Gene expression and antibiotic resistance in Escherichia coli from Swedish inland waters

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Abstract

Extensive use of antibiotics both from human-medicine and veterinary sources are believed to provide selective pressure on bacteria that leads to an increase in antibiotic resistance in environmental waters. Contamination of antibiotic resistant microbes will raise human health risks. Escherichia coli are Gram-negative bacilli that belong to the coliform group. E. coli are used as fecal indicators organism (FIO) to determine microbial contamination and water quality. We aimed to investigate the prevalence of antibiotic resistant bacteria in Swedish inland waters and determine the response of uropathogenic E. coli to the environmental waters. Samples were collected in different locations near Örebro Sweden at 4 different time points during 2010-2011. Waters were filtered and FIO were isolated using selective medium. The highest numbers of FIO were detected for both E. coli and enterococci in the river Svartån near the effluent from the wastewater treatment plant (WWTP). Over the two years, 42% and 24% of the antibiotic resistant strains were multi-drug resistant (MDR) E. coli and enterococci, respectively. In addition, 15% of MDR E. coli were extended spectrum beta-lactamase producing and AmpC overproducing strains. A vancomycin resistant E. faecium was also identified. Tetracycline resistance was the most common in FIO isolates. Our study suggests that WWTP distributed FIO and antibiotic resistant bacteria. In a second study we analyzed for the presence of various pharmaceutical residues from lake Mälaren in Västerås Sweden. Some pharmaceutical compounds were present at detectable levels but were removed by the drinking water treatment plant. Quantitative PCR was performed to investigate the effects on genes focused on antibiotic resistance, virulence factors and stress response. Forty one-gene array was developed and tested using tetracycline treatment or environmental water. No significant difference was found when compared to controls in the gene expression profile of bacteria grown in medium prepared with sub-MIC of tetracycline or environmental waters. We concluded that the pharmaceutical levels detected did not exert any significant effects on the E. coli strain tested. From this study, we conclude that MDR bacteria may actually persist in environmental waters in what is considered as a clean urban region. Pharmaceutical pollutants in the inland water did not exert a significant effect on the E. coli, suggesting that MDR strains are released in the effluent of the WWTP rather than induced through selective pressure by the pharmaceuticals contamination.
List of studies

I. Ibrahim Elmaghani, Sirirat Poonlapthawee, Per-Erik Olsson and Jana Jass. Antibiotic resistance in fecal indicator bacteria in Hjälmaren lake system Sweden (Manuscript)

II. Sirirat Poonlapthawee, Faisal Ahmad Khan, Per-Erik Olsson and Jana Jass. Gene expression in Escherichia coli CFT073 grown in sub-MIC tetracycline and environmental waters (Manuscript)
### Abbreviation list

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
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<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
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<td>CFU</td>
<td>Colony forming unit</td>
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<td>DHF</td>
<td>Dihydrofolate</td>
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<td>DHFR</td>
<td>Dihydropteroate reductase</td>
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<td>DHPS</td>
<td>Dihydropteroate synthetase</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DWTP</td>
<td>Drinking water treatment plant</td>
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<td>EARS-NET</td>
<td>European Antimicrobial Resistance Surveillance Network</td>
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<td>ECDC</td>
<td>European Centre for Disease Prevention and Control</td>
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<td>EEA</td>
<td>European Environmental Agency</td>
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<td>EHEC</td>
<td>Enterohemorrhagic <em>E. coli</em></td>
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<td>ESBL</td>
<td>Extended spectrum beta-lactamases</td>
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<td>Etest</td>
<td>Epsilometer test</td>
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<td>FIO</td>
<td>Fecal indicators organism</td>
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<td>LB</td>
<td>Luria-Bertani</td>
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<td>MDR</td>
<td>Multidrug resistant</td>
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<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<td>NSAID</td>
<td>Nonsteroidal anti-inflammatory drug</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<td>PABA</td>
<td>p-aminobenzoic acid</td>
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<tr>
<td>PBP s</td>
<td>Penicillin binding proteins</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>qPCR</td>
<td>Quantitative PCR</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SMI</td>
<td>Swedish Institute for Infectious Disease Control</td>
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<tr>
<td>Sub-MIC</td>
<td>Sub minimal inhibitory concentration</td>
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<tr>
<td>THF</td>
<td>Tetrahydrofolate</td>
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<td>tRNA</td>
<td>Transfer RNA</td>
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<tr>
<td>UPEC</td>
<td>Uropathogenic <em>E. coli</em></td>
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<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
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<td>VRE</td>
<td>Vancomycin resistant enterococci</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<td>WWTP</td>
<td>Wastewater treatment plant</td>
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Acknowledgements

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STUDY I

STUDY II
Aim
The aim of this study was to evaluate the prevalence of antibiotic resistant *Escherichia coli* in Swedish inland waters and to validate the gene expression in *E. coli* CFT073 under environmental conditions using a qPCR array. The three objectives constructed within this aim are to:

- evaluate the population of fecal indicator bacteria in inland waters;
- identify antibiotic resistant *E. coli* isolated from environmental waters; and
- validate gene expression in uropathogenic *E. coli* under different environmental conditions.
Introduction

*Escherichia coli* are Gram-negative, facultative anaerobic bacilli belonging to the Enterobacteriaceae family. *E. coli* are members of the coliform group which ferments lactose at 35-37°C, can be found in the environment and are present in animal feces. Most *E. coli* are harmless commensal organisms that are part of the normal microflora in the gut of humans and other warm-blooded animals. However, some strains have been found to be pathogens causing severe illness that may lead to death (Edberg et al., 2000, Kaper et al., 2004). Harmless *E. coli* help the host with vitamin K-production and food absorption, however they can cause extra-intestinal infection such as urinary tract infection (UTI) caused by uropathogenic *E. coli* (UPEC) (Moreno et al., 2006, Gruneberg, 1969). Additionally, opportunistic pathogenic *E. coli* can also cause infections in immunocompromised hosts. Pathogenic *E. coli* infections occur through the fecal-oral route of transmission by consuming contaminated food and water (Blackburn et al., 2004, Gould et al., 2009). An outbreak of hemolytic-uremic syndrome in Germany was caused by Enterohemorrhagic *E. coli* (EHEC) which is one of the more severe pathogenic strains (Rasko et al., 2011). Pathogenic *E. coli* are classified according to their serological characteristics (Kaper et al., 2004). Severity of illness depends on both the host and on the virulence factors of the bacteria. The diversity of *E. coli* strains makes them a complex model for studying infectious disease and the correlation between virulence properties and consumption of microbial matter.

Coliform *E. coli* and enterococci are used as fecal contamination indicators for water quality according to the recommendations by the World Health Organization (WHO) (Sobsey and Bartram, 2003, Jin et al., 2004). *E. coli* are typically enteric bacteria that do not grow in water, however they can survive aquatic environments for up to 4-12 weeks (Edberg et al., 2000, LeChevallier et al., 1996). Water intended for human consumption should not contain any fecal bacteria. Additionally *E. coli* should not be present at more than 1,000 colony forming unit (CFU) in 100 ml of fresh water according to the definition of good water quality by the European Environmental Agency (EEA) (European Environment Agency, 2012). Pathogenic *E. coli* in environmental waters can cause waterborne diseases in humans when consumed. (Patz et al., 2008, Rosenberg et al., 1977, Olsen et al., 2002, Chalmers et al., 2000). Thus the prevalence of *E. coli* in environmental water is used to indicate the risks for public health (Ishii and Sadowsky, 2008, Edberg et al., 2000, Harwood et al., 2005). Intestinal enterococci are alternative
fecal indicator bacteria, which are used to measure fecal pollution in raw water (Harwood et al., 2005). Enterococci are Gram-positive cocci found in the intestinal flora of warm-blooded animals and they can tolerate 6.5% sodium chloride and alkaline pH conditions (Fisher and Phillips, 2009). The advantage of monitoring enterococci is that they are more resistant to chlorination and tend to survive in water longer than *E. coli* (Jin et al., 2004, Fisher and Phillips, 2009). Therefore, they have been used to test water quality for leaks after pipe repairs or the laying of new distribution systems (World Health Organization, 2011).

In a public health study, the persistence and quantification of potentially pathogenic *E. coli* and enterococci in environmental waters are used to assess human health hazards (Ishii and Sadowsky, 2008, Fisher and Phillips, 2009, Jin et al., 2004). Soderstrom et al. (2008) reported that the presence of verotoxin-producing *E. coli* in stream waters were the cause of an outbreak of foodborne disease in Sweden (Soderstrom et al., 2008). Contaminated freshwater beaches with enterococci are associated with the occurrence of gastrointestinal illness in swimmers (Wade et al., 2010). The detection of fecal indicator bacteria in environmental waters necessitates proper risk assessment and abatement procedures (Schwartz et al., 2003, Soderstrom et al., 2008). Monitoring of indicator bacteria as a measure of quality will help to protect public health (Harwood et al., 2005).

**Antimicrobial agents**

Antimicrobial agents are natural or synthetic compounds that kill or inhibit the growth of microorganisms and are used for treating infections. Antimicrobial substances include antibiotic, anti-fungal and other compound that act against microbes. The terms antimicrobial agents and antibiotics are often used synonymously. Antibiotics are commonly grouped according to their 5 basic mechanisms of action against bacterial cells (Figure-1); inhibition of cell wall synthesis, inhibition of protein synthesis, inhibition of nucleic acid synthesis, antimetabolite activity and disruption of the cell membrane (Neu, 1992, van Hoek et al., 2011).
Cell wall inhibitors

Inhibition of cell wall synthesis is the most common mechanism for antibiotic activity. This prevents the growth of bacteria by inhibiting peptidoglycan synthesis, thus leading to bacteriolysis. β-lactam antibiotics are a major member of this group, followed by glycopeptides, fosfomycin and bacitracin (Bycroft and Shute, 1985). β-lactams are a broad class of antibiotics, consisting of those antibiotics that contain a β-lactam ring in their structures. Members of this class are divided into the following groups; penicillin (penams), cephalosporins (cephems), carbapenems and monobactams. These antibiotics block cell wall biosynthesis, by inhibiting the penicillin binding protein (PBPs) crosslinking the peptidoglycan in the cell wall (Bycroft and Shute, 1985, Tenover, 2006). Gram-negative bacteria are less susceptible to this group of antibiotics because of their lipopolysaccharide outer membrane. Antibiotics are blocked by the outer membrane of Gram-negative cells (Giedraitiene et al., 2011).

Ceftazidime and cefotaxime are members of the third generation of cephalosporins, which act against bacteria by inhibiting the cell wall synthesis. Cephalosporins are originally derived from the fungus, *Cephalosporium acremonium* in 1948 by the Italian scientist Giuseppe Brotzu (Abraham, 1979). The first generation cephalosporin targeted Gram-positive bacteria, however successive generations have increased effects against
Gram-negative bacteria (Weinstein, 1980). They are used for therapy of urinary tract infections, skin and soft tissue infections and respiratory tract infections (Weinstein, 1980). Some bacteria can produce β-lactamase enzymes that cleave the β-lactam ring and then deactivate the antimicrobial activity (Tenover, 2006). An extended spectrum beta-lactamases (ESBL)-producing organism is able to inactivate the third generation cephalosporins and monobactam (Paterson and Bonomo, 2005). Furthermore, ESBL-producing organisms appear to be able to resist quinolones (Karah et al., 2010).

ESBL-producing bacteria are mostly of the Enterobacteriaceae, primarily E. coli and Klebsiella pneumoniae (Katsanis et al., 1994). ESBL-producing E. coli have 3 different resistance mechanisms. The most common is the production of a β-lactamase which hydrolyzes the β-lactam ring in penicillins, monobactams, carbapenems and cephalosporins. The second is a point mutation that results in the reduction of β-lactam uptake through the porins, transport channels located in the outer membrane (Jacoby and Medeiros, 1991), or in altered PBPs, which reduce the number of specific binding sites for the antibiotics, thus preventing antibiotic function. The third resistance mechanism is the presence of efflux pumps. This process exports antibiotics out of the cell and maintains low concentrations in the cells. These are frequently multidrug transporters that lead to multidrug resistance (The European Centre for Disease Prevention and Control, 2012, Giedraitiene et al., 2011). For example, the AcrB-TolC E. coli efflux pump is responsible for resistance to β-lactams, tetracycline, fluoroquinolones, chloramphenicol, acriflavine and trimethoprim (Giedraitiene et al., 2011).

Imipenem and meropenem are members of the carbapenems, which have broad spectrum activity against both Gram-negative and Gram-positive bacteria. In addition, they are generally resistant to the β–lactamase enzyme which is one of the β-lactam resistance mechanisms in bacteria. Carbapenems are one of the last choice drugs for treatment of ESBL-producing E. coli and K. pneumoniae infections. The use of carbapenems is increasing as a result of the rising resistance to cephalosporin antibiotics in ESBL–producing bacteria. An alternative solution for treatment of bacterial infections by β-lactamase producing bacteria is the combination of β-lactam antibiotics with β-lactamase inhibitors such as clavulanic acid (Matsuura et al., 1980). Clavulanic acid interacts with the enzyme β-lactamase by binding the serine residue, the active site
of the β-lactamase. This complex permanently inactivates the β-lactamase, and restores the activity of the β-lactam antibiotics (Matsuura et al., 1980).

**Protein synthesis inhibitors**
Inhibitors of protein synthesis can be divided into two groups according to their target, either binding to the 30S or the 50S subunit of the ribosome to inhibit bacterial translation. Protein synthesis is an essential process for bacterial replication and survival. Antibiotics in this group are mostly bacteriostatic, that is they inhibit bacterial growth. Tetracyclines and aminoglycosides are protein synthesis inhibitors, interfering with the 30S ribosomal subunit’s function by blocking the transfer of tRNA binding to 30S ribosome-mRNA complex. Tetracycline is a broad spectrum antibiotic that is used for the inhibition of growth of both Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001). Since its discovery in 1940s, tetracycline has been used extensively in human and animal therapy. It has also been used as a nutritional supplement for animals (Chopra and Roberts, 2001). Comprehensive nonmedical applications of medically important antibiotics worldwide have promoted the development of tetracycline resistant bacteria. Pumping tetracycline out of the cell through efflux pumps and modifying the target site by using ribosomal protection proteins are the mechanism of resistance in *E. coli* (Chopra and Roberts, 2001, Connell et al., 2003).

Chloramphenicol, macrolides, clindamycin, linezolid and streptogramins are proteins inhibiting antibiotics that bind to the 50S subunit of the ribosome. Chloramphenicol, introduced in 1949, is a broad spectrum and inexpensive antibiotic. It binds the peptidyl transferase component of the 50S ribosome and blocks peptide elongation. The action inhibits protein synthesis and bacterial growth (Garrett et al., 1966). Resistance in *E. coli* is through the enzymatic degradation of antibiotics and an altered outer membrane to prevent antibiotic uptake. Chloramphenicol transferase hydrolyses the antibiotics, while mutations in the chloramphenicol resistance genes (*cmlA*) and the efflux pump system increase the removal of the antibiotics (Tenover, 2006, Bischoff et al., 2002).

**Nucleic acid inhibitors**
Antibiotics that inhibit nucleic acid synthesis are divided into 2 groups, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis inhibitors. Rifampin is a member of the RNA synthesis inhibitors, while fluoroquinolones and
metronidazole are members of DNA synthesis inhibitors. Quinolones were introduced in 1962, and nalidixic acid was the first generation quinolone that was used for Gram-negative infections. The second-, third- and fourth-generation antibiotics, were developed with a aim to broaden their antibacterial spectrum and were effective against Gram-positive bacteria. Their functional effects involved inhibiting the enzyme DNA-gyrase and topoisomerase IV during DNA synthesis in bacteria (Oliphant and Green, 2002).

Ciprofloxacin is the second generation broad spectrum fluoroquinolone used for oral administration. Ciprofloxacin is frequently used to treat *E. coli* that causes urinary tract infections (UTI). However skin, lungs, airways, bones and joint infections are also treated with this antibiotic. Prolonged therapy with quinolones can cause serious adverse effects such as phototoxicity and liver enzyme abnormalities in humans (Oliphant and Green, 2002). *E. coli* resist quinolones by point mutations in the DNA gyrase subunit A (*gyrA*) which leads to an alteration of the binding site (Namboodiri et al., 2011). In Sweden, the use of quinolones has decreased during the period 2000 to 2011 according to the statistics of antibiotic prescriptions for outpatient care per 1000 inhabitants per year (Swedish Institute for Communicable Disease Control 2011, Struwe and Olsson-Liljequist, 2009). However, the prevalence of quinolone resistant *E. coli* is on the increase, and is related to the rise of ESBL-producing strains (Karah et al., 2010).

**Anti-metabolite substances**

Folic acid inhibitors are members of the antibiotics that have antimetabolite activities, including trimethoprim and sulfamethoxazole. They are often prescribed in combination, at a ratio of 1:5 (Miller et al., 2007). Their functional effects are inhibiting the enzymes involved in the synthesis of folate. Folate is used in synthesis of DNA nucleosides and thus in DNA replication and transcription in bacteria. Sulfamethoxazole targets and competes with *p*-aminobenzoic acid (PABA) in dihydrofolate synthesis (DHF). Trimethoprim inhibits dihydrofolate reductase in the synthesis of tetrahydrofolate (THF). Both of these mechanisms are essential for the production of folic acid, a vitamin synthesized by bacteria but not humans (Huovinen, 2001, Tenover, 2006). Altering the targets of these antibiotics is a resistance mechanism. This mechanism reduces sensitivity and affinity of the enzymes dihydropteroate synthetase (DHPS) and dihydropteroate reductase (DHFR) to sulfamethoxazole and trimethoprim (Huovinen, 2001, Giedraitiene et al., 2011).
Membrane disruptors
Disruption of the cell membrane in bacteria is a mechanism of certain antibiotics and thus they interfere with the cross-membrane potential. Important and essential substances needed for bacterial survival can thus leak out of the cell (Tenover, 2006, Giedraitiene et al., 2011). These are used commonly for Gram-negative bacterial infections because of their specificity to the lipopolysaccharide in the Gram-negative outer membrane. The limited usage of these antibiotics is due to their mechanism of action, causing toxicity to the mammalian host cell (Tenover, 2006). Polymyxins B, polymyxins E and colistin are members of this group (Tenover, 2006, Giedraitiene et al., 2011). Modification of the bacterial outer membrane through alteration of the lipopolysaccharide moiety leads to resistance to these antibiotics (Falagas et al., 2010).

Antimicrobial resistance and epidemiology
Selection of antimicrobial drugs in each country has been different depending on the national regulations and on the severity of the infectious disease. In Sweden, approved antibiotics introduced for treatment of infections in humans are regulated by Swedish Association of Medicine and the Swedish Institute for Infectious Disease Control (SMI) (Struwe and Olsson-Liljequist, 2009). It is known that extensive use of antibiotics leads to selective pressures on bacteria which promote the development of resistance mechanisms. Bacteria demonstrate two types of resistance; natural or acquired. Natural resistance refers to inherent resistance to antibiotics. For example, the small porin channels in the outer membrane of E. coli protect them from vancomycin, since it cannot pass through the channel and thus acts as natural resistance (Tenover, 2006). Acquired resistance is a mechanism where changes at the genomic level of bacteria produce resistance, such as spontaneous mutations or acquisition of mobile extrachromosomal DNA elements from another bacterium via plasmids, transposons or integrons (Chopra and Roberts, 2001, Tenover, 2006). Spontaneous mutations occur rapidly, but they are rare events. They are a consequence of errors during DNA replication due to inefficiency in the repair mechanisms of DNA damage in bacteria. For example, quinolone resistant E. coli are a complex result of mutations in at least seven positions in the gyrA or three positions in parC genes (Giedraitiene et al., 2011).

Vertical and horizontal gene transfer also plays an important role in the distribution of antibiotic resistant strains in a population. Resistance genes transferred to the next generation by replication is called vertical gene transfer. Horizontal gene transfer is a
process where small packages of genes are transferred from one bacterium to another of the same or different species (Silva et al., 2006). There are 3 possible classifications of horizontal gene transfer; transduction, transformation and conjugation. Conjugation is the main mechanism of gene transfer and it requires direct cell-cell contact between two bacteria to transfer a plasmid encoded with resistance genes or via conjugative transposons (Baquero et al., 2008). Transformation is a process where bacteria take-up free-DNA or plasmids from the external environment directly. Transduction occurs when bacteria-specific viruses (bacteriophages) transfer DNA fragments between two closely related bacteria (Alekshun and Levy, 2007). Transposons or ‘jumping gene systems’ are a process to transfer genes from one plasmid to another or onto the chromosome (Alekshun and Levy, 2007, Giedraitiene et al., 2011). Transposons can be transferred by all mechanisms, conjugation, transformation and transduction. Integrons are a gene capture system with specific recombination mechanisms and contains gene cassettes that encode single or more resistant genes in same region. Essentially, integrons constitute an association between three main components, an enzyme, integrase, coded by integron’s genes called \textit{int}l, a specific recombination site called \textit{att}I, and a promoter for initiating gene transcription (Giedraitiene et al., 2011, Alekshun and Levy, 2007). For example, class 1 integrons are widely detected in \textit{E. coli}, which stimulate resistance to trimethroprim, aminoglyconsides and sulphonamides. However the differences of gene cassette arrays in the integrons depend on the original microbe (Kang et al., 2005).

The 3 main types of mechanisms that bacteria use for defense are as follows: enzymatic modification and inactivation, decreased uptake by the bacteria, or changes in the outer membrane permeability and alteration of the targets (Giedraitiene et al., 2011, Neu and Gootz, 1996). The most common is enzymatic modification. Bacteria are able to produce enzymes to hydrolyze or restructure antibiotics. For example, \(\beta\)-lactamase hydrolyzes the \(\beta\)-lactam ring in penicillin antibiotics. In addition genes that encode \(\beta\)-lactamase can be transferred by transposons or integrons (Alekshun and Levy, 2007). Efflux pumps and the outer membrane play important roles in reducing intracellular drug concentration. Genes encoding efflux pump are found on the chromosome and plasmids that are able to spread resistant determinants among bacteria via horizontal transfer (Alekshun and Levy, 2007). The mutation of \texttt{gyrA} and \texttt{parC} are responsible for modification of the enzymes topoisomerase II and topoisomerase IV,
respectively, which lead to the failure of binding by quinolone. Quinolone resistant bacteria arise from horizontal transfer (Namboodiri et al., 2011, Tenover, 2006). Antibiotic resistance is a rapidly occurring and by now a widespread phenomenon, thus a cause for global concern in clinical treatment.

The European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) have defined multidrug resistant (MDR) bacteria as those that acquired resistance to at least one agent in three or more categories of antimicrobial substances (Magiorakos et al., 2012). Bacteria may have many different antibiotic resistance genes together with a multidrug efflux system to produce MDR strains (Tenover, 2006). MDR E. coli have been reported in food and water (Ahmed et al., 2010, Silva et al., 2006, Karczmarczyk et al., 2011). ESBL-producing E. coli strains have been examined and studied worldwide since they are considered extended MDR bacteria. The European Antimicrobial Resistance Surveillance Network (EARS-NET), a part of the ECDC reported that the third-generation cephalosporin resistant E. coli increased significantly in 14 of 28 European countries during 2007-2010. EARS-NET reported in 2011 that Sweden had the lowest resistance proportions, 3%, while Cyprus had the highest resistance proportions, 36.2%, which was likely related to the higher cephalosporin consumption of outpatients in Cyprus compared to Sweden (The European Centre for Disease Prevention and Control, 2012, Versporten et al., 2011).

Epidemiological studies by ECDC at the same time have reported that more than 25% quinolone resistant E. coli were detected in 11 European countries, and 21% of all clinical isolates in the European countries were fluoroquinolone resistant E. coli (The European Centre for Disease Prevention and Control, 2012). However, the trend for quinolone resistant E. coli in Sweden is increasing due to a rising number of ESBL-producing strains (Karah et al., 2010). Antibiotic resistant E. coli is a major problem hindering the treatment of infectious diseases. Resistance against any antibiotic develops as a result of evolution of the bacterial genome under continuous selective pressure of that particular antibiotic (Baquero et al., 2008, Hernando et al., 2006, Schwartz et al., 2003). Alternative antibiotics are under development, while the genetic resistance capability of microbes threatens us with a lack of antimicrobial substances to protect the public in the future.
Pharmaceutical pollution in environmental waters

The benefits of pharmaceuticals used for treatment of different diseases in humans and animals are well known. They are also used as nutritional promoters in agriculture in some countries. Extensive use and misuse, including improper use and overdose, and disposal of unused drugs can increase the pharmaceutical pollution in the environment (Bendz et al., 2005). Many pharmaceuticals are not fully metabolized and are thus excreted into the wastewater system (Hernando et al., 2006). Pharmaceutical residues and antibiotics in waters have been studied worldwide (Xi et al., 2009, Pei et al., 2006, Kummerer, 2003, Hernando et al., 2006). However, little is known about their persistence, effects correlating with the released pharmaceuticals and antimicrobial substances into the aquatic environment. In Europe, the hazardous substances in fresh waters are regulated by the European Environment Agency (EEA) (European Environment Agency, 2011). Diclofenac is one example of a nonsteroidal anti-inflammatory drug (NSAID) which is widely used and often detected in surface waters (Loos et al., 2009). Pharmaceuticals as hazardous substances in fresh water are of concern since they have adverse effect on living organisms even after exposure at low levels (Triebskorn et al., 2007, Hoeger et al., 2005, European Environment Agency, 2011). In Sweden, pharmaceutical compounds persist in inland water in different metabolic forms (Falas et al., 2012, Bendz et al., 2005) and have shown adverse effect on the inflammatory responses of human cells in vitro (Khalaf et al., 2009). Moreover, contamination of steroidal drugs and antibiotics in the environmental water system may create selective stress on bacteria which then leads to the enrichment of antibiotic resistant strains (Kummerer and Henninger, 2003, Kummerer, 2004, Baquero et al., 2008, Schwartz et al., 2003). Falas et al (Falas et al., 2012) have studied the reduction of pharmaceuticals in contaminated waters by wastewater treatment plants (WWTP). They determined that WWTP can decrease the contamination of pharmaceuticals in the water before it is released into the environment (Falas et al., 2012).

Wastewater treatment plants

Sewage treatment plants play an important role in minimizing chemical and microbial contamination of water before it is released back into the environment (Figure 2) (Lindberg et al., 2007). Water is first sent through a coarse cleaning stage called mechanical treatment. In this stage the coarse filtration will screen and remove the particles from the waste products using grit chambers. Preliminary sedimentation by
sludge commences after filtration. The second stage is a biological stage. This stage is aerobic where the water is pumped through an aeration system together with microorganisms in active sludge. After setting, the water goes through a chemical stage which involves phosphorus and nitrogen reduction process. Flocculation of treated water is done and a last sedimentation performed. The water finally goes through a filtration process to reduce the bacterial numbers before being released into environment (Arthurson, 2008).

Figure 2. Circulation of environmental waters. The flow of inland water passes through drinking-water treatment process before supplying the consumers such as households, factories and agriculture. Waste produced will then be directed to the WWTP. Treated water will be distributed back into the environment. Waste may also be released directly without treatment to inland waters while humans and animals can consume environmental waters through recreation activities.

The operation and quality of treated water differs between each WWTP (Hendricks and Pool, 2012). Several factors decrease the quality of water released, including flooding or climate change, which increase the risks to human and animal health (Michael et al., 2013, Hendricks and Pool, 2012, Patz et al., 2008).
Methods

Water sampling

Lake water samples were collected from Örebro and Västerås, two different cities in central Sweden. Surface water samples were collected at approximately 50 cm below the surface of the water in sterile bottles. Water samples were from 6 locations in the river Svartån and lake Hjälmaren near Örebro, Sweden (Figure 3), during the summer and spring of 2010 and 2011. The second study involved the collection of water samples from surface water at Vinterviken bay by Bjönö and incoming filtrated but untreated water to Mälarenergi drinking water treatment plant in Västerås (Figure 4). All water samples were kept at 4°C until analysis was done within 48 hr.

Figure 3. A map of the sampling sites of Svartån river and Hjälmaren lake in Örebro, Sweden. Samples were collected from the river Svartån at Tekniska Kvarn (A); Svartån at Naturens hus (B) near wastewater treatment plant outlet; from lake Hjälmaren at Hemfjärden (C), the recipient of Svartån river and near the Oset bird sanctuary; in the mid part of lake Hjälmaren at Mellanfjärden (D1), connection of Mellanfjärden; (D2) Mellanfjärden and (E) StorHjälmaren, the deepest part of Hemfjärden lake. Figure extracted from Google map and modified by the author.
Figure 4. A map of the sampling sites of Mälaren lake in Västerås, Sweden. Samples were collected from (1) lake Mälaren by Bjönnö Island at Vinterviken bay; (2) incoming untreated water to Mälarenergi, the DWTP; (3) the outgoing clean drinking water. The Mälarenergi wastewater treatment plant (WWTP) is upstream in Vinterviken bay near Västerås city center and the arrow depicts the released treated effluent. Figure was modified by the author.

**Bacterial isolation**

Bacteria were enumerated and isolated using membrane filtration and grow on the selective media (LeChevallier et al., 1996). To enumerate coliform, *E. coli* and enterococci isolates, the membranes were placed onto Chromocult coliform agar (Merck, Germany) and Enterococci selective agar (Merck, Germany), respectively which are differential and selective media and cultured at 37°C for overnight. The fecal coliform and *E. coli* were quantified and reported as colony forming unit (CFU) per 100 ml of water (Byamukama et al., 2000). *E. coli* isolates were collected for confirmation by polymerase chain reaction (PCR) and used for antibiotic susceptibility testing. Bacterium isolates were stored at -80°C for future analysis.
**Susceptibility testing**

The minimum inhibitory concentration (MIC) of various antibiotics was measured using the Epsilometer test (Etest), according to the manufacturer’s instructions (BioMerieux, France). The clear zone of growth inhibition on agar plates indicated the MIC. The breakpoint for resistant, intermediate and sensitive strains was performed according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) Clinical Breakpoint v.1.3. Antibiotics from different groups that are commonly used in human and animal treatment were selected including chloramphenicol, ciprofloxacin, nalidixic acid, tetracycline and trimethoprim-sulfamethoxazole. To test for ESBL strains, the antibiotics cefotaxime, ceftazidime, imipenem and meropenem were included in study I. Furthermore suspected ESBL strains were analyzed by a confirmatory Etest with cefotaxime and ceftazidime with and without clavulamic acid (CT/CTL and TZ/TZL, respectively). ESBL strains were also verified by the department of Clinical Microbiology, Örebro University Hospital, using methods previously described (Onnberg et al., 2011).

**Primer design**

To study gene expression in *E. coli*, genes that relate to antibiotic resistance, virulence factors and stress response were selected. Specific primer pairs for PCR and quantitative PCR (qPCR) were designed using Primer3 0.4.0 (http://frodo.wi.mit.edu/) and Integrated DNA Technology PrimerQuestSM (http://eu.idtdna.com/Scitools/Applications/Primerquest/). Sequences for genes of interest were provided by the NCBI genomic database (http://www.ncbi.nlm.nih.gov/gene/). Primer sets were verified using Permier Biosoft Beacon Designer™ program (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1) to screen the specificity of the primer sets. Primer sets with reduced possibility of hairpins, primer dimer and cross dimers were selected. The primers were evaluated by gradient PCR before use in qPCR.

**Polymerase chain reaction**

*E. coli* species and the presence of antibiotic resistance genes were confirmed by PCR. Species-specific primer pairs were used to confirm *E. coli* together with universal 16S RNA as controls (Sabat et al., 2000). Chloramphenicol, nalidixic acid and tetracycline resistance genes were confirmed in antibiotic resistant *E. coli* strains. The preparation of genomic DNA was extracted by boiling several colonies in Milli-Q water. PCR
products were analyzed on agarose gel by electrophoresis and visualized by UVP transilluminator (BioDoc, Cambridge, UK).

**Sub-inhibitory tetracycline exposure**

Tetracycline was chosen as a representative of a selective pressure on bacteria in the environment. Sub-MIC of tetracycline was determined to ten times the dilutions of the MIC, 0.1 µg/ml. Uropathogenic *E. coli* CFT073 was challenged with either sub-MIC of tetracycline in Luria-Bertani (LB) broth as condition or sterilized LB broth as control, until the bacteria reached late exponential growth phase at an optical density (OD) of 1 using ultrospec 10 cell density meter (GE Healthcare Life Sciences, UK). Bacteria were pelleted and treated with RNA later solution (Ambion, USA) and kept at -80 °C for RNA isolation.

**Environmental water condition**

Incoming water from Lake Mälaren entering Mälarenergi drinking water treatment plant in Västerås, was used to provide environmental conditions to microbe. *E. coli* CFT073 was treated with either environmental waters or milli-Q water prepared with LB media (BD diagnostics, USA). The media was filter sterilized through 0.2 µm filter (Sartorius Stedium, Sweden). *E. coli* was cultured in the sterile medium until the growth reached the late exponential phase at an OD of 1 using cell density meter. Bacteria were pelleted and treated with RNA later solution (Ambion, USA) and kept at -80 °C for RNA isolation.

**Gene expression in mRNA level using qPCR**

Primer pairs were tested at 55°C, 60°C and 65°C by PCR to evaluate their suitability in qPCR. Functional primer pairs were selected and used in the qPCR array. A total of 41 genes were analysed by qPCR including 3 housekeeping genes. Uropathogenic *E. coli* CFT073 strain was selected for examination of the effect of environmental water on gene responses at the mRNA level by challenging it with two different growth conditions compared to control condition. RNA was isolated using Nucleospin RNA II (Macherey-Nagel, Germany) according to the manufacturer’s instructions. RNA concentrations were determined using the NanoVue spectrophotometer (GE Healthcare, Germany) and the integrity was evaluated by gel electrophoresis. Complementary DNA (cDNA) was synthesized by using qScript™ cDNA synthesis kit (Quanta Biosciences,
USA) according to the manufacturer’s instruction. cDNA products from 1000 ng of RNA were diluted and kept for qPCR array analysis.

Quantitative PCR was performed using Stratagene Mx3000P (Agilent Technologies, USA). cDNA was labeled and hybridized by Kapa SYBR FAST (Kapa biosystems, USA) according to the manufacturer’s protocol. The reaction mixtures were loaded by CAS-1200 PCR Setup Robot liquid handing system (Corbett life Sciences-Qiagen, Germany) to minimize technical errors. The three housekeeping genes were tested with cDNA, RNA and no template (control) as internal controls. Raw data of C\(_t\) (threshold cycle) values were used for data analysis using the \(2^{\Delta \Delta C_t}\) method (Livak and Schmittgen, 2001). The GeNorm program (http://medgen.ugent.be/genorm/) was used to estimate the stability of the housekeeping genes which were used to normalize the difference in gene expressions. Statistical analysis was done using student \(t\)-test by Microsoft Excel program. The effect on the microbe was demonstrated using GraphPad Prism5.
Results and Discussion

Study I: Antibiotic resistant fecal indicator bacteria isolated in Svartån river and lake Hjälmaren

Sweden has strict regulations for both antibiotic use and water quality as directed by Swedish Institute for Communicable Disease Control (SMI) and European Environmental Agency (EEA), respectively. The priority is to protect the public health of the community from potential pathogenic microorganism contaminating the aquatic systems. Detection of *E. coli* and enterococci isolates in the water are indicators of fecal contamination in the environment according to the World Health Organization guideline (World Health Organization, 2011). The limits for inland waters are 100 CFU/100 ml for *E. coli* and enterococci each, and 500 CFU/100 ml for total coliform according to EEA (European Environment Agency, 2012). The fecal contamination in Svartån river at Tekniska Kvarn, upstream of the WWTP, was below EEA recommendation. Hjälmaren at Mellanfjärden and Storhjälmaren which were far from the WWTP were also considered clean from fecal contamination. However, fecal indicator organisms were detected at the highest levels in Svartån river at Naturens hus followed by Hjälmaren lake at Hemfjärden directly downstream of WWTP. In addition, the greatest number of antibiotic resistant isolates was also found in these locations. Tetracycline resistance was the most common antibiotic resistance found in both *E. coli* and enterococci isolates. A multi-resistant ESBL producing and AmpC overproducing *E. coli* strain was isolated and identified from Hemfjärden in 2010. In 2011, one vancomycin resistant enterococci (VRE) strain was also identified. This study confirmed that the WWTP played an important role in distributing enteric and potential pathogenic bacteria to the aquatic environment in Sweden (Sahlstrom et al., 2004, Lindberg et al., 2007). Acquired resistance can be transfer between bacterial populations in waters, therefore this may raise a potential for human health problems (Sahlstrom et al., 2004, Kummerer, 2003, Dziuban et al., 2006). In Sweden, the incidence of ESBL- producing bacteria and VRE are increasing (Soderblom et al., 2010, Onnberg et al., 2011, The European Centre for Disease Prevention and Control, 2012). Identification of multi-antibiotic resistant bacteria in the environment forces us to be aware that this is an increasing problem even in areas considered relatively ‘clean’ in the world. Infection due to multi-resistant organisms may cause clinical problems since they are difficult to treat.
Study II: Gene expression in *Escherichia coli* treated with sub-MIC of tetracycline and environmental waters

We examined the prevalence of fecal indicator bacteria (coliform *E. coli* and enterococci) for water quality (World Health Organization, 2011, European Environment Agency, 2012) and antibiotic resistant *E. coli* in lake Mälaren from surface water in Björnö Island at Vinterviken bay and in untreated incoming water to Mälarenergi, drinking water treatment plant (DWTP) in Västerås, Sweden. Our studies have demonstrated that the water quality of environmental water was ‘excellent’ in both surface water at Bjornö in Vinterviken bay and in the untreated incoming water to the DWTP. However, one multi-antibiotic resistant and one antibiotic resistant strain were isolated from the surface waters. Hence antibiotic resistant strains can persist even in low population of fecal bacterial contamination in the waters. Pharmaceutical compounds in the environment are believed to provide a selective pressure for the development of antibiotic resistance in bacteria (Tenover, 2006, Alekshun and Levy, 2007, Mazumdar et al., 2006). Our study demonstrated that pharmaceutical compounds persisted in lake waters with detectable concentrations as others have shown (Godfrey et al., 2007, Loos et al., 2009). Caffeine, naproxen, hydrochlorothiazide, metoprolol and tramadol were present at detectable levels. To study the effect of contaminants on microorganisms, qPCR method was chosen due to its rapid, sensitive and quantitative analyses of gene expression. Thus a qPCR array was designed, to determine the effect of environmental waters on *E. coli* isolates. Specific genes that were responsible for antibiotic resistance, virulence factors and stress responses were selected. *E. coli* CFT073 was treated with either sub-MIC levels (0.1 μg/ml) of tetracycline in LB medium or incoming untreated waters to DWTP in LB medium and compared to controls. Gene expression was determined using qPCR. No significant difference in gene response was observed after treatment with sub-MIC of tetracycline or environmental waters. Pharmaceutical compounds which contaminated the water did not appear to exert a significant gene response in the pathogenic *E. coli*. Pharmaceutical contamination in the water can promote human and animal health risks however the effect of long-term exposure is yet unknown (European Environment Agency, 2011, Wennmalm and Gunnarsson, 2005, Triebeskorn et al., 2007). The antibiotic resistant strains likely originated from the WWTP rather than the selective pressure due to pharmaceutical pollutants in the water.
Conclusions

- The highest fecal \textit{E. coli} contaminated area and the presence of MDR \textit{E. coli} samples were in Svartån at Naturens hus and Lake Hjälmaren at Hemfjärden.

- ESBL/ AmpC overproducing \textit{E. coli} and vancomycin resistant enterococci have been identified from Hjälmaren lake at Hemfjärden. There are likely of human origin suggesting the WWTP contributed in bacterial contamination.

- Caffeine, naproxen, hydrochlorothiazide, metoprolol and tramadol contaminate were found in waters at detectable limits. Caffeine was found with the highest levels in the water samples.

- qPCR was used as a tool for measurement of gene expression in bacteria under different conditions. No significant gene response was observed in uropathogenic \textit{E. coli} when treated either with sub-MIC of tetracycline or environmental waters. This suggested that the low level of pharmaceutical compounds contaminating inland waters in lake Mälaren do not significantly exert a selective pressure on the bacterium. Additionally, a dose of 0.1 µg/ml of tetracycline does not provide antibiotic stress to microbe under the tested conditions.
Future studies

Validation of gene expression in a tetracycline resistant environmental strain of *Escherichia coli* with antibiotic mixture

A tetracycline sensitive strain of uropathogenic *E. coli* (UPEC) was used in our current study. No significant difference was observed between sub-MIC of tetracycline and the control. Thus by using a tetracycline resistant environmental strain of *E. coli*, validation of gene expression under tetracycline or combined antibiotic treatment could show environmental pressure for the development of multi-resistance. Tetracycline resistant genes were confirmed and observed in tetracycline resistant strains. Most of them had one tetracycline resistant gene per strain. By using a resistant strain with combination of other antibiotic groups we can identify the key genes involved in multi-antibiotic resistance using qPCR. The response of these genes will confirm the function of antibiotic resistance activity and also show the correlation with other genetic defenses such as efflux pump and outer membrane modification that relate to tetracycline resistance and other antibiotic mechanisms.

Function of tetracycline transcriptional regulators

Tetracycline resistant genes play an important role in the developing resistance to antibiotics. They belong to three resistance mechanisms including efflux pumps, ribosomal protection proteins and tetracycline inactivation. The expression of these genes is controlled by a tetracycline transcriptional regulator known as TetR. TetR is involved in suppressing other tetracycline gene activity. Little is known about the mechanism and function of TetR. Our study found that some of tetracycline sensitive strains had TetR. The presence of TetR may block the resistance mechanism of other tetracycline gene activity. Comparison of TetR sequences between sensitive and resistant strains may provide a database that will lead to a better understanding of the function of TetR. This can be done by sequencing of the TetR in tetracycline sensitive and resistant *E. coli* strains using PCR and DNA sequencing.

Evaluating the role of gene expression in environmental strains of *E. coli*

Uropathogenic *E. coli* was used in our study for treatment with environmental waters. No significant difference was found between the two conditions and their respective control. However the gene expression in an environmental *E. coli* strain treatmented with environmental water is not known. We can evaluate both antibiotic sensitive and
resistant strains for understanding the differences between gene response in bacteria treated with environmental waters. Pharmaceutical substances in the water may induce gene response in environmental strains of *E. coli*. Comparison of gene expression in pathogenic, antibiotic resistant and sensitive strains under environmental conditions could be done using the newly designed qPCR array.
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