00 - 15.85
	July	1.52 ± .206	0.00 - 6.16
	August	1.47 ± .206	0.00 - 8.25
	September	2.00 ± .206	0.00 - 20.84
	October	1.92 ± .225	0.00 - 16.58
	November	1.61 ± .215	0.00 - 18.82
	December	0.62 ± .206	0.00 - 3.98

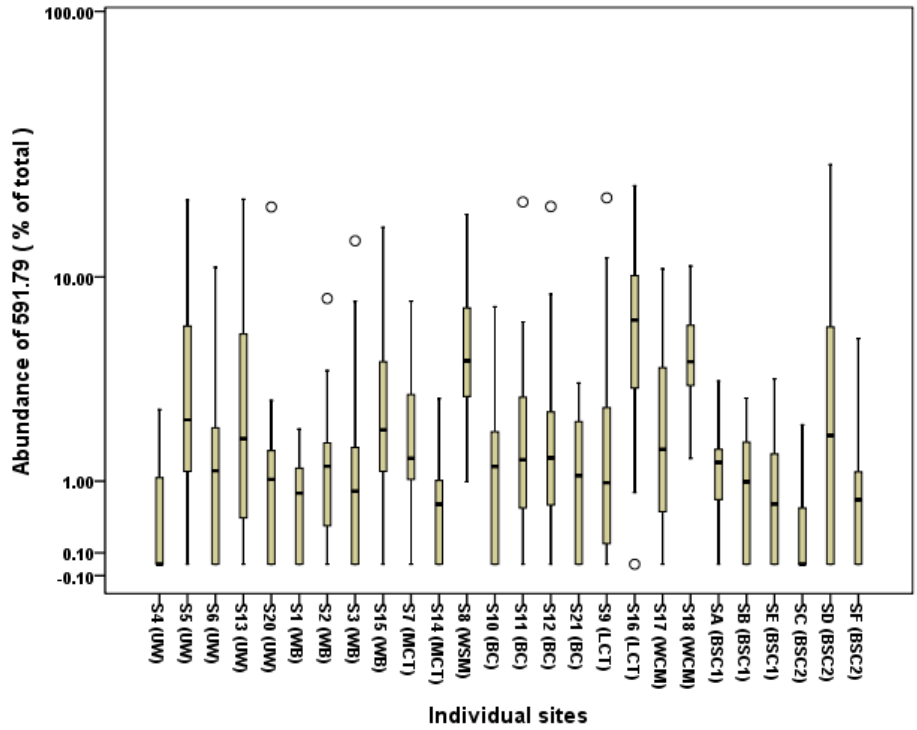

Figure A 4.3 Spatial variation of the common OTU (591.79) by sites from June 2011 to December 2012.

Table A 4.3 Mean, standard error, minimum and maximum of the abundance (%) of the common OUT (591.79) in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	0.19 ± .246	0.00 - 2.63
S5	3.40 ± .215	0.00 - 19.88
S6	1.02 ± .215	0.00 - 10.91
S13	2.65 ± .201	0.00 - 20.03
S20	0.86 ± .215	0.00 - 18.73
S1	0.53 ± .215	0.00 - 2.09
S2	1.14 ± .209	0.00 - 8.19
S3	1.07 ± .215	0.00 - 13.88
S15	3.06 ± .215	0.00 - 15.64
S7	1.68 ± .215	0.00 - 7.98
S14	0.51 ± .215	0.00 - 2.99
S8	5.04 ± .201	0.99 - 17.56
S10	1.10 ± .215	0.00 - 7.58
S11	1.73 ± .215	0.00 - 19.57
S12	1.55 ± .196	0.00 - 18.8
S21	0.90 ± .222	0.00 - 3.54
S9	1.71 ± .222	0.00 - 20.3
S16	6.78 ± .215	0.00 - 22.56
S17	1.85 ± .215	0.00 - 10.76
S18	4.75 ± .215	1.42 - 11.04
SA	0.96 ± .204	0.00 - 3.61
SB	0.63 ± .216	0.00 – 3.00
SE	0.52 ± .209	0.00 - 3.70
SC	0.13 ± .209	0.00 - 2.20
SD	3.21 ± .209	0.00 - 27.06
SF	0.69 ± .223	0.00 - 5.58

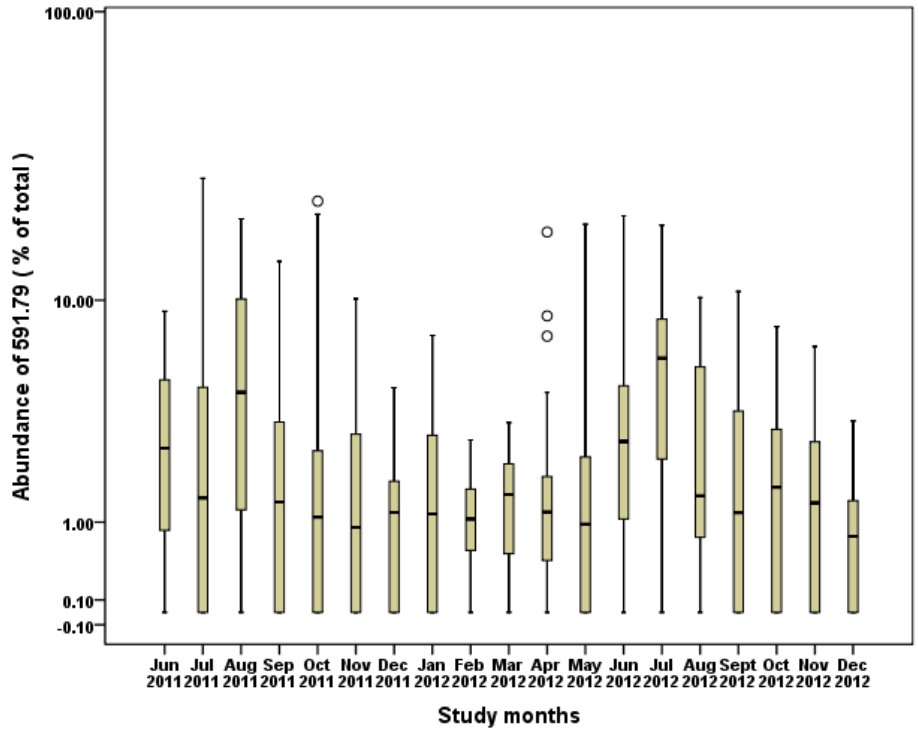

Figure A 4.4 Temporal variation of the common DNA fragment size (591.79) by months from June 2011 to December 2012.

Table A 4.4 Mean, standard error, minimum and maximum of the abundance (%) of the common OTU (591.79) in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	1.14 ± .519	0.00 - 9.10
	July	2.62 ± .227	0.00 - 27.06
	August	4.14 ± .203	0.00 - 19.57
	September	1.22 ± .180	0.00 - 13.82
	October	1.37 ± .164	0.00 - 22.56
	November	1.17 ± .172	0.00 - 10.13
	December	0.70 ± .172	0.00 - 4.62
2012	January	0.83 ± .172	0.00 - 7.40
	February	0.85 ± .172	0.00 - 2.76
	March	1.06 ± .172	0.00 - 3.30
	April	1.28 ± .172	0.00 - 17.58
	May	1.39 ± .172	0.00 - 18.73
	June	3.16 ± .176	0.00 - 20.03
	July	5.32 ± .172	0.00 - 18.57
	August	1.96 ± .172	0.00 - 10.23
	September	1.19 ± .172	0.00 - 10.76
	October	1.23 ± .188	0.00 - 7.98
	November	1.00 ± .180	0.00 - 6.72
	December	0.55 ± .172	0.00 - 3.35

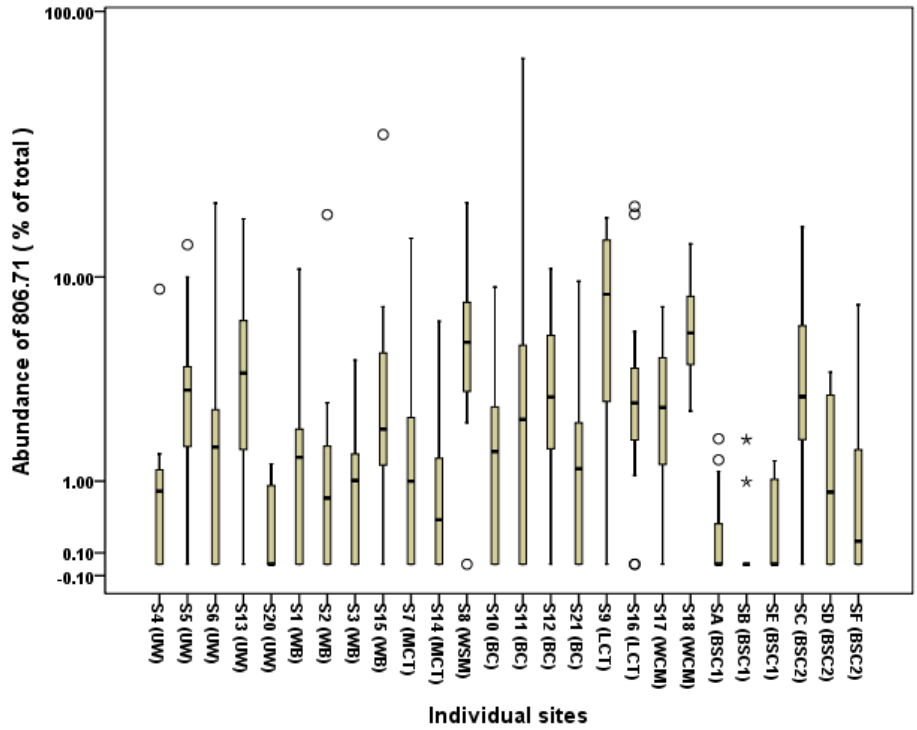

Figure A 4.5 Spatial variation of the common OTU (806.71) by sites from June 2011 to December 2012.

Table A 4.5 Mean, standard error, minimum and maximum of the abundance (%) of the common OUT (806.71) in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	0.51 ± .279	0.00 - 8.93
S5	2.78 ± .244	0.00 - 13.39
S6	1.14 ± .244	0.00 - 19.37
S13	3.71 ± .228	0.00 - 16.88
S20	0.15 ± .244	0.00 - 1.31
S1	1.35 ± .244	0.00 - 10.75
S2	0.75 ± .237	0.00 - 17.51
S3	0.59 ± .244	0.00 - 4.49
S15	3.06 ± .244	0.00 - 35.1
S7	1.01 ± .244	0.00 - 14.19
S14	0.65 ± .244	0.00 - 6.59
S8	5.92 ± .228	0.00 - 19.43
S10	1.40 ± .244	0.00 - 9.12
S11	4.32 ± .244	0.00 - 67.05
S12	2.72 ± .223	0.00 - 10.76
S21	0.80 ± .251	0.00 - 9.62
S9	6.82 ± .251	0.00 - 17.01
S16	3.27 ± .244	0.00 - 18.82
S17	2.66 ± .244	0.00 - 7.57
S18	6.36 ± .244	2.59 - 13.49
SA	0.11 ± .231	0.00 - 1.85
SB	0.01 ± .245	0.00 - 1.83
SE	0.23 ± .237	0.00 - 1.37
SC	3.72 ± .237	0.00 - 15.67
SD	0.88 ± .237	0.00 - 3.97
SF	0.63 ± .252	0.00 - 7.72

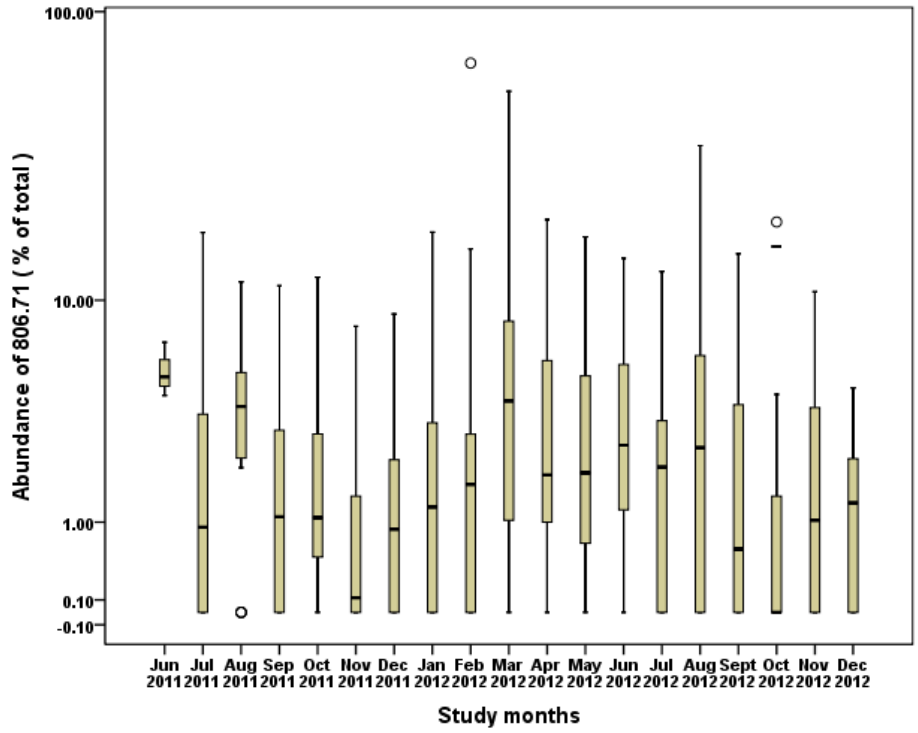

Figure A 4.6 Temporal variation of the common OTU (806.71) by months from June 2011 to December 2012.

Table A 4.6 Mean, standard error, minimum and maximum of the abundance (%) of the common OTU (806.71) in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	2.59 ± .588	4.29 - 6.96
	July	2.05 ± .257	0.00 - 17.51
	August	2.55 ± .230	0.00 - 11.66
	September	0.93 ± .204	0.00 - 11.31
	October	1.43 ± .186	0.00 - 12.12
	November	0.53 ± .195	0.00 - 8.01
	December	0.65 ± .195	0.00 - 8.90
2012	January	1.25 ± .195	0.00 - 17.58
	February	2.16 ± .195	0.00 - 67.05
	March	4.44 ± .195	0.00 - 53.76
	April	2.92 ± .195	0.00 - 19.43
	May	2.37 ± .195	0.00 - 16.88
	June	2.52 ± .199	0.00 - 14.19
	July	1.85 ± .195	0.00 - 12.72
	August	2.65 ± .195	0.00 - 35.10
	September	1.11 ± .195	0.00 - 14.72
	October	0.56 ± .213	0.00 - 19.08
	November	0.92 ± .204	0.00 - 10.75
	December	0.79 ± .195	0.00 - 4.60

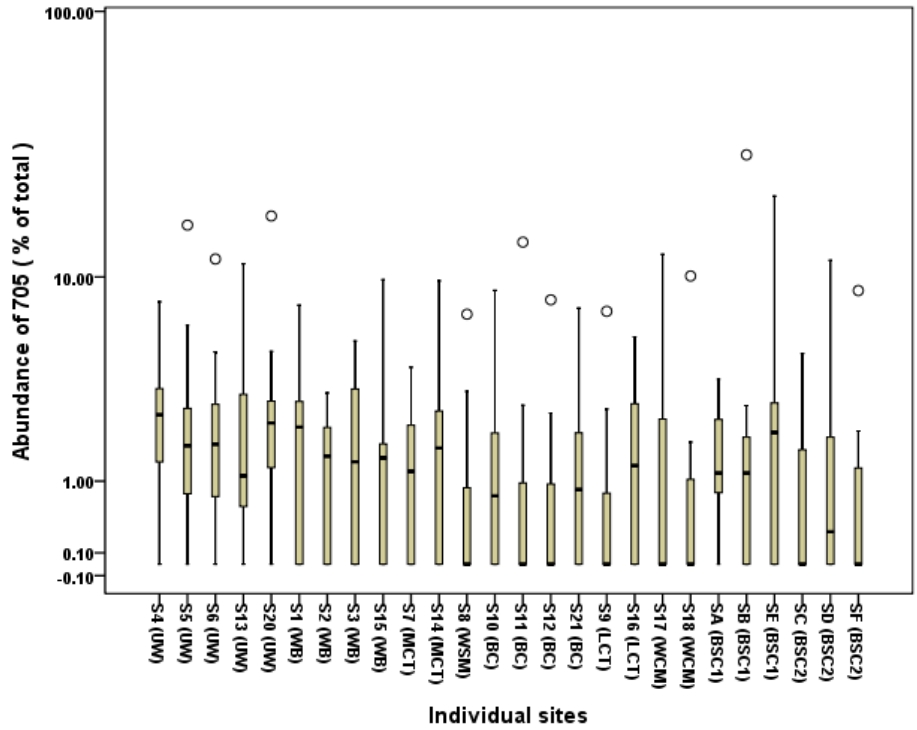

Figure A 4.7 Spatial variation of the common OTU (705) by sites from June 2011 to December 2012.

Table A 4.7 Mean, standard error, minimum and maximum of the abundance (%) of the common OTU (705) in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	1.88 ± .210	0.00 - 7.94
S5	1.93 ± .183	0.00 - 15.96
S6	1.63 ± .183	0.00 - 11.78
S13	1.86 ± .171	0.00 - 11.28
S20	2.19 ± .183	0.00 - 17.30
S1	1.34 ± .183	0.00 - 7.69
S2	0.67 ± .178	0.00 - 3.18
S3	1.11 ± .183	0.00 - 5.45
S15	0.91 ± .183	0.00 - 9.74
S7	0.77 ± .183	0.00 - 4.17
S14	1.20 ± .183	0.00 - 9.65
S8	0.30 ± .171	0.00 - 7.06
S10	0.71 ± .183	0.00 - 8.83
S11	0.38 ± .183	0.00 - 13.71
S12	0.29 ± .167	0.00 - 8.09
S21	0.90 ± .189	0.00 - 7.48
S9	0.30 ± .189	0.00 - 7.26
S16	1.10 ± .183	0.00 - 5.67
S17	0.65 ± .183	0.00 - 12.28
S18	0.39 ± .183	0.00 - 10.09
SA	1.17 ± .174	0.00 - 3.68
SB	1.10 ± .184	0.00 - 29.50
SE	1.60 ± .178	0.00 - 20.61
SC	0.35 ± .178	0.00 - 4.81
SD	0.55 ± .178	0.00 - 11.64
SF	0.33 ± .189	0.00 - 8.82

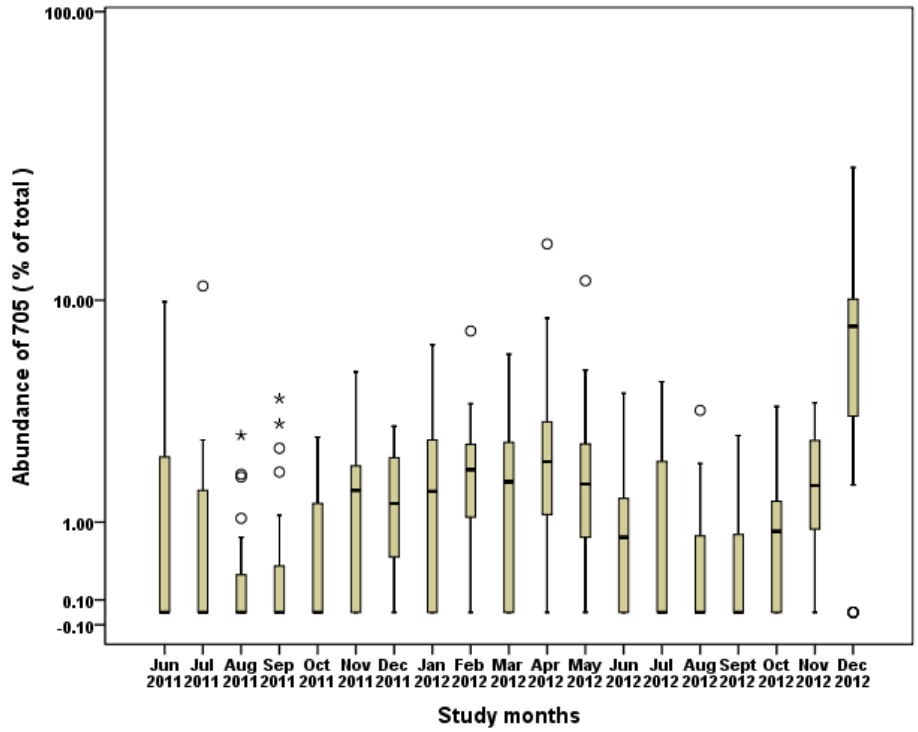

Figure A 4.8 Temporal variation of the common OTU (705) by months from June 2011 to December 2012.

Table A 4.8 Mean, standard error, minimum and maximum of the abundance (%) of the common OUT (705) in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	1.39 ± .441	0.00 - 9.88
	July	0.58 ± .193	0.00 - 11.28
	August	0.11 ± .173	0.00 - 2.90
	September	0.15 ± .153	0.00 - 4.17
	October	0.23 ± .139	0.00 - 2.84
	November	1.03 ± .146	0.00 - 5.34
	December	1.01 ± .146	0.00 - 3.18
2012	January	1.16 ± .146	0.00 - 6.82
	February	1.78 ± .146	0.00 - 7.69
	March	1.16 ± .146	0.00 - 6.27
	April	2.26 ± .146	0.00 - 15.96
	May	1.86 ± .146	0.00 - 11.78
	June	0.52 ± .150	0.00 - 4.38
	July	0.39 ± .146	0.00 - 4.88
	August	0.15 ± .146	0.00 - 3.72
	September	0.13 ± .146	0.00 - 2.89
	October	0.46 ± .160	0.00 - 3.86
	November	1.41 ± .153	0.00 – 4.00
	December	6.51 ± .146	0.00 - 29.50

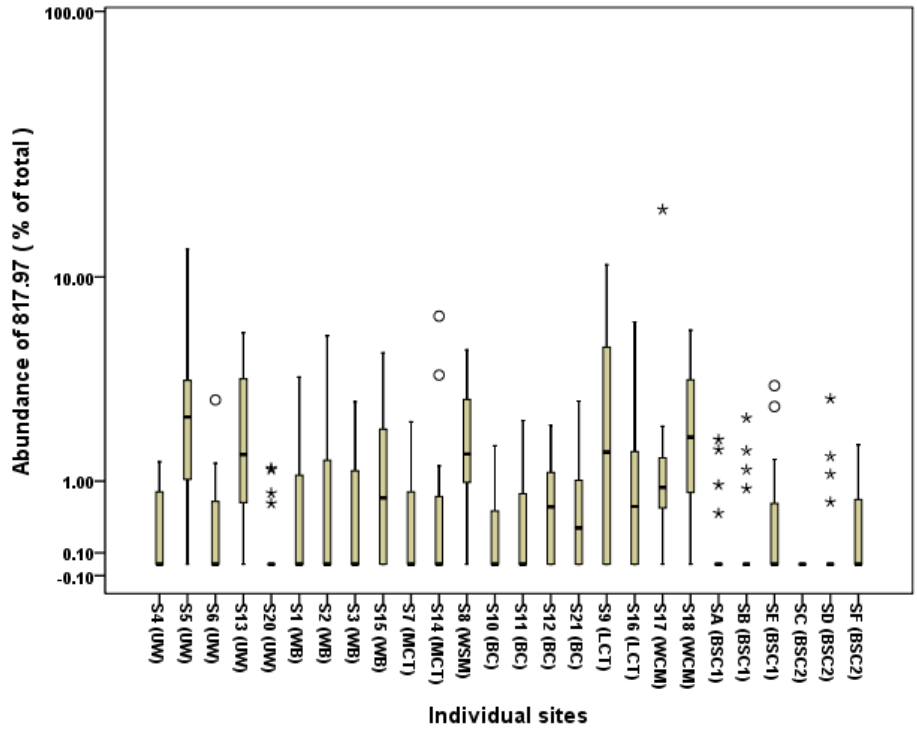

Figure A 4.9 Spatial variation of the common DNA fragment size (817.97) by sites from June 2011 to December 2012.

Table A 4.9 Mean, standard error, minimum and maximum of the abundance (%) of the common OUT (817.97) in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	0.14 ± .183	0.00 - 1.35
S5	2.21 ± .160	0.00 - 12.86
S6	0.14 ± .160	0.00 - 2.94
S13	1.60 ± .150	0.00 - 5.90
S20	0.07 ± .160	0.00 - 1.23
S1	0.29 ± .160	0.00 - 3.77
S2	0.38 ± .155	0.00 - 5.73
S3	0.29 ± .160	0.00 - 2.88
S15	0.73 ± .160	0.00 - 4.83
S7	0.14 ± .160	0.00 - 2.28
S14	0.30 ± .160	0.00 - 6.93
S8	1.61 ± .150	0.00 - 4.98
S10	0.12 ± .160	0.00 - 1.69
S11	0.17 ± .160	0.00 - 2.32
S12	0.39 ± .146	0.00 - 2.19
S21	0.34 ± .165	0.00 - 2.90
S9	1.59 ± .165	0.00 - 11.17
S16	0.49 ± .160	0.00 - 6.56
S17	1.07 ± .160	0.00 - 18.32
S18	1.82 ± .160	0.00 - 6.05
SA	0.05 ± .152	0.00 - 1.83
SB	0.06 ± .161	0.00 - 2.39
SE	0.14 ± .156	0.00 - 3.44
SC	0.00 ± .156	0.00 – 0.00
SD	0.08 ± .156	0.00 - 2.98
SF	0.10 ± .165	0.00 - 1.71

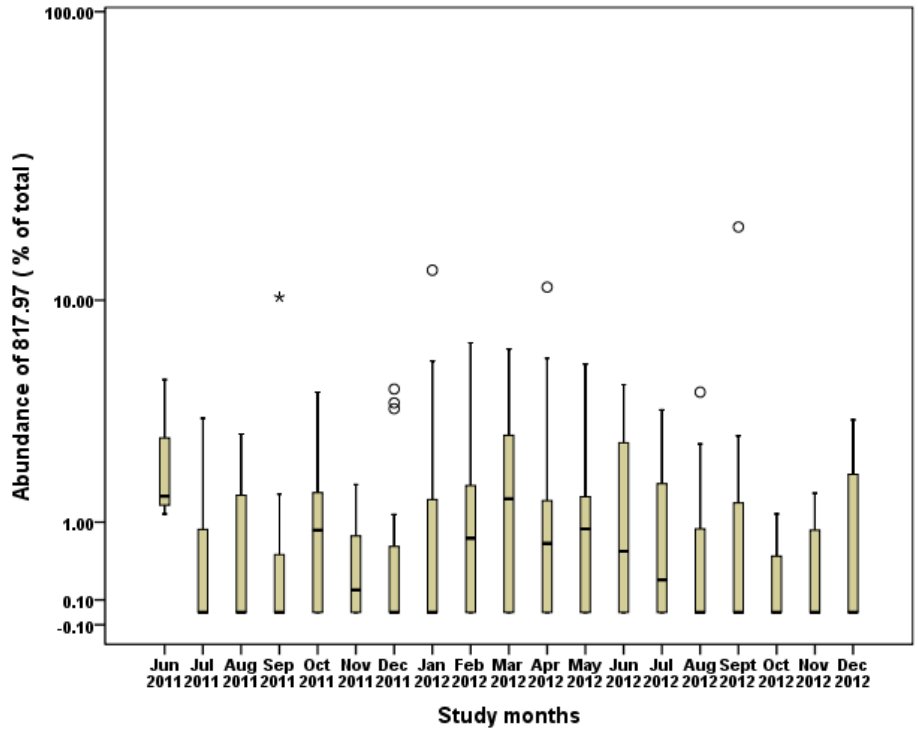

Figure A 4.10 Temporal variation of the common OTU (817.97) by months from June 2011 to December 2012.

Table A 4.10 Mean, standard error, minimum and maximum of the abundance (%) of the common OTU (817.97) in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	1.15 ± .386	1.13 - 4.98
	July	0.39 ± .169	0.00 - 3.44
	August	0.20 ± .151	0.00 - 2.94
	September	0.14 ± .133	0.00 - 10.27
	October	0.68 ± .122	0.00 - 4.43
	November	0.22 ± .128	0.00 - 1.67
	December	0.14 ± .128	0.00 - 4.56
2012	January	0.49 ± .128	0.00 - 12.86
	February	0.59 ± .128	0.00 - 6.93
	March	0.91 ± .128	0.00 - 6.56
	April	0.56 ± .128	0.00 - 11.17
	May	0.80 ± .128	0.00 - 5.73
	June	0.63 ± .131	0.00 - 4.75
	July	0.47 ± .128	0.00 - 3.74
	August	0.21 ± .128	0.00 - 4.43
	September	0.31 ± .128	0.00 - 18.32
	October	0.07 ± .140	0.00 - 1.13
	November	0.11 ± .133	0.00 - 1.50
	December	0.30 ± .128	0.00 - 3.39

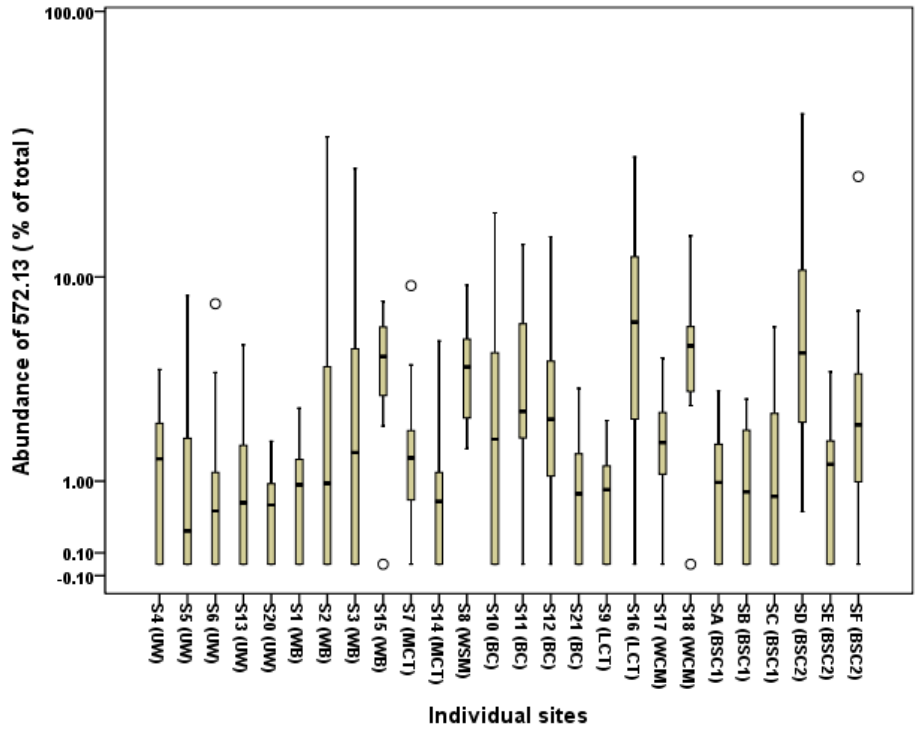

Figure A 4.11 Temporal variation of the common OTU (572.13) by sites from June 2011 to December 2012.

Figure A 4.11 Mean, standard error, minimum and maximum of the abundance (%) of the common OTU (572.13) in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	0.65 ± .251	0.00 - 4.08
S5	0.67 ± .219	0.00 - 8.41
S6	0.55 ± .220	0.00 - 7.80
S13	0.61 ± .205	0.00 - 5.24
S20	0.31 ± .219	0.00 - 1.79
S1	0.55 ± .219	0.00 - 2.68
S2	1.75 ± .213	0.00 - 34.44
S3	2.11 ± .219	0.00 - 26.20
S15	4.17 ± .219	0.00 - 7.98
S7	1.28 ± .219	0.00 - 9.23
S14	0.55 ± .219	0.00 - 5.45
S8	4.08 ± .205	1.62 - 9.27
S10	2.24 ± .219	0.00 - 17.79
S11	3.18 ± .219	0.00 - 13.42
S12	2.59 ± .200	0.00 - 14.39
S21	0.64 ± .226	0.00 - 3.34
S9	0.54 ± .226	0.00 - 2.32
S16	6.25 ± .219	0.00 - 28.93
S17	1.46 ± .219	0.00 - 4.59
S18	4.82 ± .219	0.00 - 14.51
SA	0.56 ± .208	0.00 - 3.25
SB	0.60 ± .220	0.00 - 2.97
SE	0.93 ± .213	0.00 - 3.98
SC	0.74 ± .213	0.00 - 6.24
SD	6.18 ± .213	0.55 - 41.94
SF	2.61 ± .227	0.00 - 24.44

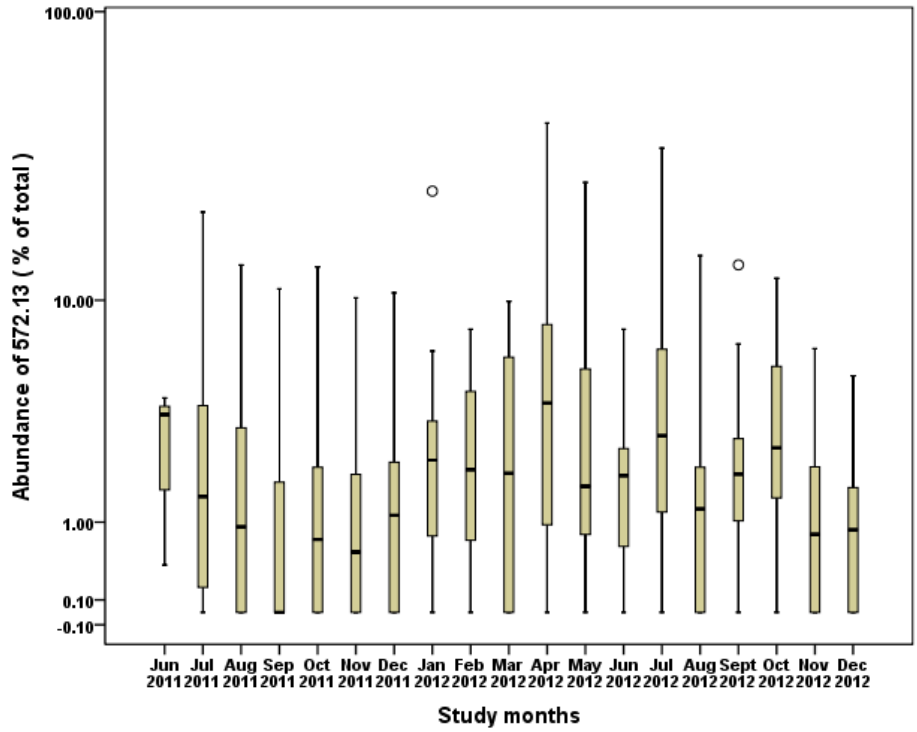

Figure A 4.12 (c) Temporal variation of the common OTU (572.13) by months from June 2011 to December 2012.

Table A 4.12 Mean, standard error, minimum and maximum of the abundance (%) of common OUT (572.13) in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	1.77 ± .529	0.44 - 4.19
	July	1.95 ± .231	0.00 - 20.65
	August	1.20 ± .207	0.00 - 13.42
	September	0.43 ± .183	0.00 – 11.00
	October	1.00 ± .167	0.00 - 13.19
	November	0.66 ± .175	0.00 - 10.20
	December	0.97 ± .175	0.00 - 10.65
2012	January	1.91 ± .175	0.00 - 24.44
	February	2.07 ± .175	0.00 - 7.80
	March	1.66 ± .175	0.00 - 9.91
	April	4.33 ± .175	0.00 - 41.94
	May	2.47 ± .175	0.00 - 26.20
	June	1.64 ± .179	0.00 - 7.81
	July	3.64 ± .175	0.00 - 34.44
	August	1.19 ± .175	0.00 - 14.51
	September	1.94 ± .175	0.00 - 13.44
	October	2.72 ± .192	0.00 - 12.03
	November	0.67 ± .183	0.00 - 6.59
	December	0.77 ± .175	0.00 - 5.15

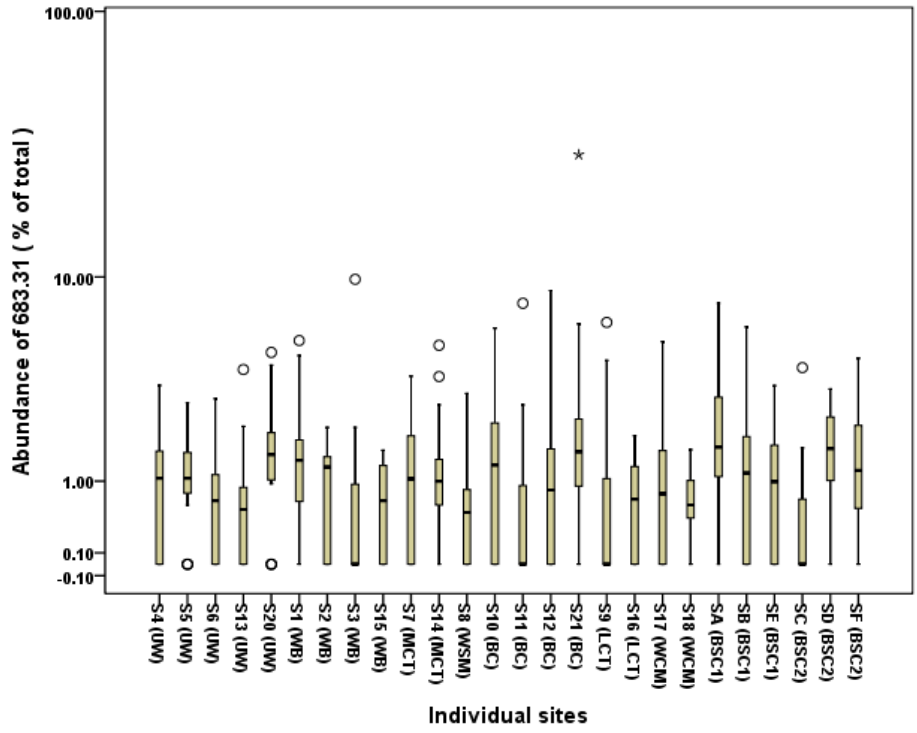

Figure A 4.13 Spatial variation of the common OTU (683.31) by sites from June 2011 to December 2012.

Table A 4.13 Mean, standard error, minimum and maximum of the abundance (%) of the common OTU (683.31) in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	0.45 ± .187	0.00 - 3.45
S5	0.84 ± .163	0.00 - 2.85
S6	0.35 ± .164	0.00 - 2.98
S13	0.44 ± .153	0.00 - 4.08
S20	1.22 ± .163	0.00 - 4.86
S1	1.11 ± .163	0.00 - 5.47
S2	0.62 ± .159	0.00 - 2.13
S3	0.37 ± .163	0.00 - 9.79
S15	0.50 ± .163	0.00 - 1.59
S7	0.68 ± .163	0.00 - 3.80
S14	0.10 ± .163	0.00 - 5.22
S8	0.37 ± .153	0.00 - 3.16
S10	0.84 ± .163	0.00 - 6.17
S11	0.29 ± .163	0.00 - 7.83
S12	0.68 ± .149	0.00 - 8.82
S21	2.07 ± .169	0.00 - 29.52
S9	0.29 ± .169	0.00 - 6.53
S16	0.46 ± .163	0.00 - 1.92
S17	0.73 ± .163	0.00 - 5.41
S18	0.56 ± .163	0.00 - 1.60
SA	1.52 ± .155	0.00 - 7.83
SB	0.81 ± .164	0.00 - 6.24
SE	0.75 ± .159	0.00 - 3.44
SC	0.12 ± .159	0.00 - 4.16
SD	1.38 ± .159	0.00 - 3.32
SF	1.10 ± .169	0.00 - 4.58

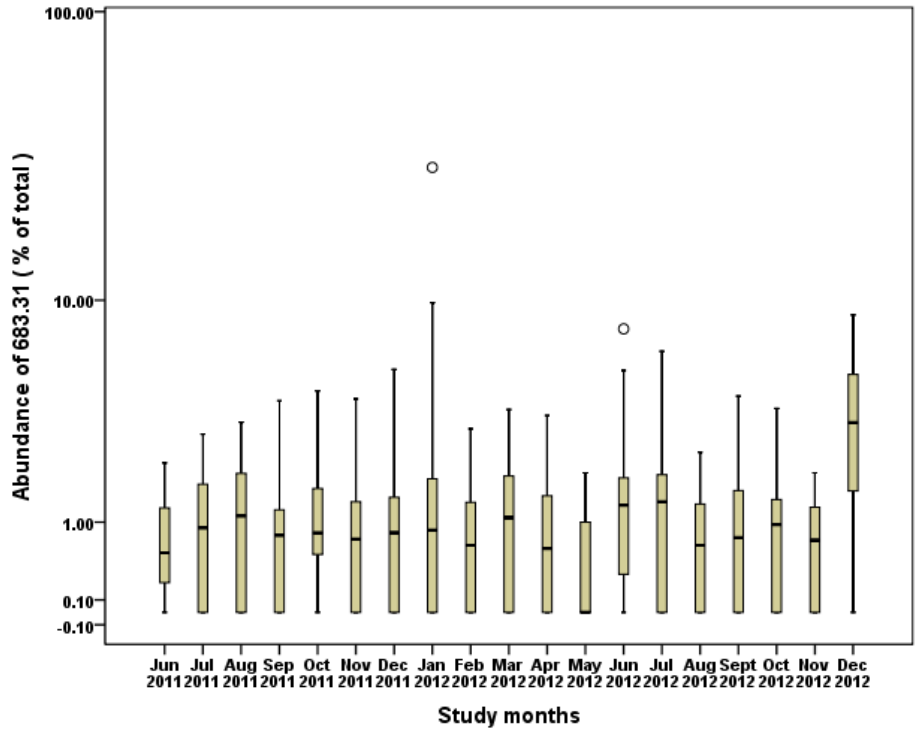

Figure A 4.14 Temporal variation of the common OUT (683.31) by months from June 2011 to December 2012.

Table A 4.14 Mean, standard error, minimum and maximum of the abundance (%) of common OUT (683.31) in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	0.77 ± .395	0.00 - 2.15
	July	0.46 ± .173	0.00 - 2.93
	August	0.82 ± .154	0.00 - 3.31
	September	0.43 ± .137	0.00 - 4.08
	October	0.73 ± .125	0.00 - 4.48
	November	0.56 ± .131	0.00 - 4.16
	December	0.60 ± .131	0.00 - 5.47
2012	January	1.00 ± .131	0.00 - 29.52
	February	0.51 ± .131	0.00 - 3.10
	March	0.78 ± .131	0.00 - 3.75
	April	0.48 ± .131	0.00 - 3.54
	May	0.23 ± .131	0.00 - 1.92
	June	1.07 ± .134	0.00 - 7.83
	July	0.97 ± .131	0.00 - 6.43
	August	0.43 ± .131	0.00 - 2.42
	September	0.67 ± .131	0.00 - 4.27
	October	0.53 ± .143	0.00 - 3.79
	November	0.41 ± .137	0.00 - 1.92
	December	2.85 ± .131	0.00 - 8.82

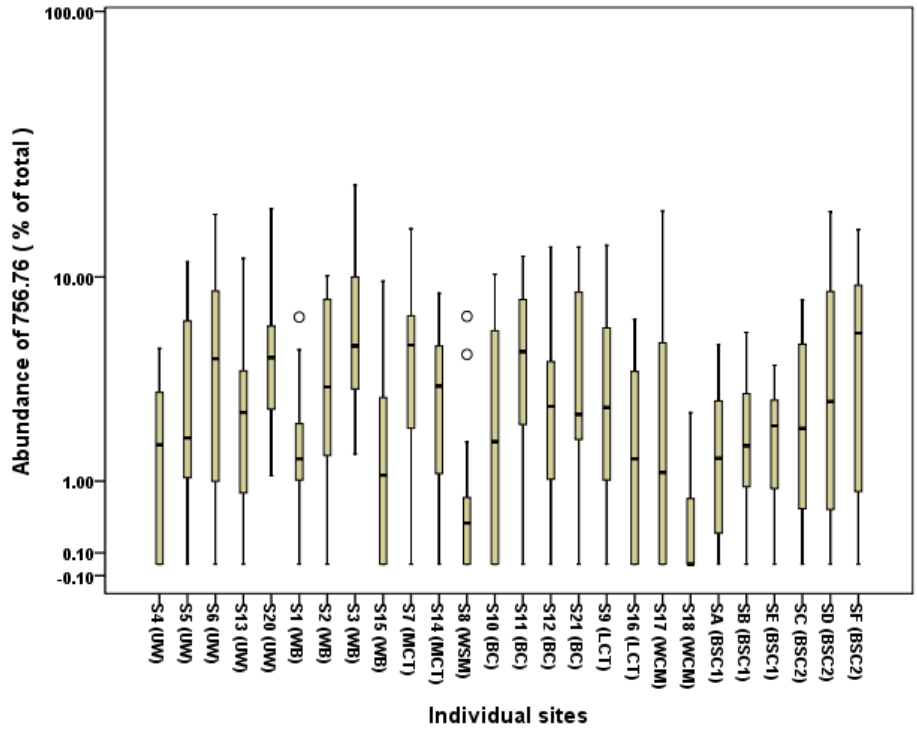

Figure A 4.15 Spatial variation of the common OTU (756.76) by sites from June 2011 to December 2012.

Table A 4.15 Mean, standard error, minimum and maximum of the abundance (%) of the common OUT (756.76) in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	1.41 ± .220	0.00 - 5.06
S5	2.88 ± .192	0.00 - 11.49
S6	4.07 ± .193	0.00 - 17.55
S13	1.96 ± .180	0.00 - 11.83
S20	4.56 ± .192	1.09 - 18.45
S1	1.32 ± .192	0.00 - 6.87
S2	3.12 ± .187	0.00 - 10.11
S3	6.22 ± .192	1.51 - 22.64
S15	1.07 ± .192	0.00 - 9.61
S7	3.88 ± .192	0.00 - 15.45
S14	2.53 ± .192	0.00 - 8.60
S8	0.39 ± .180	0.00 - 6.92
S10	1.88 ± .192	0.00 - 10.24
S11	4.18 ± .192	0.00 - 12.09
S12	2.24 ± .176	0.00 - 13.12
S21	3.68 ± .198	0.00 - 13.12
S9	2.80 ± .198	0.00 - 13.31
S16	1.03 ± .192	0.00 - 6.72
S17	2.10 ± .192	0.00 - 18.07
S18	0.16 ± .192	0.00 - 2.54
SA	0.99 ± .182	0.00 - 5.26
SB	1.54 ± .193	0.00 - 5.93
SE	1.47 ± .187	0.00 - 4.26
SC	1.67 ± .187	0.00 - 8.05
SD	2.76 ± .187	0.00 - 17.99
SF	3.92 ± .199	0.00 - 15.31

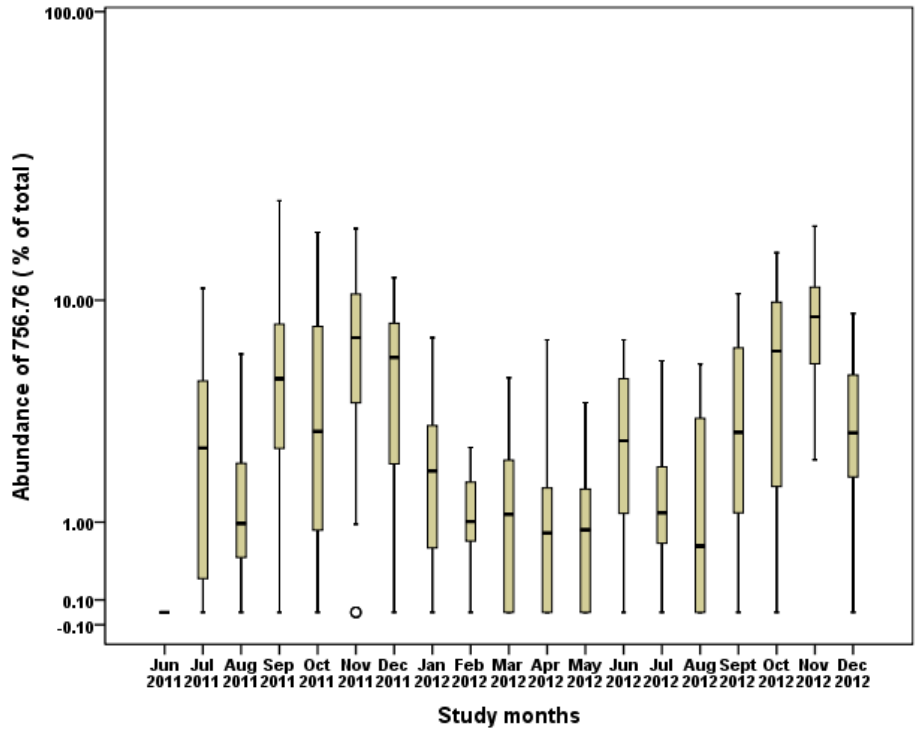

Figure A 4.16 Temporal variation of the common OTU (756.76) by months from June 2011 to December 2012.

Table A 4.16 Mean, standard error, minimum and maximum of the abundance (%) of the common OTU (756.76) in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	0.10 ± .464	0.00 – 0.00
	July	2.42 ± .203	0.00 - 11.05
	August	1.04 ± .182	0.00 - 6.28
	September	5.03 ± .161	0.00 - 22.64
	October	3.07 ± .146	0.00 - 17.55
	November	6.22 ± .154	0.00 - 18.07
	December	4.84 ± .154	0.00 - 12.09
2012	January	1.51 ± .154	0.00 - 7.26
	February	0.91 ± .154	0.00 - 2.55
	March	0.82 ± .154	0.00 - 5.06
	April	0.72 ± .154	0.00 - 7.12
	May	0.63 ± .154	0.00 - 4.01
	June	2.16 ± .157	0.00 - 7.12
	July	1.12 ± .154	0.00 - 5.91
	August	0.84 ± .154	0.00 - 5.74
	September	3.13 ± .154	0.00 - 10.56
	October	5.07 ± .168	0.00 - 14.85
	November	8.33 ± .161	0.00 - 18.45
	December	2.87 ± .154	0.00 - 8.94

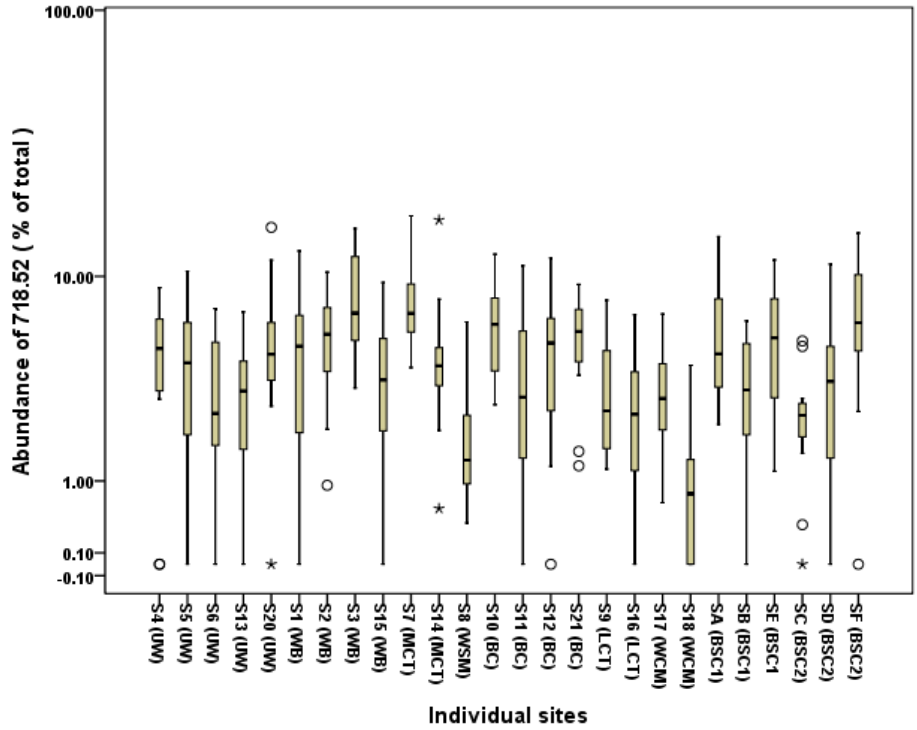

Figure A 4.17 Spatial variation of the common OTU (718.52) by sites from June 2011 to December 2012.

Table A 4.17 Mean, standard error, minimum and maximum of the abundance (%) of common OTU (718.52) in the River Wensum by sites from June 2011 to December 2012.

Individual site	Mean ± standard error	Minimum and maximum
S4	4.07 ± .193	0.00 - 9.01
S5	3.71 ± .168	0.00 - 10.5
S6	3.03 ± .169	0.00 - 7.40
S13	2.64 ± .158	0.00 - 7.17
S20	4.79 ± .168	0.00 - 15.56
S1	4.11 ± .168	0.00 - 12.59
S2	5.50 ± .163	0.93 - 10.4
S3	7.76 ± .168	3.34 - 15.39
S15	3.43 ± .168	0.00 - 9.45
S7	7.73 ± .168	4.15 - 17.21
S14	4.50 ± .168	0.59 - 16.59
S8	1.83 ± .158	0.41 - 6.53
S10	6.23 ± .168	2.78 - 12.24
S11	3.01 ± .168	0.00 - 11.03
S12	4.44 ± .154	0.00 - 11.81
S21	5.53 ± .173	1.27 - 9.29
S9	3.17 ± .174	1.21 - 8.01
S16	2.55 ± .168	0.00 - 6.99
S17	2.92 ± .168	0.67 - 7.05
S18	0.63 ± .168	0.00 - 4.25
SA	5.49 ± .160	2.21 - 14.33
SB	2.75 ± .169	0.00 - 6.58
SE	5.04 ± .164	1.17 - 11.62
SC	2.16 ± .164	0.00 - 5.43
SD	3.29 ± .164	0.00 - 11.18
SF	6.71 ± .174	0.00 - 14.78

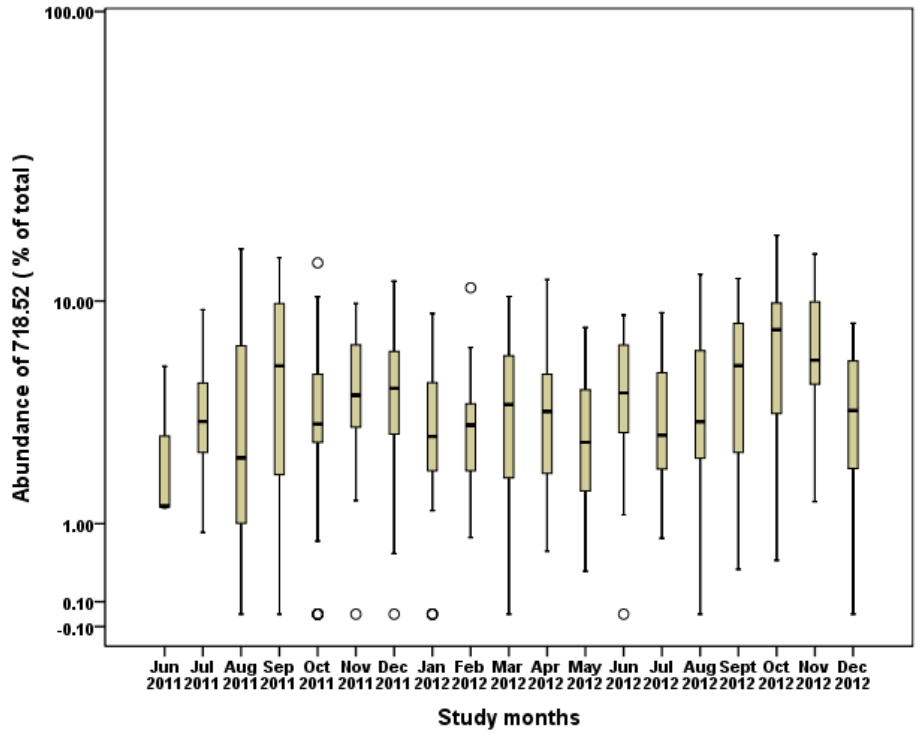

Figure A 4.18 Temporal variation of the common OTU (718.52) by months from June 2011 to December 2012.

Table A 4.18 Mean, standard error, minimum and maximum of the abundance (%) of the common OTU (718.52) in the River Wensum by months from June 2011 to December 2012.

Year	Month	Mean ± standard error	Minimum and maximum
2011	June	3.35 ± .406	1.26 - 5.67
	July	3.50 ± .178	0.87 - 9.29
	August	3.14 ± .159	0.00 - 15.39
	September	4.68 ± .141	0.00 - 14.33
	October	3.43 ± .128	0.00 - 13.73
	November	4.34 ± .135	0.00 - 9.80
	Dec ember	4.41 ± .135	0.00 - 11.81
2012	January	2.82 ± .135	0.00 - 9.01
	February	3.10 ± .135	0.80 - 11.18
	March	3.53 ± .135	0.00 - 10.40
	April	3.60 ±.135	0.62 - 11.96
	May	2.82 ± .135	0.39 - 7.99
	June	4.34 ± .137	0.00 - 8.88
	July	3.46 ± .135	0.79 - 9.06
	August	3.49 ± .135	0.00 - 12.51
	September	5.07 ± .135	0.41 - 12.08
	October	6.98 ± .147	0.51 - 17.21
	November	6.62 ± .141	1.37 - 14.78
	December	3.08 ± .135	0.00 - 8.28

Table A 4.19 Fragment sizes making the most contribution to the dissimilarity between bacterial community composition upstream and downstream sites on the River Wensum

Fragment size	Relative abundance (%)		Contribution to dissimilarity (%)	Cumulative contribution to dissimilarity (%)
	Upstream	Downstream		
702.09	0.45	4.49	4.55	4.55
795.42	0.52	3.24	3.12	7.67
806.71	0.30	2.46	2.44	10.1
591.97	0.82	2.21	1.57	11.68
810.12	0.08	1.41	1.5	13.18
572.13	0.83	2.10	1.47	14.65
575.27	1.41	0.20	1.43	16.08
713.78	0.45	1.42	1.42	17.5
718.52	2.09	1.09	1.35	18.86
565.24	1.56	0.40	1.35	20.2
724.25	0.50	1.63	1.31	21.52
817.97	0.29	1.29	1.25	22.77
559.55	1.18	0.21	1.19	23.96
694.57	0.32	1.19	1.19	25.15

Table A 4.20 Fragment sizes making the most contribution to the dissimilarity between bacterial community composition in December 2011 and December 2012 of the River Wensum.

Fragment size	Relative abundance (%)		Contribution to dissimilarity (%)	Cumulative contribution to dissimilarity (%)
	December 2011	December 2012		
638.7	3.51	0.91	3.17	3.17
705	1.01	2.55	2.25	5.43
702.09	1.41	1.17	1.39	6.82
683.31	0.77	1.69	1.38	8.2
729.94	0.35	1.31	1.37	9.57
724.25	0.70	1.43	1.29	10.86
848.48	1.29	0.44	1.28	12.14
756.76	2.20	1.69	1.21	13.36
779.95	1.29	0.89	1.21	14.57
782.92	0.33	1.09	1.2	15.77
718.52	2.10	1.75	1.11	16.88
795.42	0.83	0.79	1.11	17.99
557.13	1.10	0.52	1.08	19.07
806.71	0.81	0.89	1.06	20.13
727.07	1.15	0.52	1.05	21.18
776.61	0.31	1.00	1.04	22.22
694.57	0.32	0.98	1.02	23.24

Table A 4.21 Fragment sizes making the most contribution to the dissimilarity between bacterial community composition in September 2011 and September 2012 of the River Wensum.

Fragment sizes	Relative abundance (%)		Contribution to dissimilarity (%)	Cumulative contribution to dissimilarity (%)
	September 2011	September 2012		
702.09	2.99	2.36	3.25	3.25
795.42	1.70	1.41	2.20	5.46
806.71	1.04	1.05	1.65	7.11
756.76	2.27	1.77	1.60	8.71
572.13	0.70	1.39	1.59	10.3
591.97	1.16	1.09	1.57	11.87
727.07	1.62	1.49	1.46	13.33
718.52	2.18	2.25	1.45	14.78
711.23	0.97	1.04	1.43	16.21
742.92	1.83	1.48	1.29	17.5
162.59	0.5	1.04	1.26	18.76
565.24	0.62	1.13	1.25	20.01
753.56	0.99	0.61	1.24	21.26
697.29	0.97	0.38	1.24	22.5
678.11	0.70	0.99	1.22	23.72
638.7	0.85	1.12	1.22	24.94

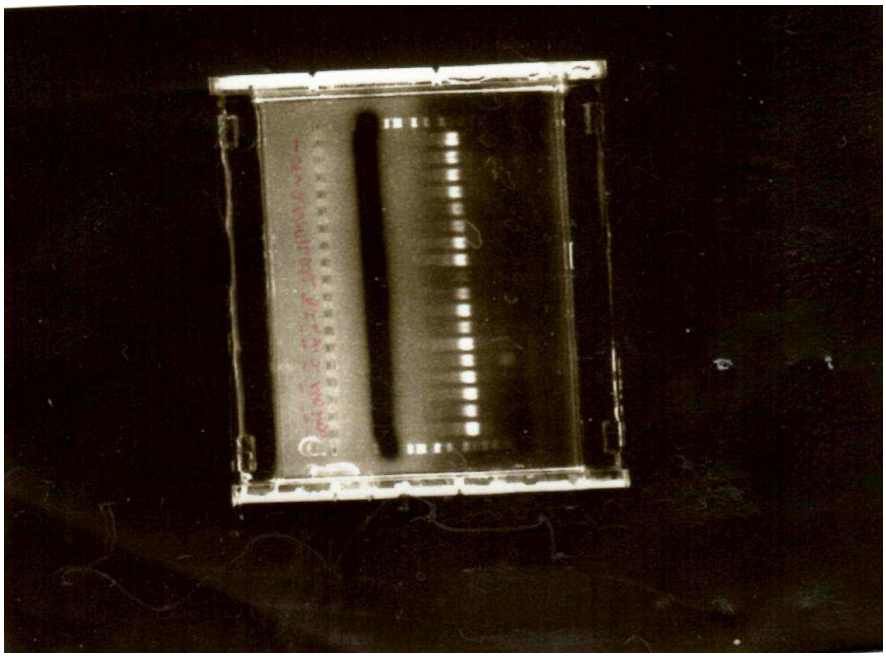

Table A 4.22 Photograph of bacterial DNA bands in 17 samples from February 2012 in the River Wensum, using the UV transilluminator.

Appendix to Chapter Five

Table A 5.1 Samples, reverse primers and barcodes used to tag each one of the 36 PCR products.

Sample	Reverse primer	Barcode
S1	338R1	ACACGACGACT
S2	333R2	ACACGTAGTAT
S3	338R3	ACACTACTCGT
S4	333R4	ACGACACGTAT
S5	338R5	ACGAGTAGACT
S6	333R6	ACGCGTCTAGT
S7	338R7	ACGTACACACT
S8	333R8	ACGTACTGTGT
S9	338R9	ACGTAGATCGT
S10	333R10	ACTACGTCTCT
S11	338R11	ACTATACGAGT
S12	333R12	ACTCGCGTCGT
S13	338R13	AGACTCGACGT
S14	333R14	AGTACGAGAGT
S15	338R15	AGTACTACTAT
S16	333R16	AGTAGACGTCT
S17	338R17	AGTCGTACACT
S18	333R18	AGTGTAGTAGT
S20	338R19	ATAGTATACGT
S21	333R20	CAGTACGTACT
SA	338R21	CGACGACGCGT
SB	333R22	CGACGAGTACT
SC	338R23	CGATACTACGT
SD	333R24	CGTACGTCGAT
SE	338R25	CTACTCGTAGT
SF	333R26	GTACAGTACGT
S1D	338R27	GTCGTACGTAT
S8D	333R28	GTGTACGACGT
S18D	338R29	ACACAGTGAGT
S20D	333R30	ACACTCATACT
SAD	338R31	ACAGACAGCGT
SBD	333R32	ACAGACTATAT
SCD	338R33	ACAGAGACTCT
SDD	333R34	ACAGCTCGTGT
SED	338R35	ACAGTGTCGAT
SEF	333R36	ACGAGCGCGCT

Key symbols: S= sample site. Numbers and letters refer to site names, D after a site name indicates December 2012.

Table A 5.2 The commonest bacterial phyla and their taxonomic affinities in the River Wensum from February and December 2012, based on 454 pyrosequencing of the 16S rRNA gene, using the Mothur programme

OTU 1 Bacteria(100);unclassified(97);unclassified(97);unclassified(97);unclassified (97);unclassified(97);unclassified(97);unclassified(97);unclassified(97);

OTU 2 Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Comamonadaceae(100);Rhodoferax(64);unclassified(64);unclassified(64);unclassified(64);

OTU 3 Bacteria(100);Bacteroidetes(100);Flavobacteria(100);Flavobacteriales (100);Flavobacteriaceae(100);Flavobacterium(100);unclassified(100);unclassified(100);unclassified(100);

OTU 4 Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Oxalobacteraceae(100);unclassified(70);unclassified(70);unclassified(70);unclassified(70);

OTU 5 Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Comamonadaceae(100);unclassified(60);unclassified(60);unclassified(60);unclassified(60);

OTU 6 Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);

OTU 7 Bacteria(100);Cyanobacteria(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);

OTU 8 Bacteria(100);Bacteroidetes(100);Flavobacteria(100);Flavobacteriales(100);Flavobacteriaceae(100);Flavobacterium(100);unclassified(100);unclassified(100);unclassified(100);

OTU 9 Bacteria(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);

OTU 10 Bacteria(100);Bacteroidetes(100);Flavobacteria(100);Flavobacteriales(100);Flavobacteriaceae(100);Flavobacterium(100);unclassified(100);unclassified(100);unclassified(100);

OTU 11 Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Comamonadaceae(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);

OTU 12 Bacteria(100);Bacteroidetes(100);Flavobacteria(100);Flavobacteriales(100);Flavobacteriaceae(100);Flavobacterium(100);unclassified(100);unclassified(100);unclassified(100);

OTU 13 Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Comamonadaceae(91);unclassified(80);unclassified(80);unclassified(80);unclassified(80);

OTU 14 Bacteria(100);Bacteroidetes(100);Sphingobacteria(100);Sphingobacteriales(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);

OTU 15 Bacteria(100);Proteobacteria(100);Epsilonproteobacteria(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);

OTU 16 Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Burkholderiaceae(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);

OTU 17 Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Comamonadaceae(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);

OTU 18 Bacteria(100);Bacteroidetes(100);Flavobacteria(100);Flavobacteriales(100);Flavobacteriaceae(100);Flavobacterium(100);unclassified(100);unclassified(100);unclassified(100);

OTU 19 Bacteria(100);Bacteroidetes(100);Flavobacteria(100);Flavobacteriales(100);Flavobacteriaceae(100);Flavobacterium(100);unclassified(100);unclassified(100);unclassified(100);

OTU 20 Bacteria(100);Actinobacteria(100);Actinobacteria(100);Actinobacteridae(100);Actinomycetales(100);Frankineae(100);Sporichthyaceae(100);hgcI_clade(100);unclassified(100);

OTU 21 Bacteria(100);Actinobacteria(100);Actinobacteria(100);Actinobacteridae(100);Actinomycetales(100);Micrococcineae(100);Microbacteriaceae(100);unclassified(100);unclassified(100);

OTU 22 **Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);**

OTU 23 **Bacteria(100);Bacteroidetes(100);Flavobacteria(100);Flavobacteriales(100);Flavobacteriaceae(100);Flavobacterium(100);unclassified(100);unclassified(100);unclassified(100);**

OTU 24 **Bacteria(100);Bacteroidetes(100);Flavobacteria(100);Flavobacteriales(100);Flavobacteriaceae(100);Flavobacterium(100);unclassified(100);unclassified(100);unclassified(100);**

OTU 25 **Bacteria(100);Actinobacteria(100);Actinobacteria(100);Actinobacteridae(100);Actinomycetales(100);Frankineae(100);Sporichthyaceae(100);hgcI_clade(100);unclassified(100);**

OTU 26 **Bacteria(100);Proteobacteria(100);Alphaproteobacteria(100);Rhodobacterales(100);Rhodobacteraceae(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);**

OTU 27 **Bacteria(100);Proteobacteria(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);**

OTU 28 **Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Comamonadaceae(100);Polaromonas(100);unclassified(100);unclassified(100);unclassified(100);**

OTU 29 **Bacteria(100);Proteobacteria(100);Deltaproteobacteria(98);unclassified(98);unclassified(98);unclassified(98);unclassified(98);unclassified(98);unclassified(98);**

OTU 30 **Bacteria(100);Cyanobacteria(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);**

OTU 31 **Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);unclassified(87);unclassified(87);unclassified(87);unclassified(87);unclassified(87);unclassified(87);**

OTU 32 **Bacteria(100);Proteobacteria(100);Gammaproteobacteria(100);unclassified(99);unclassified(99);unclassified(99);unclassified(99);unclassified(99);unclassified(99);**

OTU 33 **Bacteria(100);Bacteroidetes(100);Flavobacteria(100);Flavobacteriales(100);Flavobacteriaceae(100);Flavobacterium(100);unclassified(100);unclassified(100);unclassified(100);**

OTU 34 **Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);Rhodocyclales(100);Rhodocyclaceae(100);unclassified(96);unclassified(96);unclassified(96);unclassified(96);**

OTU 35 **Bacteria(100);Cyanobacteria(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);**

OTU 36 **Bacteria(100);Proteobacteria(100);Epsilonproteobacteria(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);**

OTU 37 **Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Comamonadaceae(98);unclassified(98);unclassified(98);unclassified(98);unclassified(98);**

OTU 38 **Bacteria(100);Proteobacteria(100);unclassified(95);unclassified(95);unclassified(95);unclassified(95);unclassified(95);unclassified(95);unclassified(95);**

OTU 39 **Bacteria(100);Cyanobacteria(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);**

OTU 40 **Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Comamonadaceae(100);unclassified(100);unclassified(100);unclassified(100);unclassified(100);**

Table A 5.3 The distribution of the abundance (%) of the commonest bacterial phyla (OTU1 to OTU20) in all sites of the River Wensum from February and December 2012.

Site	OTU1	OTU2	OTU3	OTU4	OTU5	OTU6	OTU7	OTU8	OYU9	OTU10	OTU11	OTU12	OTU13	OTU14	OTU15	OTU16	OTU17	OTU18	OTU19	OTU20
S1	0.21	3.45	0.32	3.72	0.53	2.12	0.00	3.34	3.03	0.64	0.21	2.34	0.42	0.11	0.27	0.16	1.86	1.65	0.00	0.00
S10	0.80	2.28	1.12	1.80	2.92	0.19	0.96	1.12	2.47	0.22	0.32	0.80	1.35	0.48	0.22	0.80	0.35	0.51	0.22	0.96
S11	11.96	1.67	1.96	0.55	1.39	2.22	0.67	1.32	1.24	3.01	16.87	0.36	1.03	0.53	1.24	0.26	0.48	1.51	0.45	0.45
S12	4.44	2.86	2.35	1.46	1.89	3.08	0.90	1.77	1.26	2.50	1.31	0.51	1.07	0.61	1.24	0.61	0.95	1.60	0.29	0.75
S13	33.68	2.94	0.98	1.00	1.31	0.41	0.90	0.82	0.67	0.88	0.33	0.57	0.80	0.54	2.37	0.72	0.64	0.28	0.00	0.41
S14	1.78	4.06	0.31	2.79	0.26	1.58	2.04	1.73	2.74	0.75	0.52	0.91	1.73	0.23	0.75	2.84	0.80	0.31	0.05	0.05
S15	2.68	3.44	2.13	1.61	4.41	3.35	13.61	2.58	0.87	1.14	0.32	0.45	0.57	0.97	1.19	1.76	0.42	1.14	0.27	1.64
S16	0.53	2.90	2.06	1.67	3.78	0.26	3.03	1.14	0.57	0.83	0.70	0.70	0.88	1.36	0.83	1.80	0.48	0.13	0.88	3.78
S17	0.39	2.83	12.00	1.99	3.22	25.52	0.66	1.15	0.70	2.17	0.14	0.45	1.09	0.52	0.75	0.91	0.41	0.57	0.27	0.39
S18	2.59	2.02	25.47	0.52	7.18	0.25	2.39	1.53	0.25	5.77	0.49	0.22	0.20	4.20	1.01	1.14	0.47	1.78	4.20	1.97
S18D	0.03	3.67	5.20	2.55	1.46	0.36	1.89	2.35	0.70	0.83	1.22	1.29	0.70	2.71	6.16	1.85	0.76	1.36	0.50	0.96
S1D	0.06	1.48	0.45	0.51	0.58	0.19	1.35	1.86	1.60	0.58	0.32	0.45	1.09	0.19	4.04	0.13	0.38	0.19	0.06	0.90
S2	0.85	3.74	0.27	3.45	0.71	0.06	0.74	1.59	1.80	0.21	0.35	0.82	1.88	0.41	0.74	0.24	0.56	0.41	0.09	0.12
S20	2.16	3.31	0.02	3.78	0.31	0.78	1.22	2.20	1.36	0.16	0.18	3.14	1.02	0.02	0.49	0.07	0.44	0.40	0.00	0.00
S20D	0.00	9.57	0.47	15.55	0.10	0.03	0.10	1.85	0.67	0.27	1.88	1.85	0.67	0.07	0.54	0.03	1.81	0.30	0.00	0.10
S21	0.00	2.78	3.98	1.45	1.60	3.33	3.33	1.05	2.57	1.73	0.13	0.83	2.01	0.53	1.26	1.30	0.66	0.64	0.06	0.41
S3	0.56	2.25	0.34	1.06	0.93	13.23	1.86	1.06	1.86	0.27	0.34	0.53	1.30	0.13	6.31	1.25	0.74	0.21	0.00	0.13
S4	0.06	2.62	0.03	3.14	0.40	0.40	1.20	2.34	2.99	0.31	0.29	2.88	1.06	0.03	0.29	0.63	0.37	1.17	0.03	0.06
S5	2.38	3.11	0.63	1.85	1.39	0.12	1.82	0.78	1.70	0.41	0.53	0.56	0.90	0.92	0.34	0.36	1.68	0.61	0.19	0.75
S6	3.85	3.90	0.08	2.33	0.68	3.52	0.13	0.89	1.27	0.25	1.74	0.51	1.52	0.00	0.51	3.13	0.64	0.17	0.04	0.04
S7	1.31	2.37	0.52	3.45	1.36	1.31	1.63	1.41	1.77	0.41	0.76	1.12	1.36	0.33	0.33	0.24	0.44	0.05	1.58	0.03
S8	2.79	2.66	10.77	1.10	5.14	1.00	5.48	1.53	1.00	2.04	0.31	0.41	0.41	2.63	0.47	2.00	0.69	0.94	2.25	1.69
S8D	0.40	1.88	4.57	1.09	1.38	0.69	2.17	2.36	1.01	0.47	0.43	0.65	0.76	2.64	0.72	0.54	0.29	0.91	0.25	1.96
S9	0.79	1.67	2.05	0.41	20.07	0.02	1.12	0.41	0.86	2.10	0.38	2.32	0.69	8.60	0.48	0.86	0.53	0.36	8.55	1.74
SA	0.00	4.92	0.08	5.03	0.19	3.45	0.06	3.59	5.47	0.19	0.14	2.79	0.83	0.25	0.17	0.33	0.83	0.30	0.00	0.00
SAD	0.00	3.96	3.30	3.18	1.23	0.06	0.24	3.12	1.83	0.36	1.71	2.64	1.68	0.75	0.03	1.68	1.80	0.72	0.18	0.54
SB	0.00	4.31	0.00	5.71	0.23	0.42	0.00	3.63	2.55	0.02	0.28	1.91	1.17	0.00	0.34	0.00	0.76	0.45	0.19	0.00
SBD	0.00	14.65	0.22	5.08	0.54	0.11	0.00	1.89	2.27	0.11	2.97	1.68	1.24	0.05	0.76	0.27	6.65	0.27	0.05	0.05
SC	41.18	1.76	5.68	0.22	4.62	0.34	1.88	7.34	0.36	6.79	0.17	0.22	0.51	0.12	0.34	0.48	0.07	4.53	1.04	0.02
SCD	0.05	3.45	4.75	1.89	1.28	0.36	0.43	3.02	1.35	0.25	0.34	0.92	0.61	0.02	0.74	4.41	1.08	0.50	0.32	0.54
SD	0.00	1.60	1.50	0.84	1.37	0.00	0.50	1.34	0.77	1.00	0.20	0.33	1.90	2.17	0.40	0.10	0.43	0.47	0.30	1.27
SDD	0.89	2.91	1.48	0.58	1.52	0.04	22.93	0.45	0.40	0.67	0.22	0.13	1.21	1.65	0.13	0.49	0.36	1.39	0.27	1.74
SE	0.00	3.37	0.00	3.53	0.26	2.79	0.16	3.66	3.66	0.45	0.03	1.94	0.42	0.16	0.62	0.10	1.13	0.42	0.03	0.00
SED	0.08	10.62	0.87	4.11	0.61	0.08	0.00	2.27	1.53	0.29	2.08	1.58	1.58	0.32	0.50	0.66	4.85	0.32	0.03	0.16
SF	5.76	0.99	2.05	0.30	1.36	0.00	0.45	2.12	1.29	2.27	0.00	0.83	2.27	0.83	0.08	0.08	0.30	2.73	1.06	0.68
SFD	0.43	6.86	1.29	2.54	0.92	0.20	6.33	2.37	1.02	0.26	1.19	0.82	1.25	0.43	0.16	1.45	2.37	0.73	0.07	0.59

Table A 5.3 (continued) The distribution of the abundance (%) of the commonest bacterial phyla (OTU21 to OTU40) in all sites of the River Wensum from February and December 2012.

Site	OTU21	OTU22	OTU23	OTU24	OTU25	OTU26	OTU27	OTU28	OTU29	OTU30	OTU31	OTU32	OTU33	OTU34	OTU35	OTU36	OTU37	OTU38	OTU39	OTU40
S1	0.11	0.11	0.96	0.96	0.00	0.21	1.59	0.69	0.16	1.01	0.32	0.32	0.69	0.16	0.00	0.00	0.21	0.16	0.00	0.05
S10	0.48	0.64	0.26	0.03	1.09	0.26	0.45	0.64	1.00	0.13	0.83	0.64	0.29	0.19	0.00	0.06	0.32	0.61	0.93	0.16
S11	0.17	0.50	0.17	0.07	0.48	0.79	0.12	0.24	0.31	0.17	0.24	0.07	0.10	0.24	0.26	0.02	0.22	0.45	0.19	0.14
S12	0.49	0.70	0.49	0.10	0.73	0.46	0.19	0.36	0.41	0.24	0.58	0.27	0.17	0.29	0.22	0.07	0.51	0.51	0.24	0.51
S13	0.72	0.23	0.28	0.28	0.36	0.36	0.13	0.18	0.23	1.08	0.23	0.41	0.23	0.05	0.59	0.77	0.26	0.00	0.39	0.26
S14	0.03	0.52	0.65	0.52	0.03	0.23	0.36	0.16	0.57	0.28	0.18	0.62	0.75	0.36	0.00	0.18	0.28	0.03	0.00	0.41
S15	1.29	0.40	0.22	0.15	1.16	0.32	3.57	0.69	0.40	0.62	0.15	0.27	0.35	0.10	0.37	0.10	0.15	0.10	0.40	0.05
S16	0.61	0.48	0.26	0.31	3.07	1.41	0.26	0.97	0.35	0.88	0.44	0.35	1.98	0.13	1.41	0.22	0.13	0.04	1.05	0.04
S17	0.39	0.20	0.52	0.25	0.29	0.16	0.36	0.34	0.48	0.77	0.14	0.20	0.57	0.07	0.00	0.25	0.25	0.00	0.20	0.18
S18	1.95	0.10	0.05	0.00	1.36	0.77	0.25	0.44	0.12	0.47	0.27	0.07	0.22	0.02	0.39	1.18	0.12	0.00	0.84	0.12
S18D	1.03	0.43	0.26	0.13	0.83	0.66	0.33	0.23	0.03	0.03	0.53	0.13	0.20	0.56	0.10	4.10	0.17	0.23	0.40	0.40
S1D	0.38	0.77	0.51	0.00	0.19	0.45	0.64	0.26	0.26	0.00	0.71	0.19	0.45	0.83	0.00	0.71	0.32	0.06	1.22	0.77
S2	0.27	1.47	0.56	0.53	0.06	0.41	0.38	0.24	0.91	2.59	0.38	0.44	0.65	0.35	0.00	0.15	0.74	0.00	0.03	0.35
S20	0.00	0.22	0.29	4.56	0.00	0.20	0.91	0.20	0.82	2.25	0.27	1.00	0.56	0.11	0.00	0.00	0.24	0.00	0.11	0.16
S20D	0.17	0.03	0.91	0.30	0.03	0.67	0.10	1.14	0.07	0.00	0.17	0.37	0.77	0.60	0.00	0.94	0.24	0.00	0.03	1.01
S21	0.98	0.49	0.62	0.47	0.11	0.36	0.41	0.71	0.51	0.17	0.51	0.60	0.41	0.13	0.00	0.09	0.43	0.11	0.15	0.26
S3	0.58	0.82	0.34	0.19	0.13	0.19	0.21	1.54	0.48	0.13	0.27	0.37	0.37	0.21	0.05	0.03	0.19	0.00	0.00	0.37
S4	0.11	0.40	0.43	0.31	0.00	0.29	1.34	0.29	1.00	0.23	0.29	0.37	0.74	0.00	0.00	0.48	0.20	0.03	0.00	0.29
S5	1.73	0.22	0.29	0.49	0.83	0.32	0.27	0.27	0.34	0.49	1.09	0.56	0.36	0.05	6.37	0.10	0.24	0.07	0.12	0.22
S6	0.13	0.47	0.30	0.76	0.04	0.30	0.21	0.08	0.59	0.64	0.76	0.55	0.30	0.30	0.00	0.17	0.51	0.04	2.24	0.13
S7	0.22	1.39	0.76	0.73	0.16	0.30	0.33	0.24	0.35	0.71	0.35	0.68	0.19	0.24	0.00	0.03	0.19	0.00	0.52	0.22
S8	1.79	0.13	0.34	0.03	1.69	0.56	1.10	0.85	0.19	0.81	0.22	0.19	0.22	0.13	0.47	0.00	0.06	0.00	0.81	0.09
S8D	1.20	0.43	0.18	0.07	1.12	0.72	1.01	0.51	0.54	0.33	0.58	0.04	0.33	0.29	0.18	0.25	0.54	0.18	0.47	0.69
S9	0.33	0.55	0.05	0.07	0.07	2.13	0.05	0.36	0.19	0.14	1.15	0.07	0.17	0.29	0.00	0.19	0.24	0.02	0.14	0.17
SA	0.03	0.36	1.08	0.41	0.00	0.25	0.28	0.41	0.80	0.00	0.44	1.55	0.86	0.03	0.00	0.00	0.22	0.17	0.00	0.03
SAD	0.72	1.20	1.68	0.03	0.09	0.75	0.18	0.33	0.33	0.00	0.51	0.36	0.57	3.93	0.00	0.24	0.81	0.00	0.00	1.59
SB	0.00	0.19	1.27	2.91	0.00	0.21	1.23	0.38	1.02	0.00	0.40	1.38	0.62	0.00	0.00	0.00	0.47	0.00	0.00	0.19
SBD	0.11	0.49	1.03	0.11	0.00	0.59	0.32	1.78	0.49	0.00	0.27	0.16	0.16	0.59	0.00	0.76	0.43	0.00	0.00	0.97
SC	0.26	0.31	0.05	0.07	0.00	0.00	0.05	0.02	0.17	0.58	0.00	0.10	0.00	0.00	0.00	0.05	0.07	0.07	0.41	0.00
SCD	0.99	0.95	1.04	0.16	0.34	0.45	0.14	0.11	0.45	0.00	0.88	0.25	0.36	0.83	0.00	0.68	0.18	6.28	0.02	0.29
SD	0.13	1.54	0.17	0.07	1.97	0.94	0.10	0.37	0.30	0.00	0.60	0.23	0.03	0.40	1.44	0.00	1.74	0.03	0.13	0.10
SDD	0.58	0.63	0.40	0.00	3.08	1.43	0.13	0.09	0.09	0.00	0.27	0.18	0.22	0.31	0.00	0.09	0.58	0.00	2.15	0.18
SE	0.00	0.23	0.75	1.20	0.00	0.16	0.45	0.42	1.10	0.00	0.29	0.68	0.55	0.19	0.00	0.26	0.19	0.06	0.00	0.13
SED	0.26	0.74	1.03	0.11	0.05	0.63	0.18	1.74	0.13	0.00	0.47	0.13	0.37	1.19	0.00	0.53	0.47	0.00	0.00	1.00
SF	0.08	1.29	0.38	0.08	0.99	0.61	0.23	0.08	0.08	0.08	0.08	0.45	0.08	0.08	0.30	0.08	1.14	0.00	0.38	0.30
SFD	0.69	0.89	0.92	0.20	0.59	0.92	0.23	0.56	0.20	0.00	0.23	0.07	0.36	0.73	0.00	0.46	0.30	0.89	0.76	0.82

Table A 5.4 Correlation between environmental parameters and common OTUs in the upstream sites of the River Wensum from February and December 2012, using Spearman's rank correlation.

	Common OTUs at upstream section						
	OTU2	**OTU4**	**OTU9**	**OTU12**	**OTU23**	**OTU29**	**OTU32**
Temperature (˚C)	N	N	N	N	N	N	N
pH	N	N	N	N	-.290* .043	N	N
Total nitrogen TN (mg/L)	.433** .004	.399** .008	N	N	.288* .044	N	N
Total phosphorus TP (µg/L)	N	N	N	-.414** .006	N	N	-.298* .039
Total carbon TC (mg/L)	.298* .039	.383* .020	.422** .005	.412** .006	.540** .000	N	N
Total organic carbon TOC (mg/L)	N	N	N	N	N	-.324* .027	-.454** .003
Total suspended solid TSS (mg/L)	N	-.304* .036	-.290* .043	N	N	N	-.390** .009
Arable land (%)	N	.330* .025	.418** .006	.380* .011	.294* .041	.288* .044	.367* .014
Improved grass (%)	N	N	N	N	N	N	N
Other grass (%)	N	N	N	N	N	N	N
Urban area (%)	N	N	-.478** .002	N	-.476** .002	N	-.280* .049
Rainfall (mm)	.352 .018	N	N	N	.352* .018	-.346* .019	-.478** .002
Sewage treatment works (n)	N	N	N	-.364* .014	N	N	N
Stream order (n)	-.309* .033	-.375* .012	-.431** .004	-.486** .001	-.512** .001	N	N
River flow (m^3/s)	.352 .018	N	N	N	.352* .018	-.346* .019	-.478** .002

** Correlation is significant at the 0.01 level, * Correlation is significant at the 0.05 level, N= not significant, (n) between brackets= numbers. Inside each square are Spearman's rank correlation and *p* values.

Table A 5.5 Correlation between environmental parameters and common OTUs in the downstream sites of the River Wensum from February and December 2012, using Spearman's rank correlation.

	Common OTUs at downstream section								
	OTU1	**OTU3**	**OTU10**	**OTU14**	**OTU19**	**OTU20**	**OTU21**	**OTU25**	**OTU26**
Temperature (˚C)	N	N	N	N	N	N	N	N	N
pH	N	N	N	.288* .045	N	N	N	N	N
Total nitrogen TN (mg/L)	-.315* .031	-.287* .045	-.401** .008	N	-.457** .003	N	N	N	N
Total phosphorus TP (µg/L)	N	.312* .032	N	N	.288* .044	N	N	.293* .041	N
Total carbon TC (mg/L)	-.390* .031	N	-.280* .049	-.287* .045	-.340* .021	N	N	-.295* .041	N
Total organic carbon TOC (mg/L)	-.317* .030	N	N	N	N	N	N	N	.419** .006
Total suspended solid TSS (mg/L)	N	N	N	.286* .021	.361* .015	.456** .003	N	.361* .015	.547** .000
Arable land (%)	-.365* .014	-.509** .001	-.473** .002	-.339* .021	-.362* .015	-.317* .030	-.317* .015	-.425** .005	N
Improved grass (%)	.419** .005	.330* .025	.380* .011	N	N	N	N	N	N
Other grass (%)	N	N	N	N	N	N	N	N	N
Urban area (%)	N	.281* .048	N	.356* .016	N	.345* .020	N	.331* .024	N
Rainfall (mm)	-.429** .005	N	-.293* .042	N	N	N	N	N	.472** .002
Sewage treatment works (n)	.297* .039	N	.375* .012	N	N	N	.368* .014	N	N
Stream Order (n)	.594** .000	.463** .002	.443** .003	.551** .000	.442** .004	.530** .000	.536** .000	.651** .000	N
River flow (m^3/s)	-.429** .031	N	-.293* .042	N	N	N	N	N	.472** .002

** Correlation is significant at the 0.01 level, * Correlation is significant at the 0.05 level, N= not significant, (n) between brackets= numbers. Inside each square are Spearman's rank correlation and *p* values.

Table A 5.6 Correlations between environmental parameters and common OTUs in the River Wensum from December 2012 (high rainfall and river flow rate), using Spearman's rank correlation.

	Common OTUs in December 2012				
	OTU2	**OTU17**	**OTU26**	**OTU34**	**OTU40**
Temperature (˚C)	**N**	**N**	**N**	**-.382***	**N**
				.011	
pH	**N**	**N**	**N**	**-.499****	**-.366****
				.003	**.014**
Total nitrogen TN (mg/L)	**.433****	**N**	**N**	**.316***	**.411****
	.004			**.030**	**.006**
Total phosphorus TP (µg/L)	**N**	**N**	**N**	**N**	**N**
Total carbon TC (mg/L)	**.298***	**.476****	**N**	**.371***	**.499****
	.039	**.002**		**.013**	**.001**
Total organic carbon TOC (mg/L)	**N**	**N**	**.419****	**.686****	**.501****
			.006	**.000**	**.001**
Total suspended solid TSS (mg/L)	**N**	**N**	**.547****	**.543****	**.347***
			.000	**.000**	**.019**
Arable land (%)	**N**	**N**	**N**	**N**	**N**
Improved grass (%)	**N**	**N**	**N**	**N**	**N**
Other grass (%)	**N**	**N**	**N**	**N**	**N**
Urban area (%)	**N**	**-.311***	**N**	**N**	**N**
		.032			
Rainfall (mm)	**.352***	**.287***	**.472****	**.729****	**.636****
	.018	**.045**	**.002**	**.000**	**.000**
Sewage treatment works (n)	**N**	**N**	**N**	**N**	**N**
Stream Order (n)	**-.309***	**N**	**N**	**N**	**N**
	.033				
River flow (m^3/s)	**.352***	**.287***	**.472****	**.729****	**.663****
	.018	**.045**	**.002**	**.000**	**.000**

** Correlation is significant at the 0.01 level, * Correlation is significant at the 0.05 level, N= not significant, (n) between brackets= numbers. Inside each square are Spearman's rank correlation and *p* values.