Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Fakulteten för landskapsplanering,
trädgårds– och jordbruksvetenskap

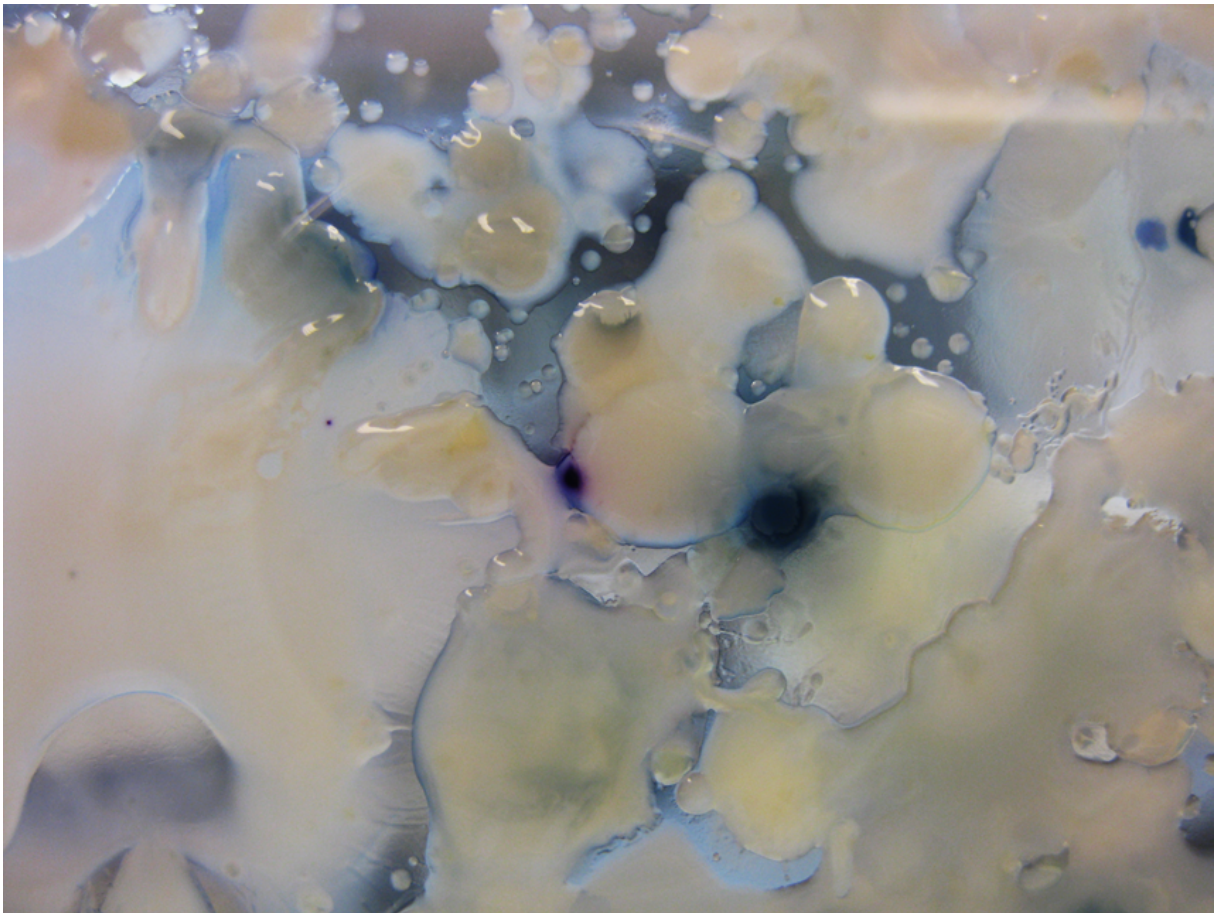
Antibiotic resistance associated with bacteria in irrigation water

– a case study of irrigation ponds in Southern Sweden

Antibiotikaresistens hos bakterier i bevattningsvatten

– en fallstudie från två bevattningsdammar södra Sverige

Maria Grudén



Självständigt arbete vid LTJ-fakulteten, SLU
Examensarbete 30 hp, Hortonomprogrammet, Alnarp 2013

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Fakulteten för landskapsplanering,
trädgårds– och jordbruksvetenskap

Antibiotic resistance associated with bacteria in irrigation water - a case study from Southern Sweden
Antibiotikaresistenshos bakterier i bevattningsvatten – en fallstudie från södra Sverige

Maria Grudén

Handledare: *Beatrix W. Alsanius*, SLU, Institutionen för biosystem och teknologi

Examinator: *Malin Hultberg*, SLU, Institutionen för biosystem och teknologi

Kurstitel: Examensarbete inom hortonomprogrammet
Kurskod: EX0544
Omfattning: 30 hp
Nivå: E
Fördjupning: A2E

Program/utbildning: Hortonomprogrammet
Ämne: Biologi

Serienamn: Självständigt arbete vid LTJ-fakulteten, SLU
Utgivningsort: Alnarp
Utgivningsmånad och -år: Februari 2013
Elektronisk publicering: http://stud.epsilon.slu.se

Omslagsbild: Maria Grudén

Nyckelord: *Animal husbandry, Enterobacteriaceae, ESBL, Etest, exogenous isolation, irrigation water, hygiene standards in water, water quality*

Ansvarig institution:
SLU, Sveriges lantbruksuniversitet
Fakulteten för Landskapsplanering, trädgårds- och jordbruksvetenskap
Institutionen för växtförädling

Medansvarig institution:
Institutionen för arbetsvetenskap, ekonomi och miljöpsykologi
Institutionen för biosystem och teknologi
Institutionen för växtskyddsbiologi

Abstract

The focus of this project was to characterize the occurrence of ESBL-producing bacteria in two irrigation water ponds in Southern Sweden. Samples were taken from two ponds nearby each other, from which analyses were made based on levels of community and individual isolates. Community samples were used for attempts for characterization of resistance patterns in irrigation water using the Omnilog PM-plate system. The growth of community microbiota in the presence of the following antibiotic substances was assessed; amikacin, gentamicin, kanamycin, penicillin G, ampicillin, piperacillin, trimethoprim, potassium tellurite and ciprofloxacin. Isolates were pure cultured using semi-selective media: LB, Rainbow Agar, mEnterococcus, 0.1 TSA and VRBD. A total of 177 isolates were isolated randomly. Each isolate was identified using the Omnilog GENIII-system of biochemical characterization. Further, the isolates were tested on Brilliance ESBL-agar for their ability to grow. Out of the total number of isolates, 30 were selected, preferably *Enterobacteriaceae* or *Pseudomonas spp.,* based on their high similarity to library strain during the identification in the Omnilog-system. These were further characterized using Etest-sticks. Three selected strains were picked for further analyses using exogenous isolation for attempts to simulate horizontal gene transfer, and Omnilog PM-panels containing various antibiotics of interest. Selections of samples were chosen for molecular tests targeting plasmid-borne resistance genes CTX-M1, CTX-M2 and SHV. The method for assessing community-resistance patterns showed ambiguous data that was difficult to interpret; further optimization is recommended. For all tested PM-plates, generally greatest differences between substances were found among wells with the strongest concentration of antibiotic substance.

A total of 175 isolates displayed ability to grow on ESBL-agar. Etests confirmed ability of isolates to resist many β-lactam antibiotics and many were clinically resistant to certain substances. No plasmids were transferred during exogenous isolation. During molecular tests, a few matches were found for the CTX-M1 gene. Isolates analyzed with Omnilog PM-plates showed resistance to most compounds tested. The isolates showed greatest resistance toward penicillin G, ampicillin, amikacin, kanamycin and trimethoprim. In the study, it was established that resistance among bacteria was frequently occurring in the irrigation water, and β-lactamase producing bacteria was very common. Extended studies are needed to assess the frequency- and occurrence of horizontal gene transfer (HGF) in this environment. Although some potential corresponding data is found, it is at this point not possible to determine whether the resistances are due to antibiotics used in veterinary medicine, human medicine or one additional reason is travel and trade.

Sammanfattning

Målet med detta projekt var att karaktärisera förekomsten av ESBL-producerande bakterier i bevattningsvatten i södra Sverige. Prover togs från två närbelägna bevattningsdammar, från vilka analyser gjordes på enskilda isolat och direkta prover. De prover som användes utan att selektera fram enskilda isolat användes för att testa- och utvärdera en metod för karakterisering av resistensmönster i bevattningsvatten med hjälp av Omnilogs system med PM-plattor. I detta test bedömdes tillväxten av mikroorganismerna i närvaro av följande antibiotiska ämnen: amikacin, gentamicin, kanamycin, penicillin G, ampicillin, piperacillin, trimetoprim, kalium tellurit och ciprofloxacin. Ur samma prover isolerade enskilda isolat på följande medium: LB, Rainbow Agar, mEnterococcus, 0,1 TSA och VRBD. Totalt isolerades 177 stammar vilka plockades baserat på utseende på media, där heterogenitet i insamlat material var målet. Varje isolat identifierades med användning av Omnilog GENIII-system av biokemisk karakterisering. Vidare var isolaten testades på Brilliance ESBL-agar för deras förmåga att växa genom att producera betalaktamaser. Av det totala antalet isolat var valdes 30 stycken, företrädesvis *Enterobacteriaceae* eller *Pseudomonas spp.,* baserat på likhet med modellorganismer under identifieringen i Omnilog-systemet. Dessa isolat karaktäriserades ytterligare med Etest-stickor. Utifrån dessa tester valdes tre stammar för ytterligare analyser där försök gjordes att överföra plasmider i en ansats att simulera horisontell genöverföring. Isolaten testades också med Omnilog PM-paneler med olika antibiotika av intresse. Ett antal isolat valdes även för molekylära tester med PCR där man sökte efter de plasmidburna resistensgenerna CTX-M1, CTX-M2 och SHV. Data från tester på den totala mikrofloran var svår att tolka; för att utveckla metoden till ett tillförlitligt verktyg krävs vidare optimering. För alla test med PM-plattor (total microbiota och enskilda isolat) visades största skillnader mellan olika antibiotika i brunnar med starkast koncentration.

Totalt uppvisade 175 isolat förmåga att växa på ESBL-agar. E-tester bekräftade resistens hos isolat mot β-laktam-antibiotika. Många av dessa var kliniskt resistenta. Inga plasmider överfördes under exogen isolering. Under molekylära tester har några träffar för CTX-M1 genen hos de utvalda isolaten, men inte för de andra generna. Isolaten som analyserades med Omnilog PM-plattor visade resistens mot de flesta testade substanserna. Isolaten visade störst resistens mot penicillin G, ampicillin, amikacin, kanamycin och trimetoprim. I studien konstaterades att resistens bland bakterier var vanligt i bevattningsvattnet och att bakterier resistenta mot β-laktam-antibiotika var mycket vanligt. Vidare studier behövs för att bedöma frekvens-och förekomsten av horisontell genöverföring (HGF) i denna miljö. Även angående utredning av orsak till uppkomsten av resistens krävs mer undersökningar. Data som erhållits skulle kunna visar att resistens finns mot substanser som är vanliga inom veterinärmedicin (t ex penicillin G). Dock är dessa substanser även vanliga inom humanmedicin vilket gör att det inte går att dra någon slutsats angående resistensens ursprung utifrån data i denna studie. Spridning av bakterier med ESBL-resistens har även visats ske via människor som exponeras- och blir bärare av resistenta bakterier via exempelvis resor. Studier visar också att resistenta bakterier sprids via transport- och handel med varor och djur.

Innehåll

1. Introduction

1.1 Antibiotics

Antibiotics are substances produced by microorganisms with the aim of reduce the growth of other microorganisms in order to favor the growth of the own species (Hogg, 2005). Clinically used antibiotics are either the secondary metabolites produced by microorganisms, chemical derivatives identical to these substances, or purely synthetic. In health care settings and in veterinary medicine, antibiotics are used in order to control growth of pathogenic microorganisms. The introduction of antibiotics for medical treatment of humans and animals in the middle of the twentieth century has had huge impact on the history of medicine since it has provided a way to control previously uncontrolled infectious diseases in humans and animals (Hogg, 2005). The immediate result upon the introduction of antibiotics during the 1940s was fewer incidences of disease and more opportunities to cure the already diseased (Mazel & Davies, 1999). The three main pathways through which antibiotics act on bacteria are:

1. Inhibition of cell wall synthesis
2. Interference with protein synthesis
3. Interference with nucleic acid synthesis
4. Interference with folate synthesis

(Walsh, 2003)

However, due to the enormous genetic flexibility in microbes, introduction of clinical use of antibiotics has led to the increase of achieved resistance in bacteria toward antibiotics (Heuer & Smalla, 2007; Mazel & Davies, 1999) thus challenging practice and development of modern medicine and the successful treatment of infectious diseases.

1.2.1 Antibiotic resistance

Since antibiotics are substances produced by certain microorganisms, in order to survive these bacteria need to be resistant to their own antibiotic excreted. Subsequently, these organisms have in their genome genes that already encode antibiotic resistance, leading to antibiotic resistance genes as a naturally occurring trait in the bacterial gene pool (Walsh, 2003). Antibiotic resistance is inherent in certain bacteria but since the introduction of clinically used antibiotics, the conditions for arising of antibiotics resistance have been altered, favoring the presence of genes encoding antibiotic resistance (Baquero *et al*., 2008). Due to the presence e.g. by clinical use of antimicrobials and therefore applied selection pressure of antibiotics in certain bacterial communities, targeted

organisms will, over time, achieve resistance. Small changes in their genetic setup will make them immune to the actions and mechanisms of the antibiotic in question. Hence, only cells that have obtained these changes in traits will survive and multiply (Hogg, 2005). Resistance may be attained through several ways, i. e. through mutation, transfer of resistance plasmids, or through transposons. Currently known mechanisms of resistance are: enzymatic breakdown or modification inactivating antibiotics, modification of receptor site, activation of efflux pumps and alteration of metabolic pathways (Hogg, 2005). It has been shown that antibiotic resistance is more prevalent in nutrient rich sites such as in soil, in sewage sludge and sediments. The root-zone in soils is a very common location for resistance to arise (Seveno *et al*., 2002).

Table 1. The different classes of antibiotics along with their targets and triggered resistance mechanisms as presented by Walsh (2003).

Antibiotic	Target	Resistance mechanism
Cell wall:		
β-lactams	Transpeptidases/transglycolases (PBP's)	β-lactamases, PBP mutants
Vancomycin	D-Ala-D-Ala termini of lipid II	Reprogramming of D-Ala-D-Ala
Teicoplanin	D-Ala-D-Ala termini of lipid II	Reprogramming of D-Ala-D-Ala
Protein synthesis:		
Erthromycins	Peptidytransferase/ribosome	rRNA methylation/efflux
Tetracyclines	Peptidytransferase	Drug efflux
Aminoglycosides	Peptidytransferase	Drug modification
Oxazolodinones	Peptidytransferase	Unknown
DNA-replication/repair:		
Fluoroquinolones		Gyrase mutations

1.2.2 Risks imposed by achieved antibiotic resistance in microorganisms

The introduction of clinical and prophylactic application of antibiotics in humans and in factory farmed animals more than half a century ago has triggered the evolution of antibiotic resistance in pathogens and non-pathogens respectively. Studies have shown that the discontinuation of a certain antibiotic can lead to a decrease in resistance from 80 % to 8 %. However, despite the lowering of percentage of resistant bacteria, the trait is established in the gene pool and is very unlikely lost. Hence, if necessary, genes may be transferred to a larger percentage of the species at re-introduction of the antibiotic and thus hinder the successful treatment of disease if needed (Seveno *et al*., 2002).

The human-animal link is especially interesting to look at in the arising of antibiotic resistance in pathogens since it is recognized that farmed animals and manure from these places are pools that harbor resistance genes (Carattoli, 2008). The intestines of these animals are found to be the reservoir of zoonosis such as *Salmonella spp., Campylobacter spp.,* and certain *E. coli.* Studies have found resistances to multiple antibiotics, including extended-spectrum-β-lactam-antibiotics, in Vero-toxin producing *E. coli,* including the sero-type causing the outbreak in Germany in the spring of 2011 (Bielaszewska *et al.,* 2011). Vero toxin-producing *E. coli* have also frequently been shown to be resistant to potassium telllurite (Bielaszewska *et al.,* 2005).

Due to frequent exposure to antibiotics, bacteria residing (natural microbiota as well as potential pathogens) in the gut of the animals will become resistant. Through gene transfer, resistance will be shared among by pathogens, opportunistic pathogens or non-pathogens, within the same species and between species and genera. Resistance is thus transferred among all species in the gut and can reach humans either through contamination during slaughter or through being excreted through the animals' feces and adhere to crops all the way through the production line either through untreated manure or irrigation water (Seveno *et al*., 2002).

Bacteria from animal husbandry are transferred through animal workers and are able to reach health care clinics via the community. The other main path is where resistant bacteria are carried from animal husbandry via manure to soil or other environmental sites such as irrigation water and crops. From the environment, it travels to the community and end up in hospital settings (Heuer *et al.*, 2011).

Antibiotic resistances in opportunistic pathogens also play an important role in this context. The scenario could be that this bacterium is part of a person's microbiota and will cause disease in the event the person becomes immune-compromised or this bacterium reaches an already immune-compromised person and will then cause disease. The former is often seen in e.g. infections of surgical wounds (Seveno *et al*., 2002).

In 2007, estimates show that 25.000 people died within the EU due to infection caused by multi-resistant bacterial strains and 2.5 million extra hospital days were needed, which, correspond to in-hospital expenses of about EUR 900 million (ECDC/EMEA, 2009).

1.2.3 Plasmids and horizontal gene transfer

A number of plasmids encoding antibiotic resistance have been discovered (r-plasmids). These plasmids are transferred either through conjugation or move to the new host cell as the old cell dies and disintegrates. In order to travel between cells, the plasmid is required to display a set of genes in a *tra*- region of its circular formation. These genes will encode for proteins that will enhance the DNA-transfer between cells (Madigan *et al.*, 2012). Most plasmids are determined to only transfer within the same genus, i.e. *Enterobacteriaceae,* although conjugative plasmids from *Pseudomonas spp.* have a broad host range and may function in far-related cells as well (Madigan et al., 2012).

It has been showed in studies that this type of resistance is yet another way for bacterial species to survive in environments where their competitors produce this substance. Hence, this phenomenon has most likely existed long before the clinical use of antibiotics started, in order for microbes to survive hostile environments (Hogg, 2005).

The evolutionary functions of horizontal gene flow are considered a main pathway in order for bacterial communities to adapt and survive in times of changed circumstances (Shape, 1994). Since bacteria lack the ability to reproduce sexually and therefore do not exchange genetic material the same way eukaryotes do, one way to adapt in order to survive in a changing environment is through the exchange of genes by means of what is called horizontal gene flow (HGF), i.e. through the exchange- or uptake of plasmids (Heuer & Smalla, 2007).

1.3 Veterinary use of antibiotics

A number of studies point out the important role played by antibiotics used in the treatment of husbandry animals. Antibiotics are, in these settings, used as for therapeutic purposes as well as growth promoters. Animals carrying infections are treated with full-dose during the required time. However, sub-therapeutic, long term treatments are in many settings administered as growth promoters and as a prophylactic measure and distributed through the feed, for the whole herd (Dibner & Richards, 2005). The latter creates optimal conditions for the rising and spreading of resistance (Witte, 1998).

In literature, examples of the rising resistance related to farm factories is illustrated by the following example; fluoroquinolones have been used in the U.S. for treatment of humans since 1986, but resistance in *Campylobacter* was not seen until it was authorized for veterinary treatment of animals in 1995. Similar types of scenarios have been seen in other countries as well, e. g. Spain and the Netherlands. In countries that have not authorized the use of fluoroquinolones in animal settings, i.e.

Australia, no resistance is seen in domestically acquired infections (Molbak, 2004; Gupta *et al*., 2004). In addition, the spreading of resistance from animals to humans is further supported by a German study where pigs, in the 1980s, were fed antibiotics exclusively used for animals. After a number of years, resistant bacteria were isolated from the gut of healthy pig-farm workers, as well as from their family members and other healthy members of the community. Similarly, *E. coli* strains were also isolated from individuals with urinary tract infections and the resistance was seen in pathogens such as *Shigella* and *Salmonella* (Molbak, 2004).

The use of antibiotics for growth-promotion of animals was banned in the European Union in 1996 (IP/05/1687, 2005), but growth-promoting antibiotics are still applied liberally and with less stringent regulations outside the European Union (Dibner & Richards, 2005). However, due to i.e. travelling and trade with animals, resistance ultimately becomes a world-wide phenomenon (Tängdén *et al*., 2010).

After the banning of antibiotics as growth promoters, a decrease of resistance has been seen in animals, humans and food products within the EU (Molbak, 2004). The decrease of resistance indicates that antibiotic itself functions as a selective force for these microbes. Since the genes encoding resistance are costly for the organism to hold on to and the bacteria are given no benefits for keeping the gene/genes when not exposed to the antimicrobial agent. According to statistics, the consumption of antibiotics in animals in Sweden has decreased from 50 000 kg to 16 000 (2005). Treatment of groups of animals has decreased and the treatment of individuals has increased. However, the use of fluoroquinolones and β-lactam antibiotics has increased (Bengtsson *et al*., 2006).

In the present study, the water tested for antibiotic resistance is located close to a pig farm. According to *Sveriges Veterinärmedicinska Sällskap* (Lindberg & Wallgren, 2011), the most frequently used substances within pig farming are trimethoprim-sulfa (diarrhea), benzyl-penicillin (respiratory issues) and tetracycline (farrowing fever). Bengtsson *et al.* (National Veterinary Institute, 2006) published data regarding sales of antibiotics for veterinary use during the years 2000-2006. According to this article, penicllin G was the most frequently sold antibiotic in 2005 (7 571 kg), followed by sulphonamide (2 535 kg), macrolides (1 080 kg) and fluoroquinolones (184 kg).

1.4.1 β -lactam-antibiotics

According to the WHO, cephalosporins are considered one among the most important groups of antibiotics. It is a β-lactam-substance which carries a β-lactam-ring (figure 1) that is more stable than previous generations of β-lactam-antibiotics and is therefore active in a broader spectrum as well

(Egervärn & Lindmark, 2009). In general, β-lactam-antibiotics act by inhibiting cell wall synthesis of the peptidoglycan layer of the bacterial cell wall through binding irreversibly to the active site of the Penicillin Binding Protein (PBP) otherwise responsible for the final cross linking of the peptidoglycan layer. Thus, this interruption of cross linking triggers an excessive amount of peptidoglycan precursors which signals the bacterial cell to destroy the left over peptidoglycan strands without the production of new ones, a process which will lead to cell death (Nordmann *et al*., 2012). Structurally, all β-lactam antibiotics have a β -lactam ring, but are divided into four different groups depending on different bacterial targets (table 2); penicillinases break down penicillins but do not degrade cephalosporins, azotrezam or carbapenems. Cephalosporinases do only affect cephalosporins whereas ESBLs degrade all β-lactams with the exception of carbapenem, which is only affected by carbapenemases (Nordmann *et al*.; 2012, Cha *et al*., 2008). In order to trespass potential clinical resistances, substances such as clavulanic acid, sulbactam or tazobactams can be added to the β-lactam antibiotic. These substances have scarce antimicrobial activities, but carry a β-lactam ring and will, when added together with the antibiotic, become the target in place of the antibiotic thus leaving the antibiotic active (Nordmann *et al*., 2012).

Bacteria that have acquired the ability of producing β -lactams have, through different mechanisms, the ability to catalyze the β -lactam in question. The β –lactamases are divided into four classes (A-D) depending on their different approaches in the degradation of β –lactam antibiotics (Cha *et al*., 2008) and also have a preference in the degrading of certain β-lactams.

Table 2. Each of the four classes of β-lactams is specialized in the degradation of certain types of antibiotics, as is shown in the figure (Cha, *et al.,* 2008).

β-lactam	**Antibiotic type/types**
Class A	Penicillins
Class B	Cephalosporins
Class C	Broad range; including Carabapenems
Class D	Oxacillins

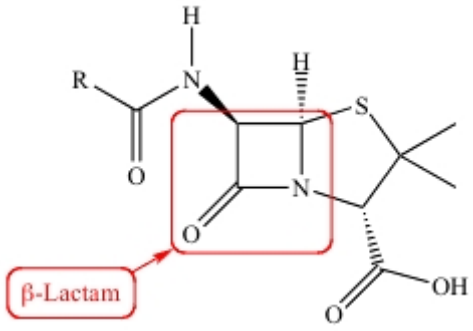


Figure 1. The molecular structure of the β-lactam ring present in all β-lactam antibiotics is encircled in the picture (Illustrated Glossary of Organic Chemistry, 2012).

1.4.2 Extended spectrum β-lactamase (ESBL)

ESBL-producing bacteria break down the β-lactam ring that in all β -lactam antibiotics, i.e. penicillins, cephalosporins and is also resistant to carbapenems (Walsh, 2003). These strains are generally susceptible to treatment using β-lactam substance in combination with β-lactam-inhibitor. However, strains displaying resistance toward inhibitor substances, rendering the organisms even more resistant are also seen (Bradford, 2001).

Genetically, this resistance has its origin on a plasmid and is often accompanied by additional non- β-lactam resistances (Mena *et al*., 2006). ESBL-producing *Enterobacteriaceae* was first found in Western Europe in the nineteen sixties, which is also where this group of antibiotics were initially put in use for clinical treatment. However, it did not take many years before they were found in North America and Japan as well (Bradford, 2001). In 2007, duty to report any finding of β-lactam-producing *Enterobacteriaceae* was implemented in Sweden (SMI, 2011). During the first 18 months after the implementation, 2390 cases were reported to the Swedish Institute for Communicable Disease Control (SMI) and is by that stated far more spread than the multi-resistant-staphylococcus-aureus (MRSA) that has, in Sweden, been monitored closely since year 2000 (SMI, 2012). At this time, ESBL is the most frequently reported type of antibiotic resistance and the number of reported cases increases every year (Gustafsson, 2011). The resistance has been seen to be acquired in hospital settings as well as in the community (Mäkitalo & Sällström, 2012).

ESBL-producing *Enterobacteriaceae* often cause problems since they are commonly found in the gut microbiota of humans and animals and at the same time act as opportunistic pathogens, causing infections when a person becomes immunosuppressed (post-surgery, elderly people, young children, etc.). β-lactam-antibiotics are often relied upon for treatments of post-surgical infections, infections in long-time hospitalized persons and at intensive care units. Resistance found in these settings may lead to deaths due to otherwise preventable diseases and will perhaps also put limitations to

development and application of new medical techniques (Nordmann *et al*., 2012). Isolates displaying β-lactam resistance are found in burn units, in rehab facilities, urology- and neonate care units, where they in many cases hinder efficient treatment (SMI, 2011). β –lactam-resistance has, through the years, been more common in countries other than Sweden. However, increase of travelling and trade makes resistant more equally dispersed throughout the world (Molbak, 2004; SMI, 2011; Tängdén *et al*., 2010). A study conducted in Sweden, including 104 volunteers, showed that after travel to foreign countries, 24 % of the previously tested negative individuals were tested positive for ESBL-producing bacteria upon returning. Fecal samples revealed resistances toward amdinocillin, ciprofloxacin, ertapenem, fosfomycin, gentamicin, imipenem, meropenem, nitrofurantoin, piperacillin-tazobactam, tobramycin, and trimetoprim-sulfamethoxazole. In all cases, negative pre-travel samples were confirmed (Tängdén *et al*., 2010). Additionally in Denmark, every third imported chicken has been proven carriers of gram-negative ESBL-producing bacteria. The bacteria are killed off upon preparation of the meat, but in the case the meat is not properly handled, the risk of spreading the resistance in the environment is easily done (Gustafsson, 2011)

A higher percentage of carriers of ESBLs has been found to be correlated to increased incidence of food-borne illnesses, increased risk of outbreak of resistant infections in hospital settings, longer times of hospitalization of diseased patients and longer duration of illness in the diseased person with a greater risk of complications i.e. spreading of infection to blood stream and increase of mortality (Molbak, 2004). Infections caused by resistant agents complicate empirical treatment of the patient, prolonging the onset of successful treatment until characterization of agent has been performed (Molbak, 2004). A study executed 1984-2002 suggests the fatality of a patient infected by *Salomonella* harboring ESBLs is 4.2%, in comparison with a patient infected by a sensitive sero-type, where fatality is 0.2% (Molbak, 2004).

In this context, *Enterobacteriaceae* are especially interesting to study since they are primary colonizers of the human and animal gut and are therefore exposed to all antibiotics administered orally. Thus, they serve as main carriers of resistance gene pools and will be capable of transferring this resistance to other bacteria in the normal flora as well as to human pathogens such as certain *E. coli, Campylobacter* and *Salmonella*.

The genes controlling degradation of β-lactams are, as with other β-lactam resistance, chromosomally encoded, plasmid-borne or residing on different transposable elements (SMI, 2011; Cha *et al*.) and are often found on gram negative bacteria, commonly in *Enterobacteriaceae* species such as *E. coli, Citrobacter* or *Klebsiella.* Gram-positive bacteria often rely on other mechanisms by which they defeat antibiotics (Cha *et al*., 2008). Carriers are often healthy and thus, probably,

unaware of themselves carrying- and potentially spreading resistant bacteria (SMI, 2011). These bacteria are predominantly of the genus *Enterobacteriacea* (*E. coli, Klebsiella, Citrobacter,* etc.). According to SMI, the most commonly reported infection caused by ESBL-producing gram negative bacteria is urinary tract infections, especially in older women (2008). ESBL-resistant *E. coli* and *Klebsiella* also tend to be involved in cases of pneumonia and sepsis (Egervärn & Lindmark, 2009).

1.5 Characterization of resistance genes

Genes encoding extended spectrum β-lactamases are divided into three main groups: CTX-M, TEM and SHV. In clinical practice, the diagnostic methods in use detect a) the presence of the ESBL-producing bacterium, and b) what group the resistant strain belongs to. The molecular methods can be faster administered than traditional, culture-based methods and can enable rapid onset of correct treatment of the patient (Pitout & Laupland, 2008). Furthermore, it is estimated that only about 1 % of all bacteria in the environment are able to grow in a laboratory environment, molecular methods will provide information that could be foreseen only using culture-dependent methods (Muyzer, 1999). Woodford (2010) has published protocols for detection and characterization of these genes through PCR; these instructions were used and slightly modified for the experiments upon which this paper is based on.

1.6.1 Objective of the study

Numerous studies show that contamination of human pathogens on fresh produce can be a serious threat linked to the production of fruits and vegetables (Jones & Shortt, 2012; WHO, 2012). It has been found that the two main pathways for these bacteria to reach the produce are through irrigation water or organic fertilizers (SMI, 2012). With regard to fruits and vegetables, that are often eaten raw, the pathways of infection are not clearly established. As previously mentioned, the bacteria could travel from the manure of infected animals, via water to irrigation ponds where after reaching vegetables through irrigation water, or the bacteria can reach the environment from carrying agents such as imported goods, animals or humans (Tängdén *et al*.; 2010; Molbak, 2004; SMI, 2011).

Water ponds serve as containers of vast amounts of microorganisms, including human pathogens and do also function as a platform for exchange of genetic elements (Schwartz *et al*., 2003). Therefore, it is of interest to find out the prevalence- and levels of resistance to expect in order to determine the health status in any current surface water used for irrigation of fruits and vegetables, and accordingly, this information is also appropriate in the context of work concerning public health and in the establishment of legislation concerning water quality in these settings.

In this study, water from two irrigation water ponds in Southern Sweden was analyzed using both culture dependent and –independent approaches. The ponds studied are located in the close up-range of a pig-farm, resulting in natural run off probably reaching the water continuously. Every season for several years, the ponds have regularly been tested for detection of i.e. *E. coli* as an indicator of recent fecal contamination. According to results from these tests, currently unidentified *E. coli* frequently occur in the ponds. *E. coli* is an indicator organism of recent fecal contamination, which suggests that run off from animal manure reaches the ponds (Alsanius *et al.,* 2008).

The objective of this study is to investigate occurrence of antibiotic resistance prevalent in gram negative bacteria residing the water and to evaluate methods for detection of resistance pattern present in these irrigation water ponds.

1.6.2 Hypotheses

1. Antibiotic resistance will frequently occur in the ponds
2. β-lactamase producing bacteria are common in irrigation water
3. Resistance against commonly used antibiotics will be transferred between frequently detected bacteria in the irrigation water
4. Resistances patterns in isolated bacteria will, due to run-off from nearby animal farms, correspond to antibiotics used in animal husbandry

2. Materials and methods

2.1 Flowchart of the experimental design

Samples of irrigation water from two different ponds were used for the experiments. Two different approaches were used as described in figure 2.

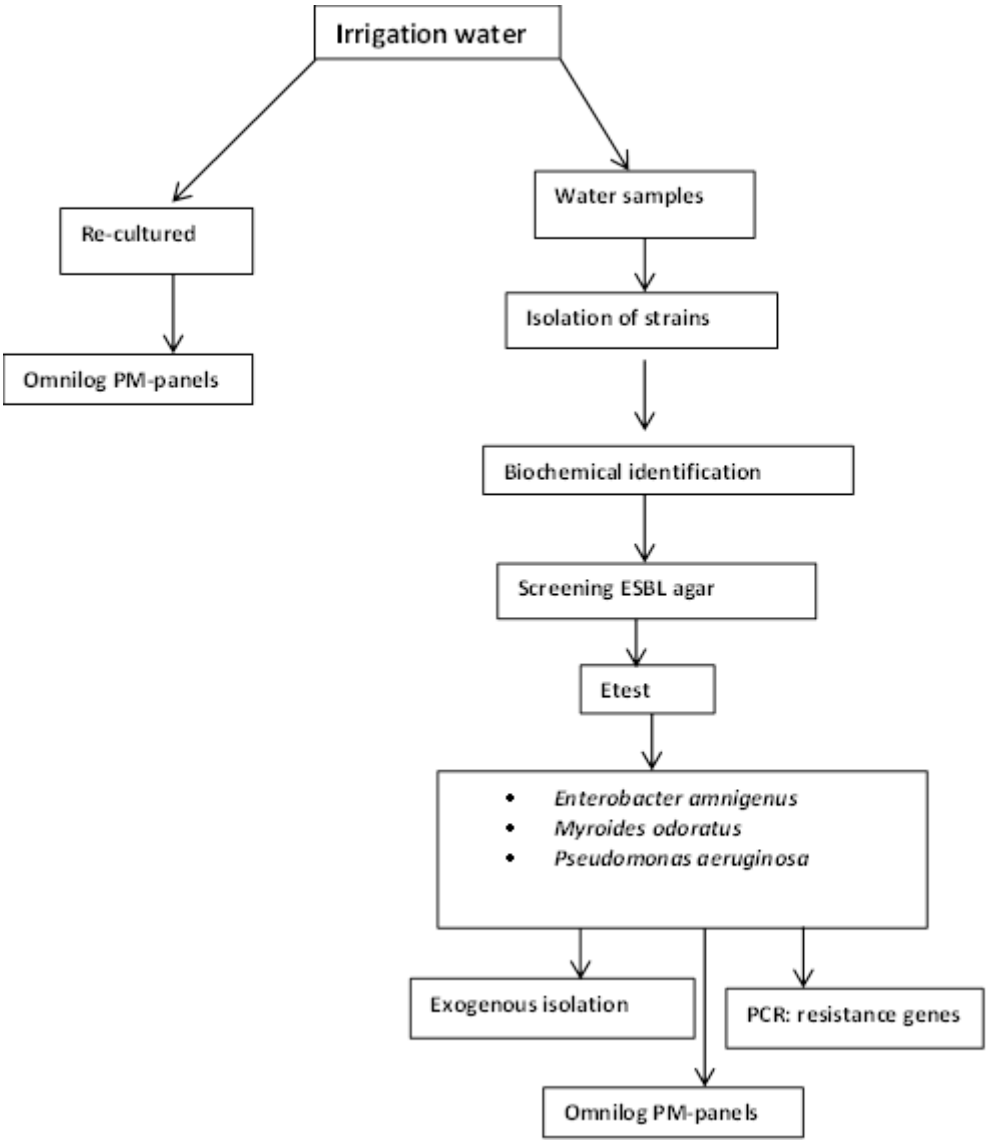


Figure 2. A flow chart of the experimental design, including the two approaches tested in the project.

2.2 Development of method for screening of antibiotic resistance in bacteria in irrigation water – screening resistance in bacterial communities using Biolog PM-panels

Water samples were taken from two different ponds (site A and site B) in Southern Sweden in October 2011 and profiled for antibiotic resistances using the Biolog PM-panels in January 23, 2012.

500 ml of water from each pond were transferred to centrifuge tubes and centrifuged at 4000 rpm for 40 minutes after which the pellets were re-suspended in 5 ml Mueller Hinton broth and incubated over night at 32°C on a shaking table. Day two, the samples were centrifuged at 4000 rpm, supernatant was decanted and pellet was re-suspended in 1 ml sterile 0.85% NaCl-buffer.

Day two, 2.7 ml sterile water was mixed with 13 ml IF-0 inoculating fluid in a sterile 20 ml glass tube and cells from the irrigation pond suspension to be tested was added until reaching a turbidity of 42%. In another sterile 20 ml glass tube, 2.3 ml sterile water was mixed with 0.35 ml Dye A, 13 ml of IF-0 and 3.2 ml of suspension with tested strain. Turbidity was checked to be 85%. To the 125 ml IF-10 inoculating fluid, 600 ml of the solution including sterile water dye A, IF-0 and irrigation pond-suspension was added. Using a multi-channel pipette, selected panels were inoculated with four replicates each, and loaded to the Biolog-incubator. Plates were incubated for 96 hours, with spectrophotometric readings determining the growth of each isolate and well every 15 minutes. The compounds analyzed were amikacin, gentamicin, kanamycin, penicillin G, trimethoprim, potassium tellurite, ciprofloxacin and amikacin.

2.3.1 Characterization of irrigation water microbiota

Water samples from two different irrigation water ponds were collected in September of 2011. From each pond, 1 l was sampled in two 500 ml sterile plastic flasks. The flasks were kept at 4 °C until analyzed 3-4 days later. A dilution series was made of the water and for each chosen dilution step (table 3), 200 µl of water was inoculated to each plate, and the plates were incubated according to table 3. After incubation, colonies were harvested and pure cultured on the following media: 0.1 TSA, mEnterococcus, LB, VRBD and Rainbow Agar, according to instructions provided by manufacturer.

2.3.2 Isolation of gram negative bacteria

After incubation of the plates, a total of 177 isolates were selected and pure cultured. Chosen colonies were picked with the aim of collecting as many species as possible, hence individuals displaying various visual characteristics, such as color or shape.

After pure culturing, a loop-full of each isolate was transferred to 5 mL single strength TSB in a sterile tube and cultured for 18 hours on a shaking table in room temperature. The tubes were centrifuged at 2000 rpm for 6 minutes at 4°C after which the supernatant was decanted. Pellet was re-suspended in 0.5 mL double strength TSB and 0.5 ml sterile glycerol. The vials were vortexed and strains were stored at - 80° C.

2.3.3 Biochemical identification

Fresh overnight cultures were prepared on 1.0 x TSA according to the Omnilog protocol and incubated for 24 hours at 33° C. Each pure colony was transferred to IFA inoculating fluid until reaching a turbidity of 90-98 % and inoculated on Biolog GENIII-plates. Panels were run 48 hours using the Omnilog system of comparing biochemical footprints spectrophotometrically to a strain library system. The results were given as percentage of similarity between the sample strain and the test strain.

2.3.4 Brilliance ESBL Agar

All isolates were tested on Brillance ESBL Agar and their individual ability to grow on this agar was noted.

Table 3. Colony colors displayed on Brilliance ESBL and their presumptive genera (Brilliance ESBL Data Sheet, Oxoid/Thermofisher).

Color	Species
Blue	*E. coli*
Green	*Klebsiella, Enterobacter, Serratia, Citrobacter*
Colorless	*Salmonella, Acetinobacter or other*
Pink	*E. coli*
Brown Halo	*Proteus, Organella, Providencia*

2.3.5 Determination of minimal inhibitory concentration (MIC) using the Etest-method

According to the lists (table 5 & 6), isolates were chosen in order to determine their individual resistance patterns with respect to β-lactam antibiotics, using Etest-sticks. Criteria for this selection were:

1. Isolate show high percentage similarity when analyzed in the Omnilog
2. Isolate is of species well known for abilities of harboring resistance

50 µl of cells were transferred from the cryo vial of each isolate to be tested and grown in 5 ml of Mueller Hinton broth over night at 35°C on a shaking table. After incubation, 200 µl of each strain was inoculated onto individual Mueller Hinton plates. For all isolates, test-strips according to table were applied and all plates were incubated over night at 35°C after which results were summarized.

Table 4. All beta-lactam antibiotics tested through the Etests presented in this table.

Test-sticks used in Etest:
Amikacin
Ampicillin + Sulbactam
Ciprofloxacin
Imipenem
Kanamycin
Levofloxacin
Meropenem
Moxifloxacin
Piperacillin + Tazobactam
Piperacillin + Tazobactam
Ticeralin + Clavulanat
Trimetoprim
Trimetoprim + Sulfamethoxazole

Table 5. . Identity of *Enerobacteriaceaceae* isolates that were profiled according to their Minimial Inhibitory Concentration (MIC) in the Etest-experiment.

Isolate	Similarity to library (%)
Citrobacter freundii	93.6
Enterobacter amnigenus	87.6
Enterobacter amnigenus	80.00
Enterobacter amnigenus	79.8
Enterobacter amnigenus	79.6
Ewingella americana	77.9
Klebsiella oxytoca	76.50
Klebsiella oxytoca	75.9
Kluyvera intermedia	75.5
Pecotvorum carotovorum ss carotovorum	73.9
Rahnella aquatilis	73.7
Raoultella planticola/ornitholytica	73.60
Raoultella planticola/ornitholytica	70.30
Raoultella terrigena	69.90
Raoultella terrigena	17.40
Serratia ficaria	68.90
Serratia ficaria	68.40

Table 6. Identity of non-*Enerobacteriaceaceae* isolates that were profiled according to their Minimial Inhibitory Concentration (MIC) in the Etest-experiment.

Isolate	Similarity to library (%)
Acinetobacter baylyi	80.00
Aeromonas bestiarium	94.60
Aeromonas bestiarium	84.70
Myroides odoratus	80.04
Pseudomonas aeruginosa	90.6
Pseudomonas putida	61.5
Pseudomonas veronii DNA-group 10	81.80
Sphingobacterium spiritovorum	81.70

2.3.6 PCR for determination ESBL-resistance genes

Isolates with resistance characterized through the Etest-method were also tested molecularly through PCR, looking for matches of the genes CTX-M1 (Woodford, 2010), CTX-M2 (Woodford, 2010) and SHV (Monstein, 2007) (table 7-9).

Table 7. Isolates used when screening for the CTX-M1-gene.

Isolate No.	ID
134	*Pseudomonas marginalis*
47A	*Serratia plymuthica*
6	*Serratia ficaria*
61	*Raoltella planticola/ornitholytica*
42A	*Pectovorum carotovorum ss carotovorum*
119A	*Aeronomas veronii* DNA group 10
130	*Pseudomonas alcaligenes*
37	*Sphingobacterium spiritovorum*
33A	*Aeromonas bestiarum*
110	*Acinetobacter ursingii*
58A	*Pseudomonas putida*
11B	*Klebsiella oxtyoca*
23	*Aeromonas bestiarum*
18	*Acinetobacter baylii*

Table 8. Isolates used for the CTX-M2-gene screening.

Isolate No.	ID
23	*Aeromonas bestiarum*
18	*Acinetobacter baylii*
11B	*Klebsiella oxtyoca*
58A	*Pseudomonas putida*
110	*Acinetobacter ursingii*
119A	*Aeronomas veronii* DNA group 10
42A	*Pectovorum carotovorum ss carotovorum*
61A	*Raoultella planticola/ornitholythica*
61	*Raoltella planticola/ornitholytica*
6	*Serratia ficaria*
56	*Pseudomonas fluorescens*
47A	*Serratia plymuthica*
134	*Pseudomonas marginalis*
33A	*Aeromonas bestiarum*
37A	*Sphingobacterium spiritovorum*
97	*Citrobacter freundii*
130	*Pseudomonas alcaligenes*

Table 9. Isolates used for the SHV-gene screening.

Isolate No.	ID
7	*Rahnella aquatilis*
10	*Myroides odoratus*
15	*Myroides odoratus*
46B	*Enterobacter amnigenus*
66A	*Kluyvera intermedia*
68	*Raoultella terrigena*
88A	*Enterobacter amnigenus*
89A	*Raoultella terrigena*
96B	*Citrobacter freundii*
111	*Ewingella americana*
120	*Pseudomonas aeruginosa*

For CTX-M1 and CTX-M2, the following protocol was used: 5 min at 94°C, 25 s at 94°C, 40 s at 52°C, 50 s at 72°C and 6 min at 72 °C. The program was repeated from '25 s at 94 °C' for 30 times (table 14). For SHV-gene-targeting, the following protocol was used: 95 °C for 15 min, 94 °C for 30 s, 56 °C for 30 s, 72°C for 2 min and 72 °C for 10 min (table 15). The program was repeated from '94 °C for 30 s' for 30 times. All primers are listed in table 10.

Table 10. The primers used for the molecular analyses are listed in the table.

Primer-set	F-primer	R-primer	No of base pairs
CTX-M1	5`-AAA AAT CAC TGC GCC AGT TC-3´	5´-AGC TTA TTC ATC GCC ACG TT-3´	415
CTX-M2	5´-CGA CGC TAC CCC TGC TAT T-3´	5´-CCA GCG TCA GAT TTT TCA GG-3´	552
SHV	5´- ATG CGT TAT ATT CGC CTG TG-3	5´- TGC TTT GTT ATT CGG GCC AA -3´	747

After amplification through the PCR-program, samples were run on an agarose gel. Bands were compared to a positive control.

2.3.7 Exogenous isolation of plasmids

In order to further analyze plasmid-borne resistance, a technique of transferring plasmids to a recipient strain through co-incubation (exogenous isolation) was utilized. Day 1, the recipient strain (*E. coli* CV 601 GFP, resistant to ampicillin and kanamycin) was grown in 20 ml LB-broth supplemented with rifampicin (50 mg/l) and kanamycin (50 mg/l) and the donor, *Pseudomonas aeruginosa* (isolate no. 120; resistant to ampicillin), was incubated in 5 ml Mueller Hinton broth

supplemented with ampicillin (256 mg/l: according to level of resistance determined by Etest). Day one, 1 ml of the recipient strain was moved to a sterile Eppendorf-tube and centrifuged at 4000 rpm for 5 minutes after which it was re-suspended in 0.1 x TSB and washed two more times and re-suspended in 0.1 x TSB again. The donor strain was centrifuged at 4000 rpm for 10 minutes. The supernatant was removed and pellet was re-suspended in 1 ml 0.85% NaCl-buffer. Washing was repeated twice after which the pellet was re-suspended in 0.5 ml 0.1 x TSB. After the washing of the donor and the recipient, mating was performed by mixing 100 µl of each donor (six replicates) with 100 µl of recipient. Background control and control of the recipient was performed by taking 100 µl aliquots of each to individual Eppendorf-tubes. All mixes were centrifuged at 4000 rpm for 10 minutes. For each mix, Plate Count Agar-plates were prepared, each with a 0.45 µm membrane filter. After centrifugation, all pellets were re-suspended in 100 µl 0.1 x TSB and transferred to the filters. Plates were incubated over night at 28 °C. Day three, each filter was transferred, using a pair of sterile forceps, to sterile 50 ml-tubes filled with 10 ml 0.85% NaCl-buffer. Each filter was washed by vortexing the tube for 1 minute, dilution series using the washing suspension was prepared and samples were inoculated onto Mueller Hinton Agar supplemented with rifampicin (50 mg/l) kanamycin (50 mg/l) and ampicillin (256 mg/l) for the donor/recipient- and donor mixes. For the counting of the recipient, the ampicillin was excluded. Day four and day five, transconjugants were counted and checked for fluorescence.

2.3.8 Omnilog PM-panels screening resistance in bacterial isolates

Three isolates; *Myroides odoratus, Pseudomonas aeruginosa* and *Enterobacter amnigenus*, were further analyzed with respect to their resistance using the Biolog-system where each isolate is inoculated to panels with 96 wells containing various antimicrobial agents, each in four different concentrations. The primary substances of interest were amikacin, gentamicin, kanamycin, penicillin G, ampicillin, piperacillin, trimethoprim, potassium tellurite and ciprofloxacin.

The isolates were grown of Mueller Hinton Agar supplemented with antibiotics according to resistance seen in strain; kanamycin for *Myroides odoratus* and *Enterobacter amnigenus,* ampicillin for *Pseudomonas aeruginosa.* Day two, 2.7 ml sterile water was mixed with 13 ml IF-0 inoculating fluid (Biolog: 72268) in a sterile 20 ml glass tube and cells from the strain to be tested was added until reaching a turbidity of 42%. In another sterile 20 ml glass tube, 2.3 ml sterile water was mixed with 0.3 ml Dye A (Biolog: 72268), 13 ml of IF-0 and 3.2 ml of suspension with tested strain. Turbidity was confirmed 85%. To the 125 ml IF-10 inoculating fluid, 600 ml of the solution including sterile

water, dye A, IF-0 and strain-solution was added. Using a multi-channel pipette, all panels were inoculated with three replicates each, and loaded to the Biolog incubator.

Plates were incubated for 96 hours, with spectrophotometric readings determining the growth of each isolate and well every 15 minutes. With this experiment, the objective was to further screen the isolates for resistance to beta-lactam antibiotics.

2.4 Statistical analyses

Data from the Omnilog-tests for pure isolates were analyzed using ANOVA with Tukey-test by MiniTab 16 (Minitab; PA, USA). Differences were considered significant at $P < 0.05$.

3. Results

3.1.1 Omnilog PM-panels screening for resistance in community samples

The growth of community samples are summarized in figure 7. Standard variation varies greatly between different wells measured. The growth in wells containing ciprofloxacin displays a growth pattern that was differs from all other panels. Gentamicin is the only compound in the presence of which the microbiota grew as expected, with decrease of growth with increase of concentration. However, the overall resistance is high in wells containing β-lactam antibiotics; ampicillin, Penicillin G and piperacillin.

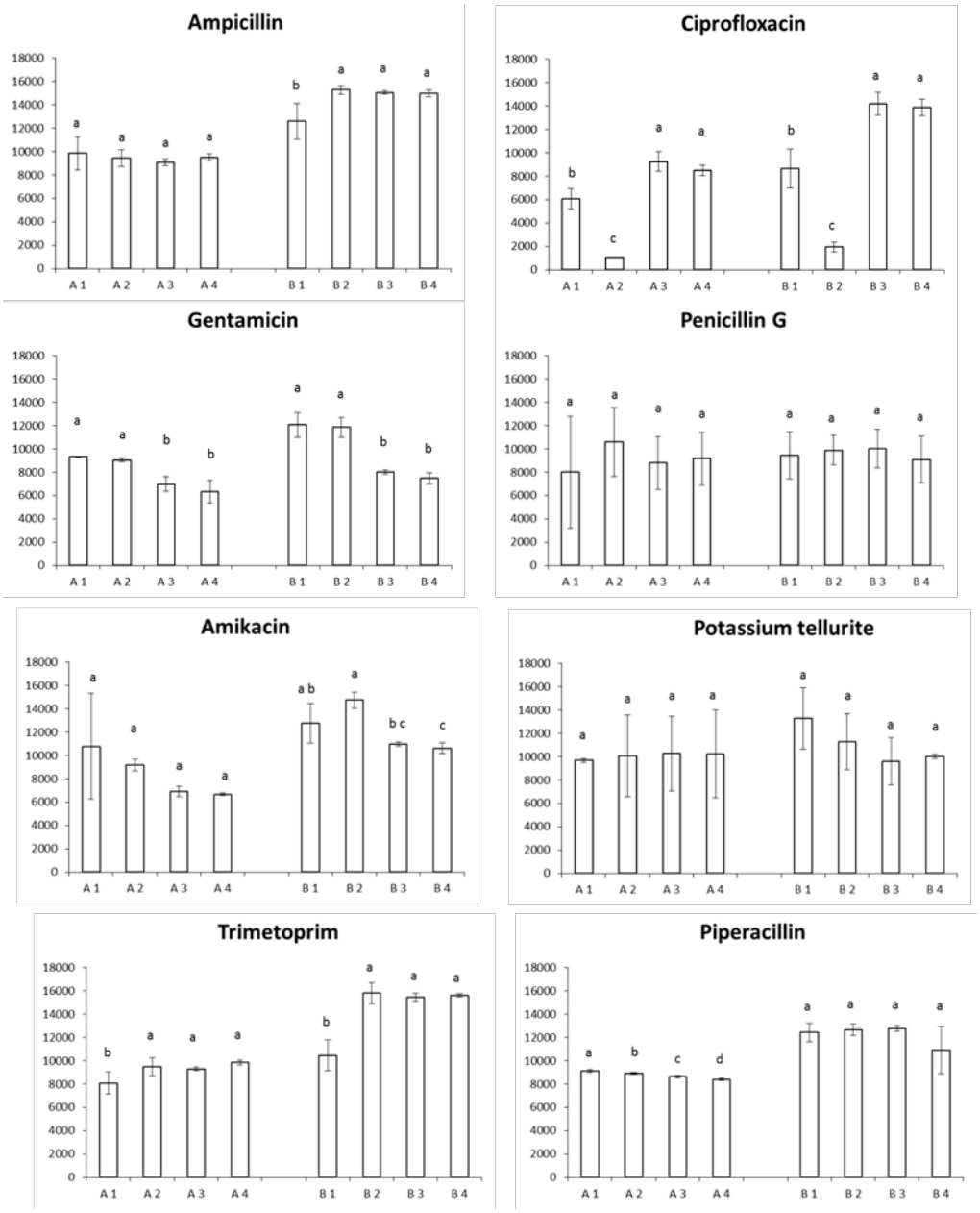


Figure 7. Re-cultured water samples were exposed to selected Omnilog PM-panels 11C, 12B, 14A, 16A and 20B, with four concentrations and four replicates per compound. Growth is expressed as the area under the curve. Y-axis is the area under the graph measured in Omnilog-units, X-axis is the wells with increasing concentrations 1-4 for pond A and pond B. Letter a-d indicate any significant differences.

3.2.1 Screening with Brilliance ESBL-agar and identification of isolates using the Omnilog GEN III-system

Out of the 177 isolates inoculated on ESBL-agar (Oxoid), a total of 175 isolates displayed growth. Isolates varied in similarity to library strain of the Omnilog-system. Strains that displayed more than 80 % similarity to library strain are shown in figure 3.

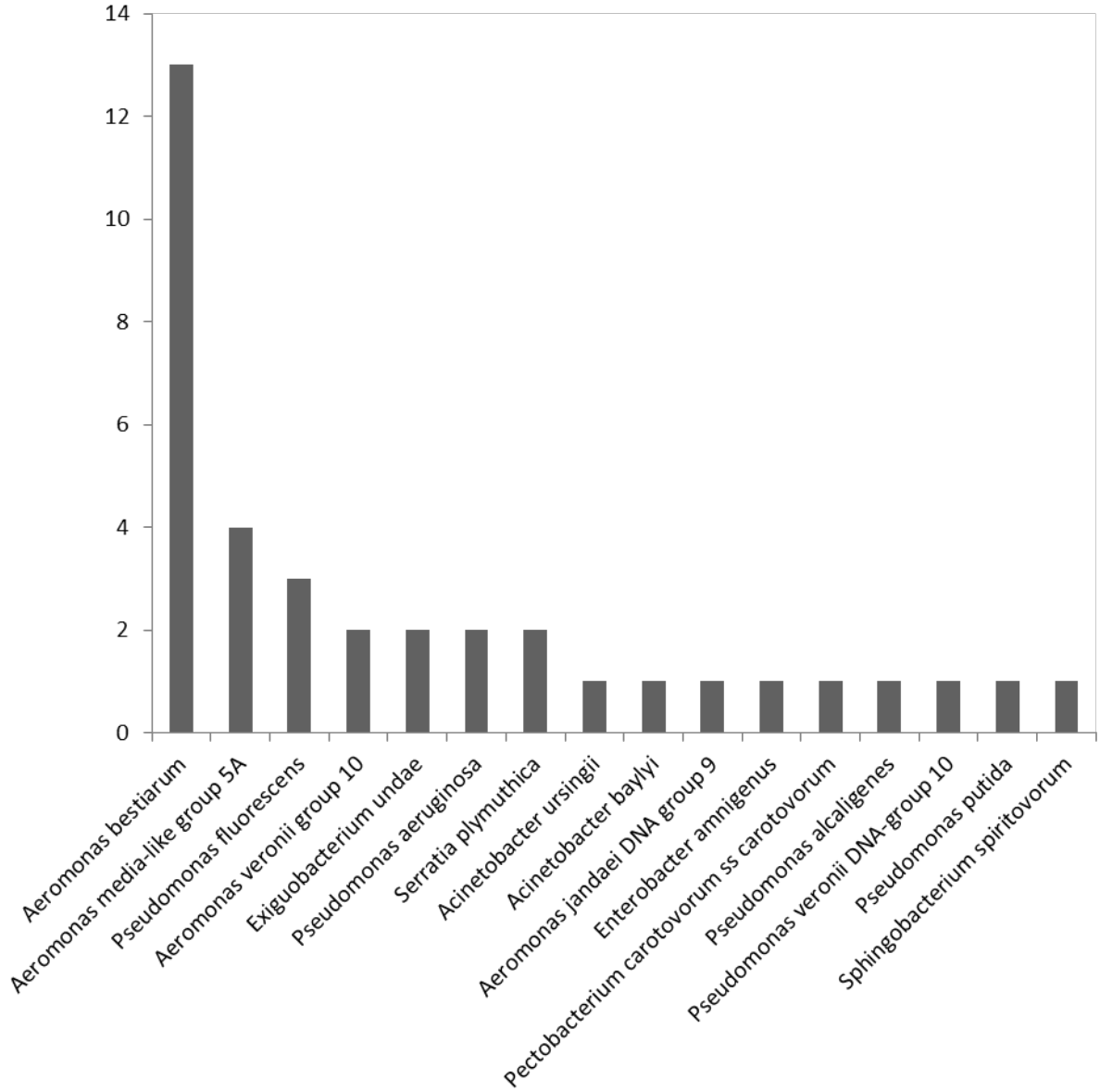


Figure 3. Out of the total of 177 isolates, 175 displayed growth on ESBL-agar. Isolates with more than 80 % similarity to Omnilog GENIII-library strain are in the diagram. The number of isolates is indicated on Y-axis.

Results from the screening of selected isolates on ESBL-medium, as well as their individual similarities when compared to library strains in the Omnilog GENIII-system is shown in table 12 and 13.

Table 12. Seventeen isolates from*Enterobacteriaceae*-genus were chosen (out of the total of 177 harvested) for further analyses after screening with Brilliance ESBL Agar. 'Similarity to library' indicates the similarity when compared to library strain in the Omnilog GENIII-system, 'ESBL' indicates ability to grow on ESBL agar.

Isolate	Similarity to library (%)	ESBL
Citrobacter freundii	93.6	+
Enterobacter amnigenus	87.6	+
Enterobacter amnigenus	80.00	+
Enterobacter amnigenus	79.8	+
Enterobacter amnigenus	79.6	+
Ewingella americana	77.9	+
Klebsiella oxytoca	76.50	+
Klebsiella oxytoca	75.9	+
Kluyvera intermedia	75.5	+
Pecotvorum carotovorum ss carotovorum	73.9	+
Rahnella aquatilis	73.7	+
Raoultella planticola/ornitholytica	73.60	+
Raoultella planticola/ornitholytica	70.30	+
Raoultella terrigena	69.90	+
Raoultella terrigena	17.40	+
Serratia ficaria	68.90	+
Serratia ficaria	68.40	+

Table 33. Eight isolates of non-*Enterobacteriaceae*-genus were chosen (out of the total of 177 harvested) for further analyses after screening with Brilliance ESBL Agar. 'Similarity to library' indicates the similarity when compared to library strain in the Omnilog GENIII-system, 'ESBL' indicates ability to grow on ESBL agar.

Isolate	Similarity to library (%)	ESBL
Acinetobacter baylyi	80.00	+
Aeromonas bestiarium	94.60	+
Aeromonas bestiarium	84.70	+
Myroides odoratus	80.04	+
Pseudomonas aeruginosa	90.6	+
Pseudomonas putida	61.5	+
Pseudomonas veronii DNA-group 10	81.80	+
Sphingobacterium spiritovorum	81.70	+

3.2.2 Etest

Results from the Etests are summarized in table 14. All resistances are marked according to the degree of resistance; i.e. red for clinical resistance, yellow for intermediate resistance. Green indicates susceptibility to substance despite results from Etest being notably high.

Table 44. In the table are studied strains and their resistances according to the Etests. Dots marked red indicate clinical resistance; dots marked green indicate susceptibility and dots marked yellow indicate intermediate resistance. Degrees of resistance were determined according to European Committee on Antibiotic Susceptibility Testing (EUCAST, 2012).

Species	Trimetoprim + Sulfamethoxazole	Ampicillin + Sulbactam	Piperacillin + Tazobactam	Ticeracillin + Clavulanic acid	Amikacin	Gentamicin	Kanamycin	Ampicillin	Piperacillin	Ciprofloxacin	Levofloxacin	Moxifloxacin	Imipenem	Meropenem	Trimetoprim
Enterobacter amnigenus (68.9% similarity)				●			●		●				●		●
Myroides odoratus	●		●				●		●				●		●
Aeromonas. Bestiarum								●						●	●
Sphingobacterium spiritovorum			●	●											
Raoultella terrigena (73.9% similarity)		●	●					●							
Enterobacter amnigenus (80.0% similarity)			●	●											
Raoultella terrigena (79.8% similarity)		●	●												
Pseudomonas aeruginosa		●		●				●							●

3.2.3 PCR targeting ESBL-genes

The results from PCR using primers CTX-M1, CTX-M2 and SHV are shown in Figure 3, 4 and 5. Two potential hits were found among CTX-M1 primers (figure 4). No matches were found for CTX-M2- or SHV primers.

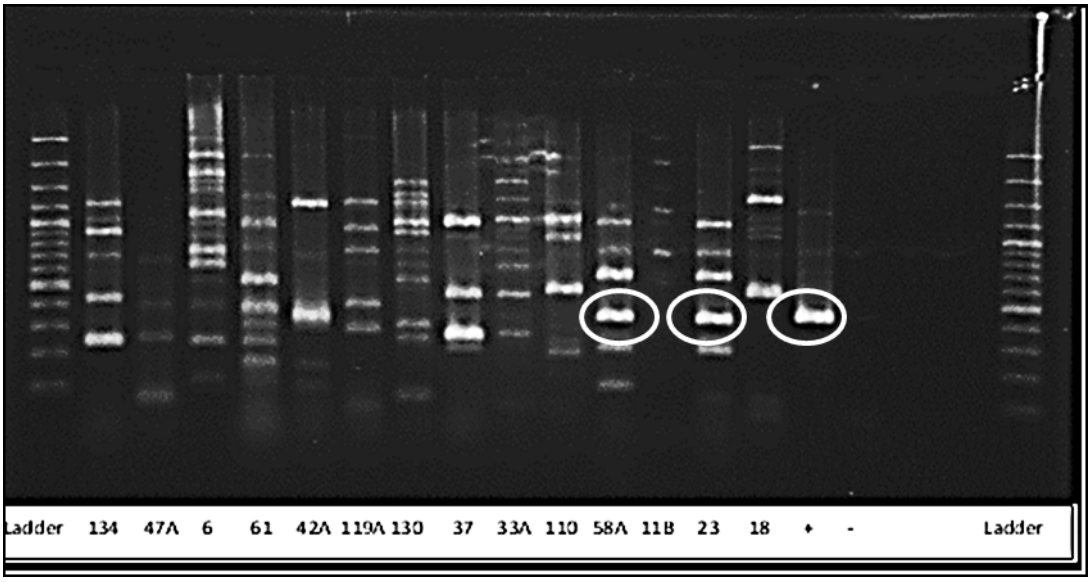


Figure 4. Gel shows results of PCR using primers CTX-M1. Potential matches, 58A and 23 are encircled in the picture. Identity of isolates are presented in table 8. The ladder that was used was O´geneRuler 100 bpPlus (Fermentas GmbH, St. Leon-Ro, Germany: SM1153).

3.2.4 Exogenous isolation of plasmids

During repeated attempts to isolate plasmids from the chosen isolates, *Pseudomonas aeruginosa*, and *Enterobacter amnigenus,* no plasmids were observed to have been transferred to the recipient strain.

3.2.5 Omnilog PM-panels screening for resistance in selected isolates

3.2.5.1 Enterobacter amnigenus (10B)

After the experiment was finished, a number files containing measurement were discovered to have been damaged and could not be restored. For *Enterobacter amnigenus,* data regarding piperacillin, gentamicin, oleandomycin, trimetoprim, ciprofloxacin and potassium tellurite was lost. For *Myroides odoratus,* all data was lost.

The results from the screening for resistance in selected isolates are summarized in the figure below (figure 5). Out of the tested antibiotics, the isolated *Enterobacter amnigenus* is affected the most by penicillin G. Ampicillin has least impact on the strain, which does not seem to be affected very much by increase of concentration of the substance. In the presence of kanamycin, the growth is rather stable regardless of the increase of concentration. Amikacin has a rather low impact on the growth as

well, but inhibition is seen that can be coupled with the increase of concentration of substance. Gentamicin has an increase of growth within concentration number three.

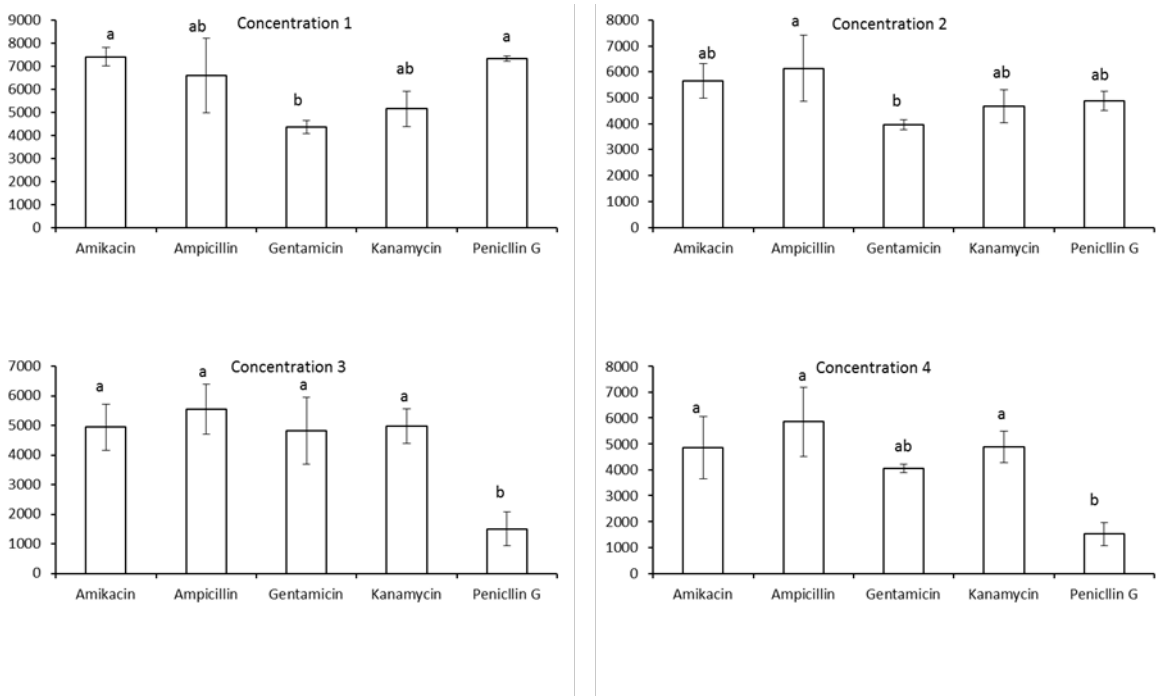


Figure 5. Impact of amikacin, ampicillin, gentamicin, kanamycin and penicillin G on *Enterobacteriaceae amnigenus*-strain 10B. The strains were exposed to compounds in the four different concentrations in Omnilog PM-antibiotic panels for 95 hours. Readings were done every 15 minutes.

3.2.5.2 Pseudomonas aeruginosa (120)

Pseudomonas aeruginosa-strain 120 was affected the most by ciprofloxacin, where the growth from the beginning is slow relative to well with other substances and is greatly diminished by increase of concentration. As for penicillin G, amikacin, piperacillin, gentamicin, trimethoprim and potassium tellurite the increased concentration seem to have no inhibiting effect on the growth; piperacillin, kanamycin and ampicillin even show an increase in growth in wells with the highest concentration of substance. In wells containing oleandomycin, the growth of strain is inhibited corresponding to increase in concentration the antibiotic substance (figure 6).

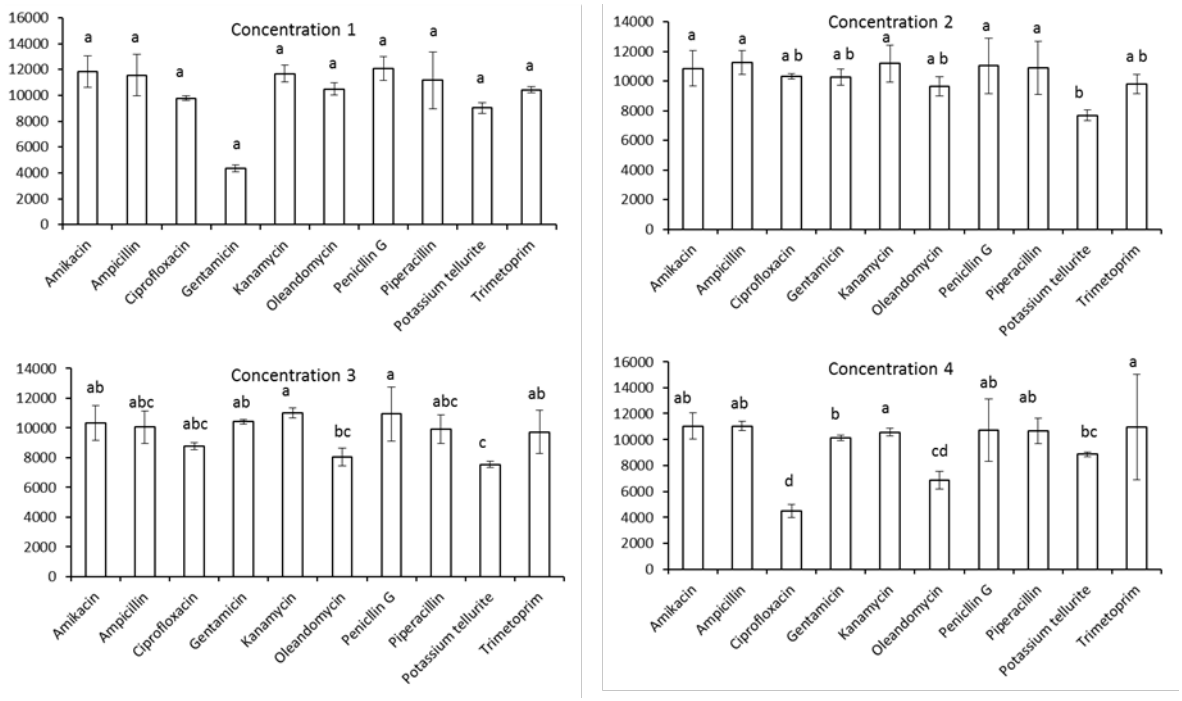


Figure 6. Impact of amikacin, ampicillin, gentamicin, kanamycin, piperacillin, gentamicin, oleadomycin, trimethoprim, ciprofloxacin, potassium tellurite and penicillin G on *Pseudomonas aeruginosa*-strain 120. The strains were exposed to compounds in the four concentrations of Omnilog PM-antibiotic panels for 95 hours. Readings were done every 15 minutes.

4. Discussion

As mentioned earlier, numerous studies have highlighted the process where intestinal bacteria from animal husbandry, through the animals' manure, reaching produce (Jones & Shortt, 2012; WHO, 2012). Irrigation water is recognized as one of the pathways through where contamination is mediated (SMI, 2012). Studies also show links between antibiotic resistance found in environmental samples and antibiotics fed to animals (Seveno, *et al.* 2002; Molbak, 2004). Resistant bacteria such as MRSA and ESBL have become a significant problem in health care settings worldwide (Nordmann, *et al.,* 2012). However, no previous studies have been made in order to investigate the potential occurrence of resistant bacteria reaching the human gut indirectly through contaminated irrigation water, which is what could be considered innovative with this study. Two different approaches were tested; one method that could be used for rapid assessment of occurrence of antibiotic resistance (community-based). The second strategy is based on several methods, including culture-dependent and –independent methods. The latter requires more time in order to perform experiments involved but has the benefit of characterize strains that would not be cultured in a laboratory environment.

4.1 Development of method for screening of resistance within microbiota of irrigation water

Results regarding the method-development for assessing antibiotic resistance within community microbiota of irrigation water show great variations in standard deviation between in the responses to the compounds tested. This suggests this method needs further optimization in order to be a helpful tool in assessment of levels of resistance in these environments. For example, it could be worth testing if only certain concentrations of antibiotics should be tested and if perhaps this level varies among different antibiotic substances. If a culture-independent approach is preferred, another method that could be used for this type of screening could be cDNA Microarray-Analysis (Curtis *et al.*, 2005).

4.2 Occurrence and frequency of β-lactamase producing bacteria

As mentioned, most of the isolates (175 of 177 tested) displayed resistance toward β-lactam antibiotics and the tested strains confirmed resistance through growth on ESBL-agar. Resistances were further characterized by Etests. An additional number of strains were tested using PCR. Out of theses strains, a few matches (1-2) were found. In order to determine the presence of the tested genes, the whole collection needs to be tested. Furthermore, as seen in the pictures of the PCR-gels, each lane displays many bands, where only a positive match or no band at all was expected. This suggests that the method needs further optimization before it is used in order to rapidly screen large amounts of isolates. However, once optimized i.e. through using new primers or reagents or adjusting the temperature or length of cycles in the PCR-program, it has the potential to be a very useful tool in this context.

4.3 Resistance against commonly used antibiotics will be transferred to bacteria of Enterobacteriaceae genus in irrigation water

Since no plasmids were transferred and the results from the molecular tests are rather incomplete so far, it is at this point not possible to determine, through the laboratory work presented in this thesis, either the presence- or frequency of horizontal gene transfer between the bacteria isolated from the irrigation ponds. However, what could be considered to speak in the favor of this type of mechanism is the large amount of isolates that displayed growth on ESBL-agar. Also, according to the results, the group of isolate that displays growth on the ESBL-agar is rather diverse, which could potentially be in the favor of occurrence of HGF since, as mentioned before; plasmids are able to move between

different species (Madigan *et al.,* 2010). Assumed that the method was performed correctly, one other possible explanation may be that the resistance is not carried on plasmids but may instead be chromosomal (Walsh, 2003). For example, *Pseudomonas aeruginosa* has been demonstrated to have a high level of intrinsic resistance due to unusually restricted outer membrane-permeability and an energy dependent multidrug efflux as well as chromosomally encoded periplasmic β-lactamases (Hancock & Speert, 2000) and as mentioned before, this bacterium is known to be very flexible in achieving resistances as well (Madigan, 2012). A recommendation for further studies would also perhaps be, when possible, to choose isolates where the intrinsic resistances are clearly established in order to obtain less ambiguity in the results. Furthermore, only two strains were tested for the exogenous isolation within the framework of this thesis. In order to determine whether the ESBL-resistance in mediated through plasmids, trials including a larger number of the isolates would be necessary but was not possible at this time due to time restraints. Further, the molecular based experiments that were made were not thorough enough in order to provide more information regarding the mechanisms of resistance. As the time was limited, a selection of isolates was tested, but *Pseudomonas aeruginosa* and *Enterobacter amnigenus* were not included to a large enough extent in order to fully determine the presence of the tested genes in these isolates. Another possible approach in order to discover the true route of resistance within specific isolates would be to use sequence-based methods.

4.4 Correspondance to antibiotics used in veterinary medicine

In *Pseudomonas aeruginosa*, an increase of growth is seen with increased concentration of ampicillin, piperacillin and kanamycin; all at higher concentrations than concentration '3'. In *Enterobacter amnigenus*, an increase is seen with increased concentration of ampicillin (after concentration '3') and gentamicin (with a peak at concentration '3'). Further analyses are needed in order to establish whether these increases of growth are an innate behavior of the isolates or if this is due to some type of error. Further, at the highest concentration, the slowest growth of *Pseudomonas aeruginosa* is seen in ciprofloxacin. According to statistics of sold antibiotics 2000-2005, published by Bengtsson et al. (2006), fluoroquinolones are the least sold antibiotics used within veterinary medicine during this period of time. This could potentially explain these figures. In the same study, penicillin G is listed as the by far mostly used antibiotic in 2000-2005. In this study, the isolated *Pseudomonas aeruginosa* is shown to grow fairly well in the presence of this substance, as opposed to the isolated

Enterobacter amnigenus, which grows slow in the presence of penicillin G, especially at the two higher concentrations. However, it has been shown that recently established *Enterobacter*-species, out of which *Enterobacter amnigenus* was one of the tested strains, it has natural susceptibility to all β-lactam-antibiotics (Stock & Wiedemann, 2002). Consequently, this information only corresponds to the behavior of *Enterobacter amnigenus* in the presence of penicillin G in this study.

Some of the data presented could potentially correspond to veterinary use of antibiotics, i.e. low degree of resistance toward ciprofloxacin which is rarely used in animals, as well as the high degree of tolerance to penicillin G in *Pseudomonas aeruginosa*. However, these figures also correspond to the use of antibiotic substances in human medicine as well, where penicillin G is the most commonly prescribed antibiotic in Sweden 2010 (Apoteksservice, 2010). At this point, it is not possible to distinguish between what resistances have arisen within the country (i.e. from use of antibiotics in human- and veterinary medicine) or what have been brought into the area from trade and travel (Tängdén et al., 2010).

5. Conclusions

1. Different types of resistances were found to be very common among isolates and in community samples.

2. Beta-lactamase-producing bacteria were found to be very common in the irrigation water.

3. Further studies need to be performed in order to confirm presence of Horizontal Gene Flow.

4. Further studies need to be performed in order to confirm if resistance patterns in the irrigation water ponds corresponds to the use of antibiotics within veterinary medicine.

6. References

ALSANIUS, B. W., GUSTAFSSON, A. K., HULTBERG, M (2010) Microbiological Aspects of Irrigation Water Quality to Field Grown Vegetables. *Acta Horticulturae 852* 53-60 (ISBN 978-90-66057-42-5)

APOTEKSSERVICE (2010) [online] Antibiotikaförsäljning öppenvård (recept+apo-dos) och slutenvård (rekvirerat) 2010. Available from: http://www.smi.se/upload/antibiotikastatistik/total/atc-grupper-06-till-10-oppen-samt-sluten.pdf [2013-01-08]

BAQUERO, F., MARTINEZ, J. L. & CANTON, R. (2008) Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology,* 19**,** 260-265

BENGTSSON, B., FRANKLIN, A. & GREKO, C. (2006) Swedish Veterinary Antimicrobial Resistance Monitoring – surveillance of resistance in bacteria from animals. *11th Symposium of the International Society for Veterinary Epidemiology and Economics.* Cairna, Australia.

BIELASZEWSKA, M., TARR, P. I., KARCH, H., ZHANG, W., MATHYS, W. (2005). Phenotypic and molecular analysis of tellurite resistance among Enterohemorrhagic *Escherichia coli* O157:H7 andSorbitol-Fermenting O157:NM Clinical Isolates. *Journal of Clinical Microbiology,* Jan 2005

BIESLAWSKA, M., MELLMAN, A., ZHANG, W., KÖCK, R., FRUTH, A., BAUWENS, A., PETERS, G., KARCH, H. (2011). Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. *The Lancet.,* September 2011, Pages 671–676

BRADFORD, P. A. (2001) Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews,* 14

CARATTOLI, A. (2008) Animal reservoirs for extended spectrum b-lactamase producers. *Clinical Microbiology and Infection,* May; 14 Suppl 5:21-4

CHA, J., LAKSHMI, P., KOTRA & MOBASHERY, S. (2008). Resistance to beta-lactam antibiotics mediated by beta-lactamases: structure, mechanism and evolution. In: Wax, R. G., Ed. *Bacterial resistance to antimicrobials*. CRC Press. Boca, FL, USA

CURTIS, K. R., ORESIC, M., VIDAL-PUIG, A. (2005). Pathways to the analysis of microarray data. *Trends in Biotechnology*, no 8, August 2005

DIBNER, J. J. & RICHARDS, J. D. (2005) Antibiotic growth promoters in agriculture: History and mode of action. *Poultry Science,* 84**,** 634-643

ECDC/EMEA (2009) [online] The bacterial challenge: time to react. A call to narrow the gap between multidrug-resistant bacteria in the EU and the development of new antibacterial agents. Available from: http://www.emea.europa.eu/ema/index.jsp?curl=pages/news_and_events/news/2009/11/news_detail_000044.jsp [2013-01-08]

EGERVÄRN, M. & LINDMARK, M. (2009) [online] Livsmedel som spridningsväg för antibiotikaresistens SLV. Available from: http://www.slv.se/upload/dokument/rapporter/bakterier_virus_mogel/Riskprofil_Livsmedel_som_spridningsvag_for_antibiotikaresistens.pdf [2013-01-08]

EUCAST (2012) [online] Clinical Breakpoints. 2012-01-01 ed., The European Committee on Antimicrobial Susceptibility Testing Available from: http://www.eucast.org/clinical_breakpoints/ [2013-01-08]

GUPTA, A., NELSON, J. M., BARRETT, T. J., TAUXE, R. V., ROSSITER, S. P., FRIEDMAN, C. R., JOYCE, K. W., SMITH, K. E., JONES, T. F., HAWKINS, M. A., SHIFERAW, B., BEEBE, J. L., VUGIA, D. J., RABATSKY-EHR, T., BENSON, J. A., ROOT, T. P. & ANGULO, F. J. (2004) Antimicrobial resistance among Campylobacter strains, United States, 1997-2001. *Emerging Infectious Diseases,* 10**,** 1102-1109

GUSTAFSSON, E. (2011) ESBL. *Smittskydd Skåne.* Lund, Region Skåne

HANCOCK, E. W. & SPEERT, D. P. (2000) Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment. *Harcourt Publishers, Ltd***,** 247-255

HEUER, H., SCHMITT, H. & SMALLA, K. (2011) Antibiotic resistance gene spread due to manure application on agricultural fields. *Current Opinion in Microbiology,* 14**,** 236-243

HEUER, H. & SMALLA, K. (2007) Horizontal gene transfer between bacteria. *Environmental Biosafety Research,* 6**,** 3-13

HOGG, S. (2005) *Essential Microbiology,* West Sussex, John Wiley & Sons, Ltd

IP/05/1687 (2005) [online] Ban on antibiotics as growth promoters in animal feed enters into effect

Brussels, European Union. Available from: http://europa.eu/rapid/press-release_IP-05-1687_en.htm [2013-01-08]

ILLUSTRATED GLOSSARY OF ORGANIC CHEMISTRY (2012) [online] Available from: http://www.google.se/imgres?q=beta+lactam+ring&hl=en&sa=X&tbo=d&biw=1366&bih=667&tbm=isch&tbnid=FFnmVWEWk4MBmM:&imgrefurl=http://www.chem.ucla.edu/harding/IGOC/B/beta_lactam.html&docid=arwfPq2ZIKf5vM&imgurl=http://www.chem.ucla.edu/harding/IGOC/B/beta_lactam01.jpg&w=239&h=166&ei=_3r1UNnOJISj4gSo0oCYAg&zoom=1&iact=rc&dur=371&sig=111734003869439805128&page=1&tbnh=132&tbnw=191&start=0&ndsp=26&ved=1t:429,r:4,s:0,i:93&tx=106&ty=73 [2013-01-13]

JONES, S. & SHORTT, R. (2012) [online] Improving On-Farm Food Safety through Good Irrigation Practices. Available from: http://www.omafra.gov.on.ca/english/crops/facts/10-037.htm [2013-01-08]

LINDBERG, M. & WALLGREN, P. (2011) Riktlinjer för användning av antibiotika till produktionsdjur. IN OLSSON, S. O., LANDIN, H. & MANSKE, T. (Eds.). Sveriges Veterinärmedicinska sällskap

MADIGAN, M., MARTINKO, J., STAHL, D., CLARK, D. (2012) *Brock Biology of Microorganisms,* San Francisco, Pearson

MAZEL, D. & DAVIES, J. (1999) Antibiotic resistance in microbes. *Cellular and Molecular Life Sciences,* 56**,** 742-754

MENA, A., PLASENCIA, V., GARCIA, L., HIDALGO, O., AYESTARAN, J. I., ALBERTI, S., BORRELL, N., PEÉEZ, J. L. & OLIVER, A. (2006) Characterization of a large outbreak by CTX-M-1-producing Klebsiella pneumoniae and mechanisms leading to in vivo carbapenem resistance development. *Journal of clinical microbiology,* 44

MOLBAK, K. (2004) Spread of resistant bacteria and resistance genes from animals to humans - The public health consequences. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health,* 51**,** 364-369

MONSTEIN, H. J., ÖSTHOLM-BALKHEM, Å., NILSSON, M.V., NILSSON, M., DORNBUSCH, K., NILSSON, L. E. (2007) Multiplex PCR amplification assay for the detection of blaSHV, blaTEM and blaCTX-M genes in Enterobacteriaceae *APMIS***,** 1400-1408

MUYZER, G. (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. *Current Opinion in Microbiology,* 2**,** 317-322

MÄKITALO, B. & SÄLLSTRÖM, E. (2012) [online] Kraftig ökning av MRSA under 2011. Available: http://www.smittskyddsinstitutet.se/nyhetsarkiv/2012/kraftig-okning-av-mrsa-under-2011/ [2013-01-08]

NORDMANN, P., DORTET, L. & POIREL, L. (2012) Carbapenem resistance in Enterobacteriaceae: here is the storm! *Trends in Molecular Medicine,* 18**,** 263-272

PITOUT, JDD., LAUPLAND, KB. (2008). Extended-spectrum β-lactamase-producing Enterobacteriaceae: an emerging public-health concern. *Lancet Infectious Diseases,* 159-66

SCHWARTZ, T., KOHNEN, W., JANSEN, B. & OBST, U. (2003) Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *Fems Microbiology Ecology,* 43**,** 325-335

SEVENO, N. A., KALLIFIDAS, D., SMALLA, K., VAN ELSAS, J. D., COLLARD, J. M., KARAGOUNI, A. D. & WELLINGTON, E. M. H. (2002) Occurrence and reservoirs of antibiotic resistance genes in the environment. *Reviews in Medical Microbiology,* 13**,** 15-27

SMI (2011) [online] Sjukdomsinformation om Extended Spectrum Beta-Lactamase (ESBL). Available from: http://www.smittskyddsinstitutet.se/sjukdomar/esbl/ [2013-01-08]

SMI (2012) [online] Sjukdomsinformation om Enterohemorragisk E. coli-infektion (EHEC). Available from: http://www.smittskyddsinstitutet.se/sjukdomar/enterohemorragisk-e-coli-infektion/ [2013-01-08]

STOCK, I. & WIEDEMANN, B. (2002) Natural antibiotic susceptibility of Enterobacter amnigenus, Enterobacter cancerogenous and Enterobacter sakazakii. *European Society of Clinical Microbiology and Infectious Diseases*

TÄNGDÉN, T., CARS, O., MELHUS, Å. & LÖWDIN, Å. (2010) Foreign travel is a major risk factor for colonization with Escherichia coli producing CTX-M-type extended-spectrum β-lactamases: a prospective study with Swedish volunteers. *Antimicrobial agents and chemotherapy,* 54

WALSH, C. (2003) *Antibiotics: Actions, Origins, Resistance,* Washington, D.C., ASM Press

WHO (2012) [online] General information related to microbiological risks in food. Available from: http://www.who.int/foodsafety/micro/general/en/index.html [2013-01-08]

WITTE, W. (1998) Medical consequences of antibiotic use in agriculture. *Science,* 279**,** 996-997

WOODFORD, N. (2010) Rapid Chacterization of B-lactamases by Multiplex PCR. IN GILLESPIE, S. H., MCHUGH, TIMOTHY D. (Ed.) *Antibiotic Resistance Protocols: Second Edition, Methods in Molecular Biology.* Springer Science Media

Appendix A.

Medium	Producer	Product No.
VRBD	Merck	1102750500
mEnterococcus	Neogen	7544
LB	Sigma-Aldrich	L2897
TSA	Neogen	7100
Brilliance ESBL Agar	Oxoid	PO5302
Rainbow Agar	Biolog	80102
Mueller-Hinton	Merck	105435
Bacto-Agar	BD	214050
TSB	Neogen	7164
Plate Count Agar	Merck	1054630500

Etest-stick (Biomeriéux)	Product No.
Amikacin	501350
Ampicillin + Sulbactam	501858
Ciprofloxacin	508650
Imipenem	513650
Kanamycin	523658
Levofloxacin	527450
Meropenem	513850
Moxifloxacin	529050
Piperacillin + Tazobactam	521550
Piperacillin + Tazobactam	521450
Ticeralin + Clavulanat	522650
Trimetoprim	523658
Trimetoprim + Sulfamethoxazole	524458

Product	Producer	Product No
Membrane filter 0.45 mm	Millipore	HPWP04700
O'geneRuler 100 bp Plus	Fermentas	SM11053

Omnilog-products (Biolog; Hayward, CA, USA):

Product	Product No.
IF-0 inoculating fluid	72268
Dye A	72268
IF-10	72268
GENIII-panel	91371
11C	12211
12B	12212
13B	12213
14A	12214
15C	12215
16A	12216
20B	12220