Perspectives on urban wastewater as a source of microbial pollution

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Cover illustration: Enterococci, Campylobacter and Vibrio by Lisa Vaccino

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ABSTRACT

Wastewater treatment plants are important links for dissemination of intestinal bacteria into surface waters. In this study, twelve mallards were exposed to treated wastewater for a period of 55 days. Faecal samples were collected and analysed for *Enterococcus spp*. and *C. jejuni*. In general, the mallard and wastewater enterococci isolates belonged to different phenotypes, although some strains were identical. Phenotypical characteristics of *C. jejuni*, including antibiotic resistance, and genetical (PFGE and MLST) patterns were compared. All STs have previously been found in both humans and wild birds. The phenotypical expression of resistance against ampicillin and cefazolin, and ability to assimilate malate and succinate, changed during the mallards exposure to wastewater.

Edible clams were collected in Maputo Bay during both the dry and rainy seasons, and number of viable counts of *V. parahaemolyticus* peaked during the rainy season. A high percentage showed haemolytic capacity but did not carry the standard set of virulence genes.

The persistence of *E. faecium* and *E. faecalis* strains in sterilized treated wastewater at 10°C and 20°C was evaluated, including if ciprofloxacin had any effect. We could conclude that *E. faecalis* had a lower DC10 (92 and 43 days) than *E. faecium* (333 and 68 days) at 10°C and 20°C, respectively. Most of the strains were unaffected of ciprofloxacin was, but there were exceptions. All strains remained culturable the whole studied period (108 days).

Keywords: W*astewater, Mallard, Anas platyrhynchos*, *Enterococcus* ssp, *E. faecium E. faecalis, Campylobacter jejuni*, *Vibrio parahaemolyticus*

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SAMMANFATTNING PÅ SVENSKA

För att kunna förhindra att människor blir smittade av bakterier är det viktigt att veta var och hur dessa sprids. I kommunala reningsverk samlas tarmbakterier från människor tillsammans med allt som människor konsumerat och utsöndrar, t ex antibiotika. Trots att reningsverk kan reducera innehållet av bakterier upp till 99%, släpps det ut stora mängder tarmbakterier i vattendragen. Många bakterier som orsakar sjukdom är zoonotiska, d.v.s. de smittar mellan djur och människa.

En del i detta arbete var att undersöka om fåglar kan ta upp mänskliga bakterier från renat kommunalt avloppsvatten. Detta undersöktes genom att exponera tolv änder för vattnet under en längre tid. Analyser av *Campylobacter jejuni* med biokemiska metoder, inklusive känslighet för antibiotika, samt med två typer av genetiska metoder, visade att andflocken bar på flera olika stammar redan vid försökets början. De genetiska resultaten jämfördes mot databaser och visade att de varianter som änderna bar på har hittats tidigare hos vilda fåglar, men även hos människa, kyckling och ytvatten. Det finns en stor oro för att reningsverk gynnar resistenta bakterier och/eller överföring av resistensgener, detta eftersom även konsumerad antibiotika hamnar i avloppsvattnet via urin och avföring. I den här studien påverkades campylobakterna inne i ändernas tarmsystem av avloppsvattnet. Före ändernas exponering för avloppsvatten visade få av isolaten resistens mot penicillin-gruppen av antibiotika, men när änderna exponerades för det renade avloppsvattnet uttryckte alla *C. jejuni* stammar resistens. Resistensen avtog igen när änderna togs bort från avloppsvattnet. Även andra förmågor ändrades då campylobakterna fick kontakt med avloppsvatten och samtidigt änderna fick tillgång till att kunna beta gräs. Från början kunde *C. jejuni* inte utnyttja äppelsyra och bärstensyra utan utryckte denna förmåga endast under ändernas exponering.

Enterokocker är tarmbakterier som förekommer hos människa, däggdjur och fåglar. Denna studie indikerade att människor och änder till en viss del bär på samma enterokockstammar. Identiska biokemiska profiler kan ibland relateras till identiska genetiska likheter och resultaten i studien indikerade att det fanns stammar i kommunalt avloppsvatten som överlappade med de som fanns hos änderna. Det kunde dock inte bevisas att änderna plockade upp enterokocker från avloppsvattnet under exponeringstiden, i princip fanns alla enterokockvarianter fanns hos änderna före exponeringen.

De flesta vibrioarter är inte sjukdomsframkallande, men vissa stammar kan orsaka tarminfektioner och är den vanligaste orsaken till magsjuka efter

konsumtion av skaldjur. Vibrio är normalt inte tarmbakterier, utan har sin naturliga miljö i kustnära hav. Infekterade människor sprider de sjukdomsframkallande stammarna till vattnen via avföring och avloppsvatten. I en undersökning av ätbara musslor från Maputo Bay, Mocambique, var *V. parahaemolyticus* den vanligaste vibrioarten. Många av stammarna som isolerades kunde förstöra cellmembran, en förmåga hos vibrio som ofta förknippas med sjukdom. Denna förmåga sågs vid odling på agar som innehöll röda blodkroppar. Nästan 70 % av alla stammar förstörde blodcellerna (hemolys). Trots detta kunde inte tdh-genen detekteras, en gen som kodar för ett protein som förstör människans tarmceller och bidrar till infektion.

Tarmbakterier utsöndras från tarmen kontinuerligt och för att överleva tills de når en ny mottaglig individ måste de tåla olika miljöfaktorer. I en experimentell laboratoriestudien överlevde enterokocker mycket länge vid 10°C än vid 20°C i sterilt renat avloppsvatten. Efter tre månader hade *E. faecium* reducerats med 30%, och *E. faecalis* med ca 90% vid 10°C, vid 20° med 70% respektive 99%. Både bakterier och resistensgenerna kan spridas med avloppsvatten. Enterokocker kan inte växa i naturen, utan överlever utanför tarmen genom att cellerna är/blir inaktiva och tåliga. Dessa inaktiva celler bör teoretiskt sett inte påverkas av antibiotika eftersom antibiotika slår mot aktiva och växande bakterieceller. I denna studie stämde detta till största del, eftersom reduceringen av enterokockceller i försöket i princip var identisk oavsett om antibiotikan ciprofloxacin tillsattes eller inte. Det fanns dock några stammar där ciprofloxacin hade en tydlig negativ inverkan på cellernas överlevnad.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. *Enterococcus spp* in Wastewater and in Mallards (*Anas platyrhynchos*) Exposed to Wastewater Wetland

II. Characterization of *Campylobacter jejuni* isolated from Mallards (*Anas platyrhynchos*) prior, during and post exposure to treated wastewater

III. Characteristics of potentially pathogenic vibrios from sub-tropical Mozambique compared to isolates from tropical India and boreal Sweden

IV. Different persistence among strains of *E. faecalis* and *E. faecium* in sterile treated wastewater microcosms – effects of temperature and ciprofloxacin

CONTENT

ABBREVIATIONS

AMP	Ampicillin
API 20NE	Biochemically identification method, gram negative bacteria
API campy	Biochemically identification method, *Campylobacter* spp
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistant gene
BHI	Brain heart infusion agar
CFU	Colony forming unit
CFZ	Cefazolin
CIP	Ciprofloxacin
Di	Diversity index
MLST	Multilocus sequence typing
MEA	M Enterococcus Agar
PCR	Polymerase chain reaction
PhP-system	Phene Plate system
qPCR	Quantitative real-time PCR
RW	Raw waster (incoming wastewater before treatment)
Sp	species (plural spp)
TCBS	Thiosulfate-citrate-bile salts-sucrose agar
tdh	Thermostable direct haemolysin gene (*V. parahaemolyticus*)
tlh	Thermolabile haemolysin gen (*V. parahaemolyticus*)

TRH	*tdh*-related hemolysin
TW	Treated wastewater
UPGMA	Unweighted pair group method using arithmetic averages
VBNC	Viable but not culturable
VRE	Vancomycin resistant enterococci
MIC	Minimal inhibitory concentration
WWTP	Wastewater treatment plant

1 INTRODUCTION

In Sweden, the wastewater management system was expanded in the 1970s thanks to new legislations and large investments by the government. This has contributed to both microbiological safety and cleaner water. Looking back 200 years, Sweden had the highest mortality rate in Europe due to poor sanitation with dirt and waste in the streets [1]. During the 1830s the bacterium causing cholera reached Sweden and a huge part of the inhabitants of larger cities died. This was the starting point for removal of the waste from the streets, but it took until the end of the 19th century to the beginning of the 20th century, until larger Swedish cities were connected to waterborne wastewater systems. This first-generation system piped the faecal material directly, without treatments, into nearest lakes or rivers and the nutrients changed ecosystems and caused eutrophication. This became the reason for investments in wastewater treatment plants (WWTP), with organic carbon and phosphorus removal, during the 1970s. Further purification regulation came in the 1990s when EU legislation required reduction in nitrogen emissions to sensitive aquatic environments [2]. The primary purpose for WWTPs was, and still is, to manage water conservation problems with visible pollution, foam formation, massive algae blooms and low oxygen levels.

Many countries worldwide lack, however, the economic conditions and the expertise to construct centralized fully functional wastewater treatment. In a way, at least in economically terms, many developing countries are in the same situation as Sweden was in the 19th century. 663 million people worldwide lack access to safe water, 50 % of these reside in Sub-Saharan Africa, predominantly in rural areas [3]. Urbanization is rapidly increasing, especially in Asia and Africa [4]. This creates huge problems with microbial safety and sanitation. Inadequate sanitation is a major cause of infectious diseases such as cholera, dysentery and other intestinal infections. Spread of faecal bacteria and other microorganisms causes human suffering, death and socio-economic decline [4].

One of the most important steps to prevent pathogens to spread between humans and animals, is to handle faeces and animal traits safely. Intestinal zoonotic bacteria can reach susceptible individuals in a number of ways, including both direct contact and indirectly via the environment. Human faecal bacteria can be transmitted through wastewater, reaching drinking water supplies and/or recreational waters. Faecal bacteria released in aquatic recipients can then

accumulate in seafood and/or infect wild birds swimming and feeding in the recipient, thus closing the loop back to humans, *figure 1*.

Figure 1. Urban wastewater treatment plants collect intestinal microbes from humans and release them into recipients where birds and other wild animals may be exposed and contribute to the spreading of pathogens or virulence genes, closing the loop.

1.1 PATHOGENIC BACTERIA FOUND IN URBAN WASTEWATER

Several pathogenic bacteria are commonly found in urban wastewater. The most important transmitted directly or indirectly by these waterborne routes are *Campylobacter* spp., *Helicobacter pylori*, *Salmonella* spp., *Shigella* spp., *Vibrio* spp. *Yersinia enterocolitica*, and enterotoxic- and enteropathogenic *Escherichia coli*. In addition, opportunistic bacteria and/or various antibiotic resistant bacteria of faecal origin may also be transmitted by this route [5, 6].

Faecal material is estimated to contain up to 9 x 10^{10} bacteria g^{-1} wet stool [7]. Removal/inactivation depends on the WWPT construction, residence time, wastewater flow, number and type of connected households and industries, intrinsic characteristics of bacteria and extrinsic factors such as UV light, temperature and salinity [8-10]. Urban WWPTs do not remove all faecal

bacteria, and these are consequently released to the recipient in large numbers. Thus, treated wastewater (TW) contains high levels of faecal pathogens as well as indicator bacteria. The reduction of enterococci, a common indicator bacterium, is reported to vary from 12% to 99,9%. Number of enterococci in effluent TW is reported to lie between 10 and 10^4 100 ml^{-1} in temperate climate [8, 11, 12]. The removal of *Vibrio* spp. is reported to be about 50% and in South Africa counts of vibrio is reported to occur at levels from 101 and 105 CFU 100 ml^{-1} in TW, with the highest counts during the hot and rainy season [13-15]. *Campylobacter* spp. in wastewater is also reported to vary with the seasons and this corresponds to incidences in the human population. The highest numbers are recovered during the summer in temperate climates [16-18]. Viable counts of *Campylobacter* spp. in TW is reported to be 10 -10^3 CFU 100 ml^{-1} and the reduction from RW to TW 35% to 99%, in Europe and South Africa [9, 13, 19].

1.2 BACTERIAL SPECIES STUDIED

1.2.1 ENTEROCOCCI

Enterococci were classified to its own genus in 1984, previously classified as group D streptococci [20]. There are now 36 species within the *Enterococcus* genus and they belong phylogenetically to the low G+C content branch of Firmicutes [21, 22]. Enterococci belong to the lactic acid bacteria, and are gram-positive ovoid cocci arranged in pairs or in short chains. They are facultative anaerobes and perform fermentation in the absence of heme, when external heme is provided they can use an electron transport chain for aerobic respiration [23]. They grow at a temperature range between 10° and 45°C, and most enterococci are halotolerant (> 6,5% NaCl W/W). They hydrolyse esculin in the presence of bile salts (40%) and all species are catalase negative.

Enterococci are widespread in nature and form an essential part of the commensal microbiota of humans and animals [24]. *E. faecium* and *E. faecalis* are the most frequently occurring species in the human intestine. In production animals like poultry, cattle, and pigs, *E. faecium* is frequent, but other species occur at higher numbers, like *E. faecalis* and *E. cecorum*. In birds *E. durans, E. hirae*, *E. faecalis. E. cecorum, E. columbae* (pigeons), *E. avium, E. mundtii* have been reported [25]. *E. mundtii* and *E. casseliflavus* form yellow pigmented colonies and are associated with plants [22].

The genus is not highly pathogenic, it is even recognized as food fermenters and probiotics due to their ability to form lactic acid and bacteriocins [26-28]. However, the genus has emerged as nosocomial opportunistic pathogens due to intrinsic resistance, but also as carriers and distributors of a variety of antibiotic

resistant genes to both gram-positive and gram-negative species [29-31]. The plasticity of the enterococcal genomes allows enterococci to rapidly respond and adapt to selective constraints by acquiring genetic determinants that increase their ability to colonize or infect the host [24, 31, 32]. This rendered vancomycin-resistant enterococci (VRE) to be included in the WHO global priority list of antibiotic-resistant bacteria in 2017 and in Sweden VRE associated infections fall into notifiable diseases; into the category Subject to mandatory contact [33, 34]. Additional factors contributing to the importance of enterococci is tolerance to various environmental stress factors [9]. The environmental stress outside the intestine can generate transformation of the enterococci cells to a starvation state, so-called persister cells, or into a viable but non-culturable (VBNC) state. Both states are triggered by nutrient depletion, especially for carbon [35, 36]. Although persisters are metabolically inactive, and thus are unlikely to multiply in sewage waters or other waters, they are culturable [37, 38]. This is one major reason of its use as an indicator of faecal contamination, especially in seawater [21, 39]. However, cells in the VBNC stage are not culturable.

1.2.2 CAMPYLOBACTER

Campylobacter is a gram-negative genus with a characteristic morphology as curved rods. The genus was discovered in the nineteenth century, but the species were permanently placed within this genus first in the seventies [40]. They were previously placed into the vibrio genus due to a resembling cell morphology [41]. Campylobacter include to date at least 26 species, several causing illnesses in humans and animals [42]. Most of *Campylobacter* spp. are microaerophilic, with a respiration metabolism [42-44]. The optimum temperature for growth for many of the species is in the range of 37–42°C, but thermophilic species such as *C. jejuni*, *C. coli* and *C. lari* thrive at 42–44°C.

Several *Campylobacter* spp. are causing diseases in humans, with *C. jejuni* as the most important. They are spread by the faecal-oral route, predominantly through insufficiently cooked poultry, but also through other types of meat as well as unpasteurized milk and water [45-47]. According the Communicable Diseases Act and the Communicable Diseases Ordinance [33], are *Campylobacter* spp. notifiable in Sweden within two categories, the Subject to mandatory contact and tracing and dangerous to public health. *C. jejuni* is the most common cause of foodborne intestinal illness among humans worldwide [48-50]. The incubation time is about of 2–4 days (range 1–10 days), probably depending on the age of the infected person and dose [48]. The dose of infection is low, 500–800 bacteria [49]. The most common symptoms are severe abdominal pain and diarrhoea, in combination with symptoms such as headache, fever and muscle pain. The duration of the illness is usually about a week [47]. In a minority of individuals, the campylobacter infection is a

precursor of immunoreactive diseases within the gastro intestinal systems and inflammatory bowel diseases [50-52], or symptoms within the neurological system such as the Guillain-Barré syndrome [53-55].

Campylobacter spp. are fastidious bacteria and features common for all species are the difficulty to cultivate them using standard plating methods. They need selective agar to be isolated and to be maintained in pure cultures [56]. However, campylobacter survive various environmental conditions and have the ability to transform into a viable but not culturable (VBNC) stage. The substantial genetic plasticity within *C. jejuni* may also support the survival of the species in unfavourable environments [57-59].

Zoonotic strains of campylobacter are mainly present among domestic animals. Wild birds usually harbour their specific strains, unrelated to human strains [60, 61]. Human associated campylobacter has however been isolated from wild birds, primarily during migration periods, but also among birds associated with human activities [62, 63].

1.2.3 VIBRIO

Vibrio spp. are gram-negative, slightly curved or curved cells that are motile. *Vibrio cholera* was one of the first waterborne bacteria to be isolated. Filippo Pacini described it for the first time, John Snow suggesting its presence in contaminated fresh water wells and prevented cholera epidemic in London by closing wells, and Robert Koch finally discovering the cause of the disease [64-66]. He determined that cholera is spread through contaminated water or food supply sources and confirmed Snow's earlier epidemiological theory.

Vibrio spp. are facultative anaerobes with a fermentative and/or respiratory metabolism. Optimum conditions for growth in their natural water habitat is temperatures of about 37°C, with a range between 10–43°C and a pH of about 7,6 (5–9,6) and a salinity of 0–35 ppt [67-69]. *Vibrio* spp. is known to enter the VBNC state at temperatures below 13°C, which means that they can be present for a long time even at low temperatures [70, 71]. The genus covers about 100 species, which are mostly of marine or freshwater origin, whereas about twelve species have been associated with diseases in humans [72-74]. Three of the species; *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* are among the most common causes of foodborne infections after consumption of contaminated seafood [47, 75]. The natural habitat of these species is coastal waters. Areas with virulent strains may cause illness if people drink contaminated fresh water or eat contaminated seafood or fish. Filter-feeding molluscs are a common cause of vibrio infections, since these animals concentrate vibrio in their tissues, and are cooked very lightly or consumed

raw [76-78]. Cholera toxin producing *V. cholerae* strains are non-invasive, but affects the small intestine via the release of the enterotoxin, whereas *V. parahaemolyticus* and *V. vulnificus* are considered invasive species affecting the colon. The bacteria can also make their way into open wounds after exposure to vibrio containing water, especially when water temperatures are above 20° C. In severe cases, they can further spread into the bloodstream causing sepsis [76, 79, 80]. *V. cholera* and *V. parahaemolyticus* are, however, usually non-pathogenic [76, 78]. *V. cholerae* infection is a notifiable disease in Sweden, falling into the categories; Subject to mandatory contact tracing and dangerous to public health. Vibrio infections excluding toxin producing *V. cholerae* (O1/O139) are as well notifiable, within the category Subject to mandatory contact [33]. With global warming vibrio infections are suggested to increase worldwide [81-83].

1.2.4 ANTIBIOTIC RESISTANCE BACTERIA IN WWTP

The use of antibiotics has led to the presence of antibiotics and antibiotic resistant bacteria in urban WWTPs and all kinds of different antibiotic determinants can be found [84-86]. Urban WWTPs are suggested to be one of the main sources of antibiotics released into the environment, as well as suggested to promote selection of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) [87, 88]. Antimicrobial resistance (AMR) in bacteria is a major threat for human health worldwide [89, 90]. AMR imply development of resistance among microorganisms to initially active antibiotics. That means that treatable diseases become untreatable and medical achievements like surgery, transplants or cancer therapies are not possible without major complications [91]. Processes contributing to emergence and dissemination is complex and not only embrace the consequences for resistance development on the molecular level, but also on human behaviour. The most important factor of rapidly emerging resistance among microorganisms is the use of antibiotics [92]. The increase of AMR is due to molecular evolution, i.e. the response of microorganisms to a changing environment, in this case the presence of antibiotics [93]. New resistance determinants appear through mutations. Antibiotic resistance is, although found among bacteria in natural environments, without anthropogenic influence. This is probably due to a response to other functions of produced antibiotics, such as signalling and metabolic roles in microbial communities [94, 95]. The increase and spread of AMR among bacteria, on the other hand, include key processes, where genes are transferred from resistant donors to susceptible recipient bacteria. The mechanisms responsible for this are in many cases plasmids transferred uptake of environment), transduction (transfer by virus), but also transfer of

resistant genes from the chromosome to the plasmid, thus generating a higher expression of the genes [96, 97].

1.2.5 BIRDS

Lately urban WWTPs have also been suggested to be a supplier of ARB and/or ARG to birds [98]. It has also been pointed out that animal-derived enterococci could be a reservoir of ARG that could be transmitted back to humans [99]. A recent hypothesis is that bacteria inhabiting the intestines of animals and humans not only exchange ARG, but might also interact with bacteria that are just passing through the colon, causing these bacteria to acquire and transmit ARG [100, 101].

Wild birds are also suggested to be reservoirs and vehicle for pathogenic bacteria [102]. Birds migrate across national and intercontinental borders. This provides a mechanism for the establishment of new pathogens at great distances from the source of the original infection, for example, waterfowl are known as a symptomatic carriers of influenza A virus, *Salmonella* spp., *Campylobacter jejuni*, and *Borrelia burgdorferi* [103-106]. Feeding habits of wild birds may influence their carriage of human-associated enteropathogenesis, for example the same serotypes of *Salmonella* spp. was found in both sewage and gull faeces. Furthermore, *E. coli* with overlapping sequence types and extended spectrum beta-lactamase (ESBL) activity have been found in wild birds [98, 107, 108]. Lately the interest in wildlife as a possible reservoir and/or potential source of ARB has increased, since identical or near identical strains have been found to circulate between wildlife, humans and domestic animals [109-111].

Mallards are widespread waterfowls worldwide, especially in the northern hemisphere, and have adapted to an extremely wide range of habitats (*figure 2*) [112, 113]. They are traditional migration birds, but since wintering conditions have become more favourable in the north, they have a delayed autumn migration and decreasing trends in migration distance [114-116]. Indeed, sometimes they don't migrate at all [117]. At temperatures below zero, and during periods of reduced availability of food and shelter, mallards are gathering in open waters, for example wastewater wetlands ponds [118]. In Kristianstad 150–300 mallards were observed from January to March within biological treatment ponds inside the WWTP, as well as in the small channel where TW is released to the recipient (unpublished data). Mallards are also

mobile within the feed seeking area, with migration distances of several kilometres during a day, between roosting and foraging areas [119].

Figure 2. During the Swedish summer, humans and mallards are sharing the same beaches and recreational waters.

2 AIM

The aims of this thesis were to generate more knowledge about the relation between wastewater and the transmission of human intestinal bacteria to waterfowl and other aquatic organism. This to be able to assess consequences of the perpetual release of microbiological waste to the aquatic environment. Thus, the scope of this thesis was to study:

- whether enterococci in urban wastewater colonize mallards intestines in terms of abundance, species distribution and biochemical diversity (I)
- whether *C. jejuni* in urban wastewater colonize mallards intestines in terms of abundance and genetic relatedness, and if the isolate´s susceptibility to antibiotics change.
- to investigate the seasonal presence of potential pathogenic *Vibrio* spp. in clams and water from Maputo Bay, Mozambique, and characterize the strains in terms of their antibiotic resistance, virulence and biochemical diversity.
- differences in culturability of *E. faecium* and *E. faecalis*, in sterile treated wastewater at different temperatures during >100 days and if the cultivability was affected by presence of ciprofloxacin.

See also Appendix regarding the outline and perspectives of the thesis.

3 METHODOLOGICAL CONSIDERATIONS

3.1 FIELD STUDIES (PAPERS I, II ,III)

3.1.1 DESCRIPTION OF STUDIED AREAS

Hässleholm is a small city situated in the south of Sweden with a temperate climate, coldest in January with temperatures slightly below 0° C and warmest in July with temperatures around 20°C. The Hässleholm WWTP is placed outside the town (56°09'57.4"N 13°47'18.7 E). The WWTP treats wastewater from urban and surrounding areas. The daily mean volume of wastewater varies from 12 500 m3 to 32 300 m3 and correspond to 30 000 person equivalents.

At the Hässleholm WWTP, the wastewater is first subjected to mechanical purification as it passes through a grid and screen, a sand trap, and a pre-settling basin. Here larger and heavier solid particles are removed. This is followed by a biological purification step with biological active sludge. In this step, biodegradable material is removed by biodegradation to carbon dioxide. Phosphorus is removed through chemical precipitation with iron or aluminium flocculants, and with a subsequent sedimentation and filtration. The Hässleholm WWTP also includes a constructed wetland, Magle Kärrsbäcken, serving as a nitrogen and phosphorus polishing step. Magle Kärrsbäcken connects the treatment plant to the lake Finja and via rivers the water is released in the Baltic Sea. The Baltic Sea is considered a sensitive area, why the treatment plant is subject to European Parliament and of the Council directive 1991/271/EEC of 21 May 1991, concerning urban wastewater treatment. Magle Kärrsbäcken was commissioned in February 1995 and has since then become an important habitat for waterfowl, including mallards.

Maputo, the capital of Mozambique, has a sub-tropical climate, with the peak of the wet and hottest season in February, and the driest month of the year in September. The city is situated by the Maputo Bay, at the Indian Ocean. Maputo is rapidly growing and has substandard systems of disposing faecal material. About 10% of the residents have connections to waterborne systems. Of these, only 3% pass WWTPs and the remaining 90% use septic tanks. It is estimated that more than 50% of the faecal waste flow is not safely handled and treated. This leads to contamination of the drainage system and in the end the recipient Maputo Bay [3]. The study area, Costa do Sol, is found in the northern part of the Maputo (25°54'52"S, 32°38'55"E) and during low tide it is a popular harvest site for clams.

3.1.2 EXPERIMENTAL SETUP (PAPERS I, II)

Twelve mallards were kept for 55 days in an aviary with an indoor artificial wastewater pond, *Figure 3*. TW was continuously pumped through the aviary with a flow corresponding to the inlet/outlet in the first pond of the wetland. Faecal samples were collected from the mallards prior, during, and post exposure for TW from August to October in 2004, *table 1*.

Tabell 1. Time of collection of faecal samples from the twelve mallards. During the first day three samples were taken (6, 18, 27 hour)

Exposure	Prior	During							Post
Weeks	0	1	2	3	4	5	6	7	8
Days	1, 5, 8	1, 5, 8	12, 15	19, 22	26	33	40	49, 55	7, 15

To compare enterococci isolates from mallards with potential human enterococci in urban wastewater, raw (RW) and TW was sampled and analysed during the autumn 2004 and early spring in 2005. The TW isolates (N=5) were obtained through the permanent equipment programmed for flow proportional sampling during 24 hours. RW isolates (N=2) was obtained with random sampling.

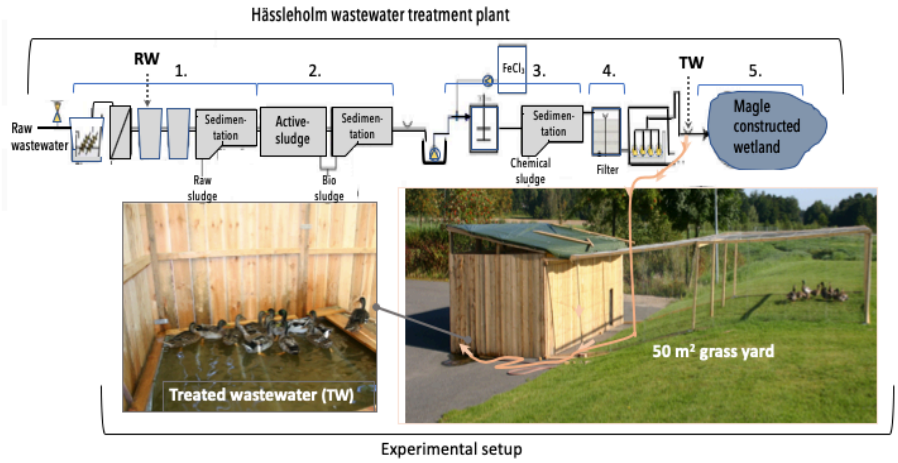

Figure 3. Experimental setup (paper I & II) and description of the different treatments at Hässleholm urban WWTP. 1. pre-aeration and sedimentation; 2. aerated activated sludge; 3. chemical precipitation; 4. filtration; 5. Magle wetland. Arrows denote collection of raw wastewater (RW) and treated wastewater (TW).

3.1.3 TREATMENT OF SAMPLES (PAPERS I, II, III)

Faecal samples for paper I and II were obtained by placing the mallards in single-used separate cardboard boxes. Thereafter, samples were collected from the droppings either using Copan sticks for individual mallard samples or by pooled samples from all 12 mallards into a single sterile Falcon tube. RW was collected in 1 litre sterile bottles (random sampling) and TW was transferred from the proportional sampling container to 1 litre sterile bottle. All samples were immediately refrigerated and cultivation took place within 8 hours.

Water samples and clams in paper III were collected at four different seasons; early rainy (November); late rainy (March); early dry (May) and late rainy season (August) from Maputo Bay, Maputo, Mozambique, during 2006 and 2007. Clams and mussel were bought from children and women that collected them during low tide. Clams were scrubbed and rinsed with distilled water before opened with a sterilized knife. All tissue, including liquid, was collected in a sterilized blender and homogenized for two minutes at maximum speed.

3.1.4 COUNTING, ISOLATING (PAPERS I, II, III)

Selective media was used for the isolation of *Enterococcus* spp.(paper I), *C. jejuni* (Paper II) and *Vibrio* spp.(paper III). Samples with high concentrations of bacteria from faecal droppings or clam tissue were subjected to serial dilutions in sterile 0,85% NaCl before plating, when needed. Water samples with lower number of bacteria were filtered through 0,45 µm pore-size membrane filters (paper I) or 0,22 µm filters (paper III). The filters were placed on M Enterococcus agar (MEA) or Thiosulphate Citrate Bile Sucrose agar (TCBS), respectively. On MEA, which is a selective substrate for enterococci, positive isolates will grow as pink to maroon colonies, and for confirmation, eight to ten enterococci colonies were sub-cultured on Bile Esculin agar at 44°C . Positive esculin hydrolysis appears as black zones around the colonies. The *Enterococcus* spp. were further analysed for lack of catalase production using 3% H_2O_2. Antibiotic resistant strains were selected for on MEA supplemented with either of the following antibiotics: ampicillin (8 mg^{-1}), ciprofloxacin (4 mg^{-1}), gentamicin (64 mg^{-1}), erythromycin (4 mg^{-1}) or vancomycin (16 mg^{-1}). When possible, two isolates were chosen for further analyses. An enrichment culture, Enterococcal broth with 32 mg l^{-1} vancomycin, was used for isolation of VRE from each mallard (IMF). After incubation for 24 hours at 37°C, 0,1 ml was spread on MEA. If colonies typical for enterococci were obtained, one isolate from each sample was chosen and colonies were tested as described above. The problem with selection of antibiotic resistant bacteria from faecal material by the mean of added antibiotics, is that faecal bacteria is growing without being resistant. 96% of the isolates from

antibiotic selective substrate showed no phenotypical resistance when tested, unlike wastewater isolates where only 20% were susceptible. The reason for this is not clear.

TCBS-agar is a selective substrate isolating vibrios. *V. cholera* grow as flat yellow colonies (2-3 mm), *V. alginolyticus* as smooth yellow colonies, *V. vulnificus* as yellow or translucent colonies, and *V. parahaemolyticus* as colourless colonies with a green centre. Other bacterial species able to grow on the plates, such as *Pseudomonas* spp. and *Aeromonas* ssp., form blue colonies. The TCBS plates were incubated at 37°C for 22± 2 hours. Bacterial colonies from each sample were transferred to hybridization filters for further molecular analyses back in Sweden. In order to isolate different vibrios from clams and mussels, pre-enrichment was performed with a culture consisting of 25 gram homogenized tissue and 225 ml alkaline peptone water, and incubated at 37°C for 22± 2 hours [120]. The cultures were then spread (0,1 ml) onto TCBS plates and incubated as described above. Suspected vibrio colonies were chosen for phenotypical identification with API 20NE. Total number of *Vibrio* spp. in clams and mussels were measured in terms of colony forming units on TCBS agar. High levels of *Aeromonas* spp. reduced the selectivity of the substrate.

For isolation of *Campylobacter* spp., each mallard faecal sample was plated onto a Campylobacter selective blood-free medium, supplemented with cefoperazone and amphotericin and incubated at 42°C in a microaerophilic atmosphere. From each plate with growth of suspected Campylobacter spp., one colony was isolated and further investigated. Isolates showing gram-negative gull-shaped cells by microscopy, and positive catalase and oxidase tests, were regarded as *Campylobacter* spp. [121]. These were further identified by the API® CAMPY test (Biomerieux, Marcy-l'Etoile, France).

3.1.5 API 20NE AND API CAMPY (PAPER II & III)

The API-system is a phenotypical identification test that depends on metabolic activities. Different biochemical tests are miniaturized and positive or negative reactions are translated into a numeric profile and identified with an identification program provided by the manufacturer (Apiweb TM Biomerieux). The method is easy to perform and is relatively inexpensive, making an ideal method to identify bacterial isolates. The accuracy of identifying *C. jejuni* is reported to about 94% and for *Vibrio parahaemolyticus* 74% [122, 123]. However, phenotypic tests are partly problematic due to inherent difficulties. Test results relies on personals interpretation and expertise, and the kits may yield poor reproducibility and discriminatory power due to variations of gene expression caused by the environmental factors the bacteria have been grown at, and age of the bacteria [124]. To avoid misinterpretations, tests difficult to read

were reinoculated with freshly grown bacteria. *C. jejuni* consist of two subspecies doylei and jejuni [125]. Potential Campylobacter jejuni subsp. doylei gave inconsistent results and were excluded for further tests. In this thesis C. *jejuni* refers to *C. jejuni* subsp. *jejuni*.

3.1.6 PROBE HYBRIDIZATION (PAPER III)

Designed and labelled probes can detect species-specific and virulence associated genes in bacterial isolates by probe hybridization to their genomic DNA. Bacterial material transferred to a filter is then lysed and hybridized onto the same filter [120]. Colony probe hybridization was mainly carried out as described in the FDA Bacteriological Analytical Manual and according to the protocol described by Thomson *et al. (1976)* [126]. The probe target gene *tlh* codes for the thermolabile haemolysin TLH. This gene is species-specific for *V. parahaemolyticus* [127]. The other probes were designed to target virulent *V. parahaemolyticus* strains, causing gastroenteritis. The virulence is associated with the two genes *thd* and *trh*, corresponding to ability to produce thermostable haemolysins [128, 129]. Many clinical strains of *V. parahaemolyticus* produce both TDH and TRH haemolysins [130]. The probe hybridization method is rapid and simple, especially when screening numerous isolates, but re-probing can be problematic. Although only one strain carried the *tdh* virulence gene, 69% of isolates showed evidence of haemolytic capacity when subjected to a phenotypical test, indicating that additional genes should be involved when screening for the occurrence of potential human pathogens.

3.1.7 THE PHENE-PLATE SYSTEM (PAPER I & III)

The PhenePlate rapid screening system was used in paper I and III for biochemical typing of enterococci (PhP-RF, paper I) and *V. parahaemolyticus* (PhP-RV IV, paper III) to discriminate between species within the genus. The system is based on the kinetics of a set of 11 biochemical reactions per assay in 96-well microplates. The identification is based on the concept that bacterial isolates belonging to the same clonal group shares identical metabolic properties. The reagents used for the PhP system is primary selected for having a high discriminatory power for all isolates within the genus, and not designed to be a species identification system. Although, by typing a set of reference strains within the PhP-plates, a preliminary species identification is possible [131]. The PhP-plates were inoculated with the chosen bacterial suspensions and incubated at 37°C. The reactions were recorded in a microplate reader after 16, 40, and 64 h. The biochemical profiles were calculated using accumulative absorbance values as previously described by Kühn et al. [132]. The relationship between isolates is visualized in a dendrogram derived from data clustering using the unweight pair group method (UPGMA). Advantages with

PhP-system are high reproducibility and that large numbers of isolates are easily typed [133]. Identity between types may have to be confirmed with a more discriminatory method like multilocus sequence typing (MLST) or whole genome sequencing (WGS), although differences between types can be regarded as true differences. Methods depending on a library of reference strains may be biased due to the range and origin of reference strains, i. e. when more clinical strains than environmental strains and strains from wild animals are included. There may be drawbacks in the possibility to distinguish between species within the genus if they have similar phenotypical pattern. In paper I, three cluster of *E. faecium* (E.fcm9, E.fcm10 and E.fcm11) were later re-identified with MALDI-TOF (Matrix Assisted Laser Desorption Ionization - Time of Flight) as *E. mundtii* and *E. casseliflavus*. Previous studies have shown that the PhP-system is more reliable for *E. faecalis* typing than *E. faecium* [134].

3.1.8 PULSED FIELD GEL ELECTROPHORESIS (PAPER II)

Pulsed Field Gel Electrophoresis is a technique based on the digestion of DNA by restriction enzymes into larger fragments. The fragments are separated by electrophoresis on an agarose gel according to their size. The orientation of electric field is changed intermittently ("pulsed") [135]. PFGE is a powerful technique for detection of microevolution and is commonly used for epidemiological studies [123]. Major disadvantages is that the method is time consuming and the necessity to have good quality chromosomal DNA [136].

3.1.9 MULTILOCUS SEQUENCE TYPING (PAPER II)

Multilocus sequence typing (MLST) is a molecular method that is used to characterize bacterial isolates by comparing sequences of coding loci of multiple housekeeping genes. A sequence type (ST) can be defined through the sequences present within each house keeping gene. The nucleotide sequence at each locus is determined and the resulting sequence gets an allele number. If nucleotides differ within alleles of the same loci, they are given a different ST number, while similar sequences are assigned the same number. The alleles at each of the loci define the allelic profile or ST for a bacterial isolate. Groups of related STs can in turn be grouped into clonal complexes on the basis of four or more shared alleles [137]. Most bacterial species have sufficient variation within housekeeping genes to provide many alleles per locus, allowing billions of distinct allelic profiles to be distinguished. Allelic profiles are then compared, which shows how closely related the isolates are to each other. The more ST the isolates have in common, the more they are related. The great advantage of using MLST is that sequence data, i. e. the allelic profiles of isolates, can easily be compared to those in an international free access database (http://pubmlst.org). MLST is therefore a suitable typing

method for monitoring of global trends in e.g. *C. jejuni* populations, and has been used in a variety of epidemiological studies since its development [138].

3.2 MICROCOSM STUDY (PAPER IV)

Persistence of enterococci were investigated in longitudinal studies (108 days) by five separate microcosm studies during 2013, 2015 and 2016. The aim was to investigate the survival of enterococci in temperatures corresponding to temperatures commonly found during the breeding season for mallards in Magle constructed wetland and other wastewater ponds or recipients in southern Sweden. Several strains of *E. faecium* and *E. faecalis* were used from a collection of isolates obtained in the study described in Paper I. *E. faecium* and *E. fa*ecalis normally inhabit gastrointestinal tract of humans as well as animals and they are released from WWTPs in huge amounts every day worldwide. These two species are also of special interest since they are nosocomial species. The study relied on viable counts, i. e culturable cells. This generates several problems. The preparation of media and plates, as well as the counting of colonies, is time-consuming and labour intensive, and it requires large amounts of materials in terms of agar plates, plastics etc. This makes it impossible to do as many replicas as might be necessary to fully compensate for large standard deviations built into the method. Another problem is contamination of the microcosms. This occurs very rarely, but caused problems especially during the last days of sampling, however never in microcosms with added ciprofloxacin. This contaminating sharp yellow pigmented microorganisms (not within the MALDI-TOF library) thrived in the autoclaved microcosm water and plates where overgrown and not countable. *E. faecalis* is reported to form VBNC and PCR based methods of detection could have been used to verify this. We attempted to apply qPCR as a complement to the CFU ml^{-1} analyses, but were unable to extract high quality DNA from the majority of the persister cells, resulting in inconsistencies between CFU ml^{-1} and qPCR data.

4 RESULTS AND DISCUSSION

4.1 *ENTEROCOCCUS* SPP. IN WASTEWATER AND IN MALLARDS (*ANAS PLATYRHYNCHOS*) EXPOSED TO WASTEWATER (PAPER I)

Enterococci are ubiquitous found in human intestine and faecal material and are released in huge numbers from WWTPs [11, 12], but even if the WWTP in the small town of Hässleholm efficiently reduce 98– 99% of the enterococci, the actual number of enterococci released every 24-hours is high. About 120 billion are discharged into the Magle constructed wetland. In this study enterococci in RW was quantified to 4 x 10^4 – 2 x 10^5 CFU 100 ml^{-1} 1 x and 10^3 – 4 x 10^3 CFU 100 ml^{-1} in TW.

The experiment in paper I was performed prior the avian flu. Thus, the breeding aviary where the mallards were purchased was still open to wild birds. Mallards were sampled prior, during and post exposure to TW, with a higher frequency during the first week. Two types of sampling were performed: pooled sampling of 12 mallards' faecal droppings (PMF, N=10) and individual mallard faecal samples (IMF, N=19). The individual samples were further enriched in vancomycin broth searching for vancomycin resistant enterococci (VRE). Enterococci were found in all PMF in the range of 10^1-10^5 CFU g^{-1} wet weight. Enterococci were also isolated from the enrichment culture from all IMF but not at all sampling occasions. As previously described the faecal material lowered the selective effect of added antibiotics and only two isolates showed vancomycin resistance.

The dominating species among mallards were *E. faecalis* (31%), followed by *E. durans* (26%), *E. faecium* (20%), and other species (5%). The remaining 21% did not cluster with any of the strains in the PhP reference database [139]. Both IMF and pooled samples showed that several enterococci species flourished simultaneously within the mallard flock. The species found had previously been found in birds [140]. The most common species isolated from the wastewater was *E. faecalis* (34%), *E. faecium* (28%), and *E. hirae* (7%), and less common were the *E. gallinarum* group including other low frequent isolated species (10%). 19% did not cluster with any of the strains in the PhP reference database, a higher proportion than previously shown [139]. Analysis of the PhP-data such as similarities between isolates, diversities within samples and similarities between populations were done with the PhP software. Isolates

with correlation coefficients equal to or higher than 0.965 were assigned the same PhP-type. The relationship between isolates was visualized in a dendrogram derived from data clustering using the unweight pair group method (UPGMA) as described by Kühn *et al.* [133].

When PhP-RF typing data for 133 *E. faecalis* and 117 *E. faecium* isolates were compared, identical PhP fingerprints were seen among mallard and raw wastewater, indicating that these strains possibly belonged to the same type. Mallard strains clustering with wastewater strains were however isolated prior, during and post the mallards' exposure to wastewater. The presence prior exposure may be explained as generalist enterococci strains. Studies indicate that some enterococci types may be more widespread since the same type has been detected in pigs poultry, from healthy humans, patients and wild birds [110, 141-143]. However, other studies indicate that enterococci are relatively reluctant to colonize a new host, i. e. enterococci from animals infecting humans [144-146]. Other species may as well be important to study as reservoirs for resistance determinants or characteristics making species zoonotic. In this study identical PhP-fingerprint was recorded for *E. hirae* but also isolates within the cfg- group. *E. hirae* and species included in the cfg-group is frequently found in several mammals as well as in birds, *figure 4* [147, 148]. Isolates from TW may include isolates from visiting birds, since wild birds have access to the wastewater treatment basins and are seen within the treatment plants, especially during winter time [149].

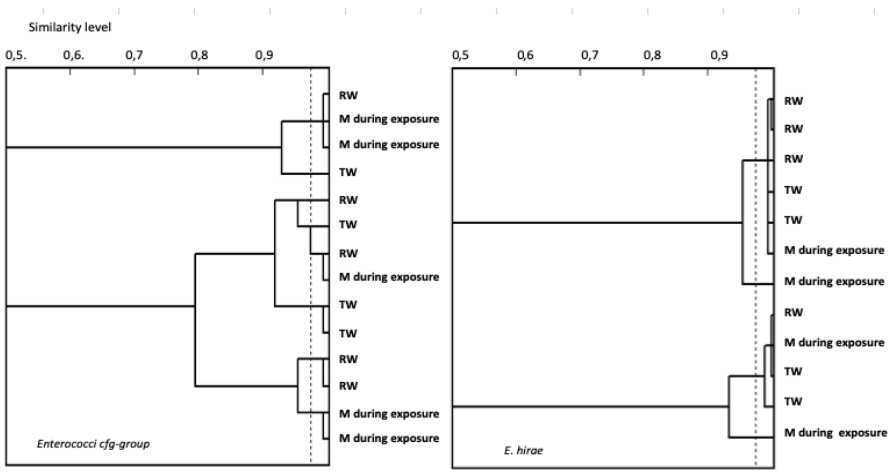

Figure 4. Isolates obtained from wastewater and mallards clustering together with E. hirae and with the Enterococcus cfg group. RW (raw wastewater); TW = (treated wastewater); M during exposure (mallard isolates obtained during exposure to wastewater).

The phenotypical resistance among the mallard enterococci isolates obtained without antibiotic additives were about 5%. Two mallard isolates were confirmed to be vancomycin resistant, one of them clustered with *E. durans* in the reference database, the second could not be typed. Ampicillin resistance was the only phenotypical resistance found among the enterococci before mallards were exposed to wastewater.

Higher frequency of resistance was seen among wastewater enterococci isolates, 28% from RW and 9% from TW. This is within the range of other studies [11, 150]. The difference between RW and TW is not significant due to few isolates and the results may be biased due to different sampling methods for RW and TW. The most ubiquitous phenotypical resistance among wastewater enterococci isolates were ampicillin and ciprofloxacin resistance. Four wastewater isolates were confirmed as vancomycin resistant when tested both by broth dilution (MIC) and disc diffusion, with an inhibition zone of < 11 mm, corresponding to an MIC value of 4 mg l^{-1}. Two of these belonged to the same phenotypical group (E.fcm1), the others to other groups, not clustering within the reference database. No wastewater samples were enriched in vancomycin broth, but vancomycin (16 mg l^{-1}) selective agar plates were used. The isolates' susceptibility was screened for ampicillin 8 mg l^{-1}, ciprofloxacin 4 mg l^{-1}, gentamicin 64 mg l^{-1}, erythromycin 4 mg l^{-1}, and vancomycin 16 l^{-1}

The low frequency of antibiotic resistant intestinal bacteria among mallards can be explained by the fact that antibiotics have been banned for growth promotion in animals in Sweden since 1986. Sweden and other Nordic countries have come far in the regulation work to restrain antibiotic resistance in the animal sector by practicing restrictive use of antibiotics in food animal production [151, 152]. The highest reported number of resistant bacteria seems in wild birds to be in raptors, placed high up in the food chain, and among birds that live close to humans [153]. In wild birds living far from human settlements, bacteria with antibiotic resistance is almost non-measurable [62].

We found no evidence for transmission of enterococci from wastewater to adult mallards during the exposure period. However, it remains possible that potential human strains could persist in the gut of the mallards at concentrations below the limit of detection, partly due to endogen enterococci microbiota masking their presence.

4.2 CHARACTERIZATION OF *C. JEJUNI* ISOLATED FROM MALLARDS PRIOR, DURING AND POST EXPOSURE TO TREATED WASTEWATER (PAPER II)

Several species of *Campylobacter* spp. are potential human pathogens, with *C. jejuni* as the most common [154]. Indeed, *C. jejuni* is the major reported bacterial cause of foodborne illness worldwide [42]. Campylobacter can be ubiquitously found in wastewater effluents as well as in surface water influenced by wastewater or agricultural runoff [155, 156]. Birds are also suggested to be a source to campylobacter in waters [61, 157]. The presence of *Campylobacter* spp. is reported to be related to incidences of campylobacteriosis in the human population as well as animal/poultry processing facilities connected to the waste water treatment plants [16]. In Hässleholm community, there are no abattoirs, why impact of domestic animal *Campylobacter* spp. can be neglected. The presence of campylobacter in the wastewater during the sampling period was not analysed. An indication of occurrence during the exposure period is the reported incidence of campylobacteriosis in Hässleholm during the time period of August to October 2004. Two cases were reported in June, three in July, and three in August (Public Health Agency of Sweden, the department of Scania). This is a low level of incidences, even if underreported cases are common. The underreporting factor for campylobacteriosis in Sweden is estimated to 1,5. This is however low compared to other countries in EU [121]. The WWTPs also remove campylobacter. Removal is reported to be between 98–99%, if an activated sludge step is included in the treatment [16]. This reduction is in the same range as for enterococci in the Hässleholm WWTP [118].

All *C. jejuni* ST-types but one, and PFGE-groups were present prior to the mallards exposure to the wastewater. This indicate that *C. jejuni* were either not present or present in low concentrations in the wastewater, or that mallards were not colonized by human campylobacter strains. It has been demonstrated that *C. jejuni* isolated from wild birds in general are restricted to birds, compared to those isolated from humans and food animals [61]. Overlapping strains between birds and humans have however been reported, primary among birds foraging in environments influenced by anthropogenic waste [98, 158, 159]. Several of the identified STs found in this study have previously been found in both humans and birds, but predominantly isolated from birds [61, 160-167].

Exposure studies including the sacrificing of birds can reveal strains present in the birds intestines, but not shredded. These analyses were however not included in the study. It has also been suggested that birds living close together are sharing *Campylobacter* spp. and/or clones. These are stabilized within the flock through inter-bird transmissions [168]. The present study exposed adult (>1 year) mallards with pre-existing campylobacter in their intestines to TW. Studies addressing chickens and colonization of campylobacter indicated that campylobacter have a "window" of colonization. After this period the microbiota is stable and alterations are more dependent on diet [169, 170]. However, if newly hatched mallards can carry human *Campylobacter* spp. strains has still to be investigated.

The results presented in paper II indicate that urban wastewater have impact on *C. jejuni* strains residing in the mallard's intestines. Isolates obtained prior to exposure/within the first week of the exposure clustered together and formed separate PFGE profiles from strains isolated later during exposure and post exposure. In addition, antibiotic resistance against ampicillin and cefazolin changed successively. The highest expressed resistance occurred during week 4-7, and declined during the post exposure period, *figure 5*.

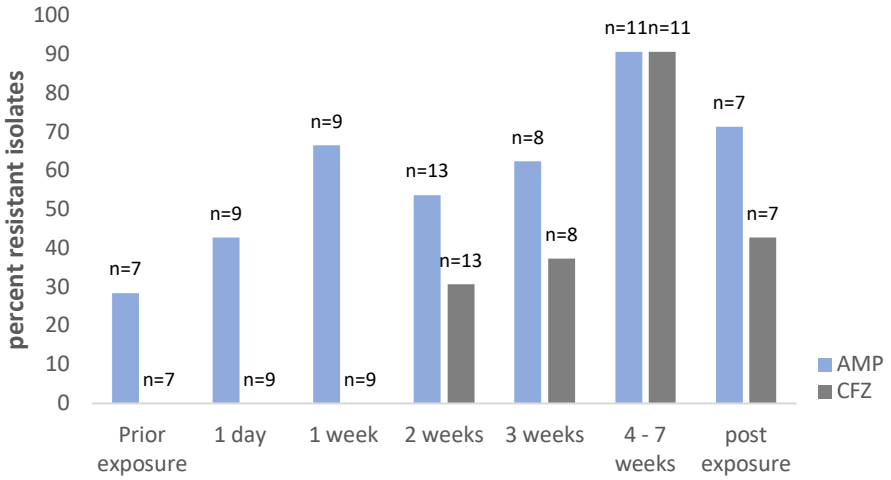

Figure 5. Percent of C. jejuni isolates resistant to ampicillin (AMP) and cefazolin (CFZ), prior, during and after the mallards exposure to wastewater.

The assimilation of malate and succinate in the isolated *C. jejuni* strains were also linked to the mallard's exposure to wastewater. The content of bioavailable carbons such, as C4-dicarboxylates, is low in TW due to the active sludge treatment step in the WWTP that remove up to 99% of all bioavailable organic material, as an effect of biodegradation. The ability of the *C. jejuni* isolates to assimilate succinate corresponded to the mallards' access to grass, as indicated by the isolates from the post exposure period then the mallards still had access. Grass contains several different polysaccharides and sugars as well as intermediates of the citric acid cycle accumulated in plant tissue, with succinate represent in as much as 10% of the dry matter, and malate for up to 1.5% [171]. *C. jejuni* is deficient in enzyme systems using C6-carbohydrates, but carbon utilization is enabled by parts of glucogenesis and tricarboxylic acid (TCA) [172-174]. Important primary energy sources have been shown to be a number of amino acids, acetate and C4-dicarboxylates, all known to be by-products of other bacteria [173, 175, 176]. This means that the observed metabolic change of the campylobacter may as well be influenced by other microbiota in mallard intestines, but also by bacteria passing through. The cause and consequences of changed phenotypical expressions of campylobacter in mallards exposed to wastewater needs to be further investigated with discriminating methods embracing patterns of individual microbiota over time.

4.3 CHARACTERISTICS OF POTENTIALLY PATHOGENIC VIBRIOS FROM SUB-TROPICAL MOZAMBIQUE COMPARED TO ISOLATES FROM TROPICAL INDIA AND BOREAL SWEDEN (PAPER III)

Vibrio spp. were present in clams during all sampling seasons in Maputo Bay, although the numbers increased during the warm (>30°C) rainy season, in concordance with other studies [14, 15]. The majority of the vibrio strains isolated were identified as *V. parahaemolyticus*. Only one of these was confirmed to carry the *tdh* gene, this in concordance with previous study of environmental isolates [126]. However, 69% of the strains exhibited phenotypical haemolytic properties. Haemolysin of *V. parahaemolyticus* has been shown to be correlated with an additional haemolysin, one of them the σ-vph gene [177]. Phenotypical comparison of strains showed similarities between strains from Mozambique and Sweden. The Indian strains had identical PhP-fingerprints with isolates from Mozambique and global dissemination of *Vibrio parahaemolyticus* has been reported [178] but has to be confirmed with more discriminating methods.

One isolate was confirmed to be *V. cholera*. This strain was not further analysed for presence of cholera related virulence genes. Cholera outbreaks have occurred in Mozambique almost every years for the past five years including the Maputo province [179]. It has been suggested that strains causing human diseases are from clades of human origin circulating in the environment and the strains of pure environmental origin are of minor importance [180].

All vibrios in this study, independent of country, expressed phenotypical resistance to beta-lactams. In Sweden strains showed additional resistance to nalidixic acid, cefuroxime and chloramphenicol. In general *V. parahaemolyticus* is commonly considered highly susceptible to virtually all antimicrobials, except to penicillin's, this recently explained by a chromosomal carbencillin hydrolysing lactamase (CARB) [181]. Lately, ESBL resistance among vibrio's isolated from clinical samples have been reported [182, 183].

Potential pathogenic strains of *Vibrio* spp. are found all over the world [184] and are predicted to be even more important as a cause of diseases in the future, as a consequence of global warming. Elevated water temperatures with more intense precipitation leads to flooding and changes in water salinity in the coastal regions, all of this may have an impact on the frequency of diseases caused by vibrios [185, 186]. Indeed, outbreaks of vibrio related infections, like bathing fever in colder climate areas, has significantly shifted lately as well as sporadic cases of *V. parahaemolyticus* towards larger outbreaks attributed to consumption of oysters harvested from northern waters. This is linked to higher mean water temperatures [82, 187]. Seabirds are suggested to contribute to the spread of pathogenic vibrio from one area to another. Birds consuming vibrio containing crustaceans and midges are of special interest [188]. But the most important source of spread is probably traveling humans and imported seafood.

4.4 CULTURABILITY OF *E. FAECIUM* AND *E. FAECALIS* IN TREATED WASTEWATER – A LONG TERM MICROCOSM STUDY AT TWO TEMPERATURES AND TO PRESENCE OF CIPROFLOXACIN (PAPER IV)

Wastewater treatment plants are good suppliers of human bacteria [12], but the persistence of the released bacteria in the environment is also of importance. The longer they survive, the greater the risk they will reach vulnerable individuals, human as well as animal. Bacteria in general survive better at low

temperatures. The temperature in Magle constructed wetland is below 10 degrees most part of the year, including the beginning of the mallards hatching period, *figure 6*.

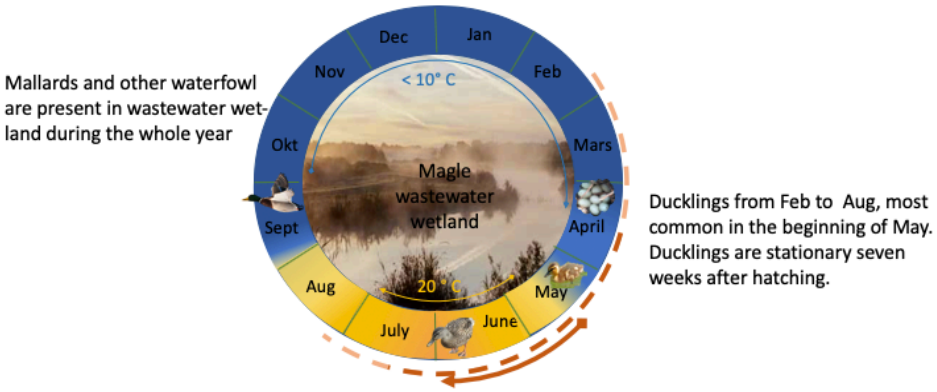

Figure 6. Water temperatures during the year in Magle constructed wetland, south of Sweden. Temperatures varies from a few degrees above zero during January and February to about 20°C during July and August [189]

The persistence of *E. faecalis* and *E. faecium* was measured as culturable cells. Persistence was higher at 10°C compared to 20°C and a significant difference was observed between *E. faecium* and *E. faecalis*. Depending on species and temperature, 0,1-0,01% of the cells remained culturable even after > 100 days. The time for one log reduction (DC10) was twice as long at 10°C compared to 20°C for *E. faecalis* (92 and 43 days) and five times higher for *E. faecium* with a DC10 of 333 and 68 days respectively. *Enterococcus* spp. are known to have the potential to survive for extended periods, especially at low temperatures. Factors such as light and predation contribute to removal in natural systems [21, 35]. *E. faecalis* is reported to survive as viable but non-culturable cells (VBNC) [190], thus the survival in the microcosm might have been more extended. PCR based methods of detection could have been used to verify this. We attempted to apply qPCR as a complement to the viable count, but were unable to extract high quality DNA from the majority of the persister cells, resulting in inconsistencies between CFU ml^{-1} and qPCR data. However, detection of enterococcal DNA doesn´t provide information regarding viability of the cells as it will detect both living and dead cells, as well as free DNA.

The effect of ciprofloxacin (CIP) on culturability of *E. faecalis* and *E. faecium* was performed with concentration of 0; 0,5; and 8 mg CIP l^{-1}. Most of the strains were not affected by CIP and the reduction of cultivable cells remained the same independent of concentrations. Although, three out of eight *E. faecium* strains analysed were sensitive to 8 mg CIP l^{-1} and one of these showed sensitivity to 0,5 mg CIP l^{-1} even if this isolate had a MIC-value of 2 mg CIP l^{-1}. One *E. faecalis* strain, with a MIC- value of 1 mg l^{-1}, was negatively affected at 0,5 mg CIP l^{-1} but not affected at 8 mg CIP l^{-1}. The ability to survive CIP in concentrations higher than the MIC-value can be attributed to the formation of persister cells, which probably are the reason for the survival of strains in this study [190, 191]. The cause and the extent of susceptibility to CIP among enterococci persister cells have still to be investigated, but the phenomena may contribute to the understanding of selection of resistant enterococci in WWTPs.

4.5 CONCLUDING REMARKS

The mallard studies were located within a WWTP in an authentic environment with treated urban wastewater with a constant flow. Twelve individuals were followed over time and exposed to wastewater for almost two months. Other studies of birds and campylobacter are either laboratory studies, often with few included individuals and parameters, or on randomly caught birds passing by, thus analyses of different individuals. Birds migrate even during the day, and the history of the birds is not recorded. The bird experiment in this thesis also included the time aspect. This enabled us to point out the changes in phenotypical patterns within the *C. jejuni* population. The significance of these results needs to be further investigated using more discriminating methods. Birds may carry several *C. jejuni* variants simultaneously and to embrace this, several isolates should have been picked and analysed. This means that this study mainly reflects dynamics of campylobacter and enterococci within the whole flock, rather than in the individual mallard. Age and weight are parameters that may have an impact on colonization and shredding of campylobacter, parameters that were not included. According to the breeder, all twelve birds were older than one year. In the campylobacter study, no samples were obtained and analysed from the wastewater. This means that it is impossible know if *C. jejuni* was present at detectable levels in the water. Wastewater isolates were however included in the enterococci study, but the results may have been biased due to few samples and different sampling methods for RW and TW, but also due to the fact that water sampling was partly performed later than the exposure of the mallards. Five of the wastewater samples were collected from TW, and visits on WWTPs during the last years

have made it obvious that mallards thrive inside WWTPs basins, primary during the cold part of the year, *figure 7*. This observation also implies that treated wastewater may include isolates from birds.

Figure 7. During the cold period of the year mallards are seen within the active sluge basins

Identification and epidemiology of bacteria strains with phenotypical methods depend on that identical phenotypical fingerprints correspond to identical genetical fingerprints. This may not necessary always be the case. The phenotypical methods may be used as indications and as tools to choose strains for further more discriminating methods. This is even more important when comparing different populations, such as mallard isolates versus wastewater isolates, or vibrios from Mozambique versus Sweden and India.

The experimental setup in this thesis to investigate the persistence of enterococci did not include additional factors important for removal of faecal enterococci in TW. Other laboratory studies have taken this into account, but most of these studies only include few strains. The study reported in paper I includes several strains of both *E. faecalis* and *E. faecium*, showing the importance of patterns and discrepancies at strain level, which is not possible to detect when only few strains of similar origin is used. The levels of nutrients, such as content of bioavailable carbon was not measured in microcosm water, nor was the intrinsic level of ciprofloxacin measured in the microcosms. The latter was assumed to be neglectable since phenotypical activity of CIP in the microcosms were measured at the last sampling days using water from analysed microcosms. Microcosm water was applicated into wells in Müller

Hilton agar inoculated with a susceptible *E. coli* strain (CCUG 8619400). No inhibiting activity from microcosms without added ciprofloxacin was seen, whereas water from microcosms with ciprofloxacin always inhibited *E. coli* growth in a zone, depending on the concentration.

The exposure study of mallards started for more than fifteen years ago, 2004. During this time very much has changed both in routines, and the availability of more discriminating tests. Today more analyses and test are affordable and can be analysed by commercial laboratories at rather low costs.

A short summary of methods that would have been appropriate to use in this study is whole genome sequencing (WGS), a method that can provide a more robust characterization of the different strains on DNA level. During the last decade the rapid development of WGS has made the technique more available for common use, partly due to reduced costs. WGS is therefore successively becoming a primary analytic method for detection of bacterial pathogens in epidemiological studies, as well as studies on virulence and antibiotic resistance [192-195]. Possible changes in the genome can also be trace with WGS. If changes in metabolic pattern among isolates are not due to genetic changes, but merely change in gene expression, other techniques are available. High resolution temporal imaging of bacterial microarrays allows a high number of individual bacterial cells to be followed [196]. If using this approach, insights into overall population behaviour as a function of time would be possible since the method can be used to study changes in transcription of genes [196, 197].

5 MAJOR FINDINGS

Enterococci are released in large amount from urban wastewater treatment plants (WWTPs), although the treatment reduction is over 98 - 99%. In the study, *Enterococcus* spp. were found to the same extent in wastewater as in mallards, with *E. faecalis* and *E. faecium* as the most abundant in wastewater, and *E. faecalis*, *E. durans* and *E. faecium* in mallards. The phenotypical sub-typing of the strains indicated that certain profiles tended to be either of mallard or human origin. However, there were some findings of identical or highly similar fingerprints, indicating a common source or transmission route for enterococci between mallards and humans. The direction of the transmission could not, however, be traced since all profiles were found before the mallards were exposed to the wastewater.

Campylobacter strains were stable within the mallard flock and the colonization was not affected by exposure to urban wastewater. The shredding of campylobacter differed largely between individual mallards. All *C. jejuni* ST-types found have been reported previously, usually from wild birds, but also from humans, poultry and recreational waters. The results in this study indicate *C. jejuni* inhabiting mallards´ intestines might change their phenotypes while exposed to wastewater, as the phenotypical expression of resistance against ampicillin and cefazolin, and the ability to assimilate malate and succinate, changed during the mallards exposure to wastewater.

V. parahaemolyticus in clams from Maputo Bay were highest during the hot rainy season. Haemolytic activity was high among isolated strains, even though the virulence genes *tdh* and *trh* were absent. This indicating that additional genes probably have to be included in the screening to identify potential pathogenic strains.

Different *E. faecium* and *E. faecalis* strains had different persistence in treated wastewater, with *E. faecium* as the more persistent. The survival of the strains was temperature dependent, with a twofold longer survival at 10°C compared to 20°C for *E. faecalis*, and a fivefold longer survival for *E. faecium* measured as DC10-values. Different die-off patterns were seen among different strains. Ciprofloxacin had no effect on most of the strains, irrespectively of susceptibility, but some *E. faecium* strains lost culturability faster when ciprofloxacin was present in levels higher than the strains corresponding MIC value.

6 FUTURE RESEARCH

Examples of future research questions and studies raised from the present thesis, are given below:

- Do Intestinal bacteria of mallards feed seeking in active sludge treatment basins resistance genes with bacteria isolates from raw wastewater?
- Are newly hatched mallards colonized by urban wastewater bacteria? A longitudinal study within a wastewater treatment plant.
- Does wastewater have impact on the presence of resistance and virulence determinants in residing microbiota of adult mallards? A longitudinal study within a wastewater treatment.
- Screening of *Enterococcus* strains of different origin for impact of ciprofloxacin on persister cells.

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APPENDIX

Schematic overview of the outline and perspectives of the thesis