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

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***Abstract*- In this study, twelve Mallards living in an artificial wastewater wetland were exposed to treated wastewater containing 1 x 10^3- 4 x 10^3 enterococci 100 ml^{-1} for a period of 55 days. Faecal samples were collected before, during and after exposure and analysed for *Enterococcus* spp. The isolates were phenotyped using the PhenePlate™ system. 270 *Enterococcus* spp. of Mallard origin were analysed, together with 116 *Enterococcus* spp. isolates from treated wastewater and from incoming raw wastewater. In general, the Mallard and wastewater enterococci isolates belonged to different phenotypes, although several sharing identical phenotypic profiles were found. One *E. faecalis* phenotype was found in Mallards before, during and after exposure to treated wastewater, as well as in raw and treated wastewater. Our results indicate that there is a common source of enterococci for Mallards and humans. We propose an increased focus on emissions of human bacteria and on systems that mediate their transfer to wild animals.**

Keywords*- *Enterococcus Faecalis; Enterococcus Faecium; Anas Platyrhynchos; Mallard; Urban Wastewater; Sewage; Wastewater Wetland

I. INTRODUCTION

Although wastewater undergoes several treatment steps during the sewage treatment process, faecal bacteria remain present in the effluent water. Consequently, human faecal bacteria are released in recipient areas [1, 2]. Some of these faecal bacteria are zoonotic, i.e. they are naturally transmissible from animals to humans and vice-versa [3]. Enterococci constitute a normal part of the intestinal bacterial community of humans and animals, although species distribution varies with host, geographical location and time [4-8]. Several studies have previously shown that animals and humans share enterococcal clones [9-12]. Although the researchers responsible employed a variety of methodological approaches, many of which were of low statistical power, the emerging general pattern is that enterococci are transmissible between animals and humans. However, some enterococcal studies examining a large number of replicates have found only sporadic transmission or even failed to detect any matching clones. [13-18].

The medical importance of enterococci is of particular interest. In the last few decades, enterococci have emerged as one of the most important organisms causing nosocomial infection and have thus been pinpointed as a specific threat to public health [19]. Their medical importance is related to their intrinsic and acquired resistance to a wide range of antibiotics and the potential risk of transmission of virulent and/or multi-drug resistant elements to other species [20, 21]. Enterococci are also always members of the human intestinal bacteria population. In this regard, municipal wastewater represents a pooled sample of the enterococcal flora of the entire human population, including enterococci from both antibiotic-treated and healthy people, resulting in a mixture of enterococci with various innate and acquired characteristics, including antibiotic resistance. Nevertheless, enterococci are released and exposed in nature in different amounts depending on the effectiveness of the wastewater treatment plant. The reduction of enterococci in wastewater plants is reported to vary from 26% to 99.99%, while the level of enterococci in effluent lies between 10 and 10^4 enterococci 100 ml^{-1} [22-26].

Wetlands often attract large numbers of birds, with constructed wastewater wetland and wastewater stabilisation ponds reported as popular habitats for waterfowl [27, 28]. Wastewater treatment wetlands are used worldwide and appear to be increasing in popularity in developing as well as developed countries [29]. One reason for their growing importance in Europe is the EU Water Framework Directive (2000/60/EC), which states that a "good ecological status" must be achieved for all European waters by 2015 [30]. There is therefore an increasing demand for constructed wetlands as they are environmentally sustainable, cost-effective and have relatively high treatment performances [31, 32]. There is also an on-going loss of natural wetlands worldwide, thus increasing the ecological value of created wastewater wetlands [29]. Indeed, the provision of wildlife

habitat is often promoted as an ancillary function of constructed wetlands [33, 34]. Nevertheless, wastewater wetlands attract large amounts of waterfowl, exposing the latter to human enterococci. Wastewater wetlands could therefore potentially act as cradles for the transmission of pathogenic enterococci and/or genetic elements to birds, increasing the risk that they will also become propagation hubs for vectors of human diseases [35].

Based on these facts we here propose the following general framework: we hypothesise that the transmission of human enterococcal clones to birds is possible and that wastewater wetlands may provide a platform for this to happen.

In this paper we address the above hypothesis by comparing enterococci from adult Mallards placed in an artificial municipal wastewater wetland with enterococci from humans. The Mallard was chosen because it is a common waterfowl living close to humans and human activities, a common model organism in ecology and commonly bred in captivity in Sweden. Enterococci were chosen because they normally inhabit the gastrointestinal tract of humans as well as animals and they are released daily in huge amounts worldwide. Enterococci are also medically important since they are intrinsically resistant to or tolerate many antibiotics and are also readily capable of acquiring resistance.

The general aim was to achieve an environment in which the ducks were subjected to high concentrations of human enterococci for a prolonged period, in this case 3 months. Epidemiological similarities and differences were studied by comparing biochemical phenotypes of the enterococcal populations. One motive for using the phenotypical method is the ability to quickly scan and compare isolates from Mallards (*Anas platyrhynchos)* with those found in municipal wastewater. In this study the biochemical relatedness of Mallard and wastewater isolates was not confirmed using genetic methods. Research comparing PhP-typing and Pulsed Field Gel Electrophoresis (PFGE) has shown that although results produced using the two techniques correlate, PFGE is slightly more discriminating than PhP [36, 37].

Two strategies were employed to sample and extract enterococci from the Mallards. The first involved comparing enterococcal phenotypes using pooled samples of faecal material from all ducks, with the normal biota of the Mallard population then unconditionally scanned during exposure to wastewater. The second involved sampling each Mallard separately and enriching the obtained faecal sample in Vancomycin broth, limiting the quantity of emerging enterococci and the selective selection of those found to be Vancomycin resistant [38].

II. MATERIAL AND METHOD

A. Site Description

The wastewater treatment plant for the city of Hässleholm is situated 10 km from the city centre and receives wastewater from both the city and its surrounding area. The daily mean volume of wastewater varies from 12,500 m^3 to 32,300 m^3. Treatment of wastewater at Hässleholm involves the following five steps: 1) pre-aeration and pre-sedimentation assisted by the production of activated sludge via chemical precipitation; 2) aerated activated sludge; 3) chemical precipitation with iron chloride; 4) filtration through a three-media filter composed of sand and plastic granules; 5) nitrogen reduction in a constructed wetland. Hässleholm represents a unique location for this type of study because of the very low background levels of enterococci from non-anthropogenic sources. No agricultural or industrial wastewater is treated in the system and stormwater is separated from the treatment plant, although some leakage may occur via either surface water movement into manholes or leakage into pipes from soil water and groundwater.

B. Experimental Design

Twelve adult Mallards (*Anas platyrhynchos*), 6 female and 6 male, were purchased from a local breeder and kept in a 50 m^2 fenced (chicken wire) grass yard according to Swedish legislation [39]. The Mallards were exposed to effluent wastewater in the form of a 2.5 m^2 pool (0.75 m^3) placed inside an 18 m^2 shelter. This treated wastewater (having passed through five treatment steps) was the only source of water in the corral and was continuously provided at a rate of 2 litres min^{-1}. The Mallards also had free access to pasteurised cereals dispensed via a food dispenser placed inside the shelter. The experimental period ran from the middle of August to October.

C. Collection of Faecal Samples

To follow variation in the Mallard enterococcal population during the study period, enterococci obtained from the collected Mallard faecal samples were phenotypically compared both to each other and to enterococci isolated from wastewater. Two strategies were employed to collect Mallard isolates. To compare enterococci during the experimental period, pooled faecal samples (PMF) from the entire group of Mallards were analysed. In addition, an individual sampling (IMF) of each Mallard was made, with the focus in this case on the presence of Vancomycin-tolerant enterococci. Since not all enterococci have an intrinsic low-level resistance to Vancomycin, this was considered a way of limiting the enterococcal yield. A single isolate per Mallard was chosen for the same reason. Mallard faecal samples were collected before, during and after animal exposure to wastewater, with the samples taken by placing the Mallards in separate boxes. IMF samples were obtained using Copan sticks (Copan Diagnostics Inc, Italy) and PMF samples by collecting as many droppings as possible from each Mallard into a single sterile Falcon tube, using sterile plastic loops. All samples were immediately stored in a refrigerated cool box, with pooled

samples analysed within 5 hours and individual samples within 24 hours. IMF samples were collected on 19 separate occasions and PMF samples on 9 occasions (Table 1). In total, 270 presumed enterococci isolates were obtained (after passing confirmation-criteria for enterococci), comprising 197 isolates from pooled samples and 73 from individual samples.

TABLE 1 SAMPLING OF INDIVIDUAL (IMF) AND POOLED (PMF) MALLARD FAECAL SAMPLES, AS WELL AS RAW (RW) AND TREATED WASTEWATER (TW), AUG–OCT 2004 AND 2005 (TW)

	Hours/ days of exposure	**IMF Nr**	**PMF Nr**	**RW & TW**	**Comments**
040811	0h	1	-	-	Control, before exposure
040816	0h	2	-	-	Control, before exposure
040818	0 h	3	1		Control, before exposure
040818	6 h	4	-	-	6 h exposure
040819	18 h	5	-	-	
040819	27 h	6	-	-	
040823	5 d	7	-	-	
040826	8 d	8	-	-	
040830	12 d	9	-	-	
040902	15 d	10	2	-	
040906	19 d	11	3	-	
040909	22 d	12	4	TW01	Flow proportional sampling (24 h)
040913	26 d	13	5	TW02	Flow proportional sampling (24 h)
040920	33 d	14	6	TW03	Flow proportional sampling (24 h), no PhP analyses
040927	40 d	15	7	-	
041006	49 d	16	-	-	
041012	55 d	17		-	
041019	No exposure	18	9	-	Mallards exposure finished
041027	No exposure	19	10	-	Mallards exposure finished
050214		-	-	RW01	Random sample
050214		-	-	TW04	Flow proportional sampling (24 h)
050415		-	-	RW02	Random sample
050415		-	-	TW05	Flow proportional sampling (24 h)

D. Collection of Wastewater Samples from the Treatment Plant

Sampling of incoming raw wastewater (RW) was undertaken twice after the completion of the experimental period, while outgoing treated wastewater (TW) was collected on three occasions during the experimental period and twice in parallel with RW. RW was collected from the incoming basin in 1 litre sterile bottles, while TW was collected via flow proportional sampling (24 hours) carried out on outgoing water (Table 1). All samples were kept refrigerated and analysed within 5 hours of collection. In total, 116 presumed enterococci isolates were obtained.

E. Isolation of Enterococcus spp on MEA with and without Antibiotics

Pooled faecal droppings were first mixed in the collection tube using an Ultra Turax T8 homogeniser (Kika Labortechnik, Germany). One gram (wet weight) was then diluted ten times in sterile NaCl (0.85%) and mixed (Vortex) at room temperature for two minutes. The samples were subsequently diluted a further ten times before 0.1 ml was spread on an *Enterococcus* agar plate (MEA). Wastewater was spread directly on MEA, with the sample subjected to tenfold serial dilution if necessary. After incubation at 37 ℃ for 48 hours, 8 to 36 typical enterococci isolates were chosen depending on the number on agar plates. The isolates were later sub-cultured on bile esculine agar and tested for catalase activity. Isolates exhibiting a typical phenotypic enterococcal pattern, i.e. esculine positive and catalase negative, were selected for further analysis.

Individual Mallard faecal samples were suspended in Todd Hewitt broth (Difco) with the addition of 32 mg l^{-1} Vancomycin, before being incubated for 24 h at 37 ℃. After incubation, 0.1 ml was spread on *Enterococcus* agar plates (MEA, Difco). If growth was obtained, one isolate from each Mallard sample was chosen and used for further analysis.

To intercept resistant enterococci present among the Mallard and wastewater populations, the pooled faecal material and wastewater were applied on MEA, each with a supplement of antibiotics consisting of one of the following: Ampicillin (8 mg^{-1}), Ciprofloxacin (4 mg^{-1}), Gentamicin (64 mg^{-1}), Erythromycin (4 mg^{-1}) or Vancomycin (16 mg^{-1}). Activity of the supplemented MEA was checked using enterococci with known sensitivity or resistance to the tested antibiotics. If growth was observed, two to three colonies were chosen for further analysis (Oxoid Ltd, Basingstoke, UK).

F. Phenotyping Enterococci with the PhenePlate™ System

Enterococcal phenotyping was performed using the PhenePlate™ rapid screening system (PhP-RF; PhPlate Microplate Techniques AB, Stockholm, www.phplate.se). In the PhP-RF plates, 11 test reagents are used to differentiate different *Enterococcus* phenotypes based on measurement of the kinetics of the biochemical reactions [40, 41]. Enterococci isolates were suspended in the first row of the microplates; 25 µl were then transferred from these wells into each reaction well via a multichannel pipette. The plates were incubated at 37 ℃ and the reactions analysed after 16, 40 and 64 h using an Elx 808 Ultra Microplate Reader (Biotek instruments, Inc, Winooski, United States) at an absorbance of 620 nm. Absorbance data obtained from each tested isolate were entered into the PhenePlate™ software in order to calculate the similarity and diversity of isolates and populations, as well as to enable species identification [18, 40, 41]. Similarities between isolates were calculated as correlation coefficients. Isolates with correlation coefficients equal to or higher than 0.965 were assigned the same PhP-type. The relationship between isolates was visualised in a dendrogram derived from data clustering using the unweight pair group method (UPGMA). A similarity with reference species data of $\geq$ 0.9 was regarded as confirming preliminary species identification [41]. The diversity of the bacterial population in each sample was calculated in terms of Simpson's diversity index (D_i), again using the PhPWin software program (www.phplate.se). D_i is a relative measure of the distribution of isolates into PhP-types. High diversity gives an index value of close to 1, whereas no diversity has a zero index value [41]. All PhP-assays included a doublet of the reference strain *E. faecium* respective *E. faecalis* and was considered credible in those cases where correlation coefficients for the respective doublet was higher than 0.98.

G. Antibiotic Resistance

The susceptibility of all isolates (386) independent of isolation method was tested using microplates containing minimal breakpoint concentrations (MIC) of antibiotics (Ampicillin 8 mg L^{-1}; Ciprofloxacin 4 mg L^{-1}; Gentamicin 64 mg L^{-1}; Erythromycin 4 mg L^{-1} ; Vancomycin 16 L^{-1}) in Iso-Sensitest Broth (Oxoid Ltd, Basingstoke, UK), as described by Iversen and Kühn [42]. Visual growth was registered after 18-20 hours, with isolates showing growth further tested via disk diffusion (Oxoid Ltd, Basingstoke, UK) as described by the Swedish Reference Group for Antibiotics (SRGA) [43].

III. RESULTS

A. Enterococcal Populations

Mallard faecal droppings contained enterococci at levels in the range of 10^1-10^5 CFU g^{-1} wet weight. A total of 270 confirmed enterococci isolates were obtained from the Mallards: 197 from pooled faecal samples, of which 147 were isolated on MEA without antibiotic supplement (hereafter called *normal isolates*) and 50 on MEA with supplement, and 73 isolates from individual samples enriched in Vancomycin broth culture. Among the 147 normal isolates the most common species identified was *E. faecalis*, followed by *E. faecium* and *E. durans*. Other species identified were *E. hirae*, *E. raffinosus* and species belonging to *E. casseliflavus*, *E. flavences* and *E. gallinarum* (the Cgf species group) [1]. 21% of enterococci isolates did not cluster with any of the strains in the reference database (PhenePlate™ software) and were thus not typable. The overall D_i value of all normal isolates was 0.91 and the geometric median of diversity indices was 0.92 (SD 0.19). However, one of the pooled Mallard samples exhibited a markedly lower D_i value of 0.38. In this case 18 out of the 26 normal isolates belonged to the same *E. faecalis* biochemical profile (BP) and thus clustered together. The low diversity of this sample affected the total diversity and standard deviation of the PMF isolate data. When this sample was excluded from the calculation, the median of diversity of the pooled samples increased to 0.95 (SD 0.08). Enterococcal diversity was lower among the IMF isolates, with a D_i value of 0.88 and a geometric median of 0.98 (SD 0.22). The enrichment broth prevented growth of *E. durans* in all except one isolate, which showed resistance to Vancomycin.

The amount of enterococci found in wastewater varied from 4 x 10^4 - 2 x 10^5 CFU 100 ml^{-1} in raw wastewater to 1 x10^3 - 4 x 10^3 CFU 100 ml^{-1} in treated wastewater. In total, 116 *Enterococcus* spp isolates were collected from wastewater, comprising 56 from raw wastewater (RW) and 60 from treated wastewater (TW), with 47 normal isolates. The relative proportion of *E. faecalis* and *E. faecium* was nearly equal in both raw and treated wastewater. A higher proportion of enterococci from raw wastewater (89%) than treated wastewater (70%) clustered with strains in the database (PhP™). The overall D_i value of all normal isolates found in raw wastewater was 0.96, while the geometric median of the diversity index was 0.90 (SD 0.15). The overall D_i value and median of isolates from treated wastewater were 0.98 and 0.95 (SD 0.001), respectively. The former value is in accordance with that of enterococci in municipal wastewater published in other studies using PhP [8, 18, 41].

B. Distribution of Isolates into PhP Types

Figures 1-3 show the clustered PhP data for the different *Enterococcus* spp isolated from Mallards and wastewater. The 386 isolates studied could be divided into 127 different biochemical phenotypes (BPs), with 50 of these containing more than one isolate. The remaining 77 BPs comprised single isolates (si). Isolates with correlation coefficients equal to or higher than 0.965 were assigned to the same BP.

Similarity level (correlation coefficient)

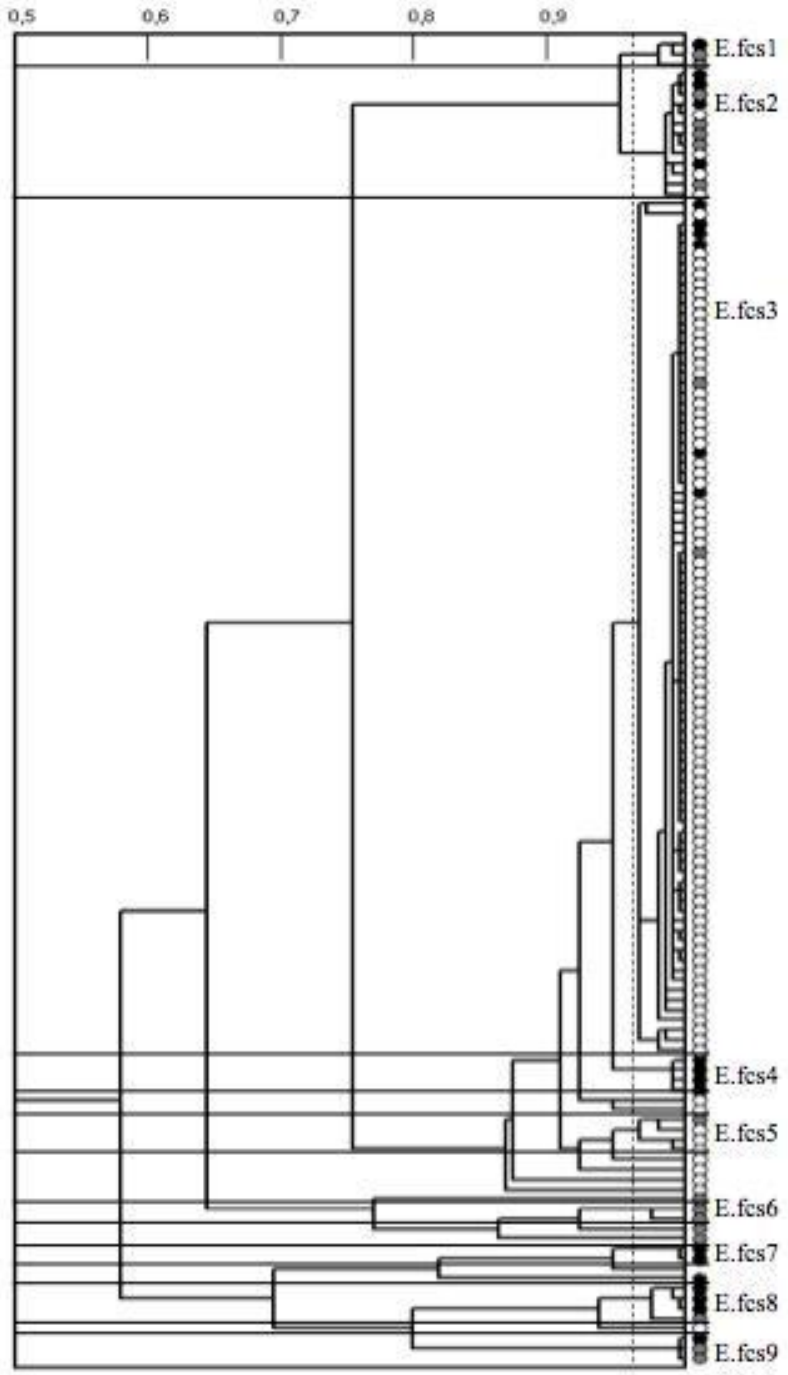

Fig. 1 Dendrogram showing UPGMA clustering of PhP-FS data based on the biochemical profile (BP) of *E. faecalis* isolated from Mallards and Swedish wastewater. Solid black circles indicate raw wastewater (RW), grey circles treated wastewater (TW) and open circles Mallards. The dotted vertical line indicates the ID-level (0.965) determined by the reproducibility of the typing method [37]. Isolates showing similarity to each other higher than this level were assigned to the same BP. 12 BP clusters. n=133

Figure 1 presents the BPs obtained for *E. faecalis* strains. Isolates belonging to this species could be divided into two main groups, which in turn could be broken down into 9 clusters and 12 single BPs. A mixed origin was found for the first group, which comprised E.fcs1- 6 and related single isolates. The second group, E.fcs7-9, seemed to be specific to wastewater although it also included one Mallard isolate. Of all *E. faecalis* BPs, E.fcs3 was the most commonly found in this study. This BP was isolated from Mallards before, during and after exposure to wastewater, as well as from both raw and treated wastewater (Table 2). It was also isolated from all 12 Mallards at least once (data not shown). Identical biochemical profiles of strains, including both Mallard and wastewater strains, were observed in BP clusters E.fcl2-3 and 5. E.fcs2 was mainly recovered from (treated and raw) wastewater, but was also found in Mallards during the period of exposure to wastewater (Table 2).

TABLE 2 BIOCHEMICAL CLUSTERING OF *E.FAECALIS* AND *E.FAECIUM* FROM MALLARDS, ISOLATED BEFORE, DURING, AND AFTER EXPOSURE TO RAW (RW) AND TREATED (TW) WASTEWATER

Species	**Mallards**			**RW**	**TW**
	Before	**During**	**After**		
E. faecalis	E.fcs**3**	E.fcs**2** E.fcs**3** E.fcs**5**	E.fcs**3** E.fcs **5**	E.fcs**1** E.fcs**2** E.fcs**3** E.fcs**4** E.fcs**7** E.fcs**8** E.fcs**9**	E.fcs**1** E.fcs**2** E.fcs**3** E.fcs**5** E.fcs**6** E.fcs**8** E.fcs**9**
E. faecium	E.fcm**1** E.fcm**2** E.fcm**3** E.fcm**7** E.fcm**8** E.fcm**9**	E.fcm**1** E.fcm**3** E.fcm**7** E.fcm**8** E.fcm**9** E.fcm**10** E.fcm**11**		E.fcm**1** E.fcm**2** E.fcm**3** E.fcm**4** E.fcm**5** E.fcm**6** E.fcm**8**	E.fcm**1** E.fcm**2** E.fcm**3** E.fcm**6** E.fcm**7** E.fcm**8**

Figure 2 presents the distribution of *E. faecium* isolates, which could be divided into 11 clusters and 15 single BPs. E.fcm1-6 and E.fcm7-11 are two clearly separate biochemical groups. E.fcm7-11 could be further subdivided into two subgroups, 7-8 and 9-11, with the latter appearing to be specific to Mallards.

Similarity level (correlation coefficient)

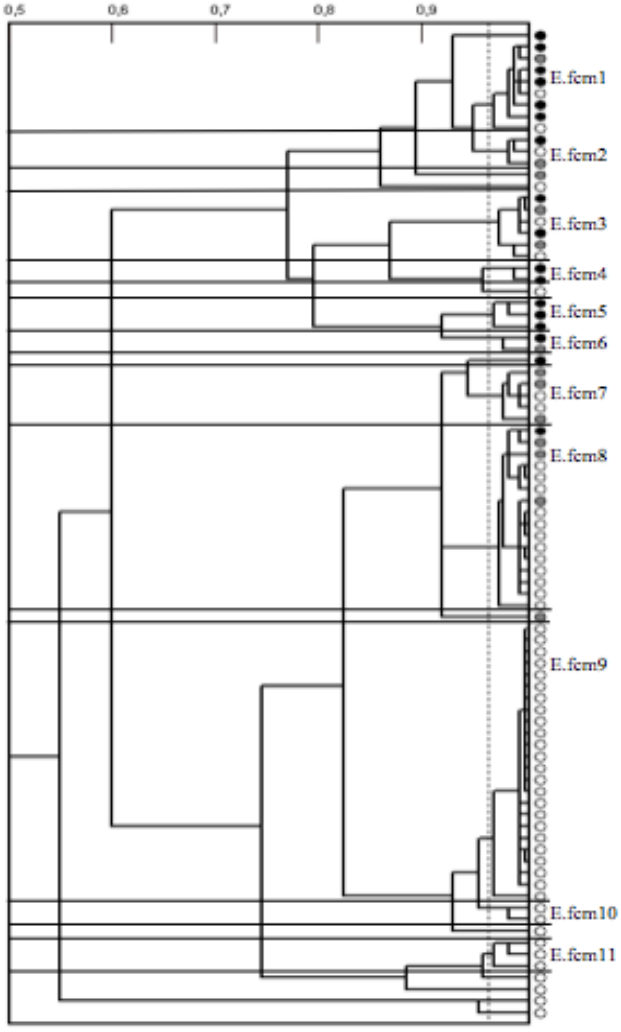

Fig. 2 Dendrogram showing UPGMA clustering of PhP-FS data based on the biochemical profile (BP) of *E. faecium* isolated from Mallards and Swedish wastewater. Solid black circles indicate raw wastewater (RW), grey circles treated wastewater (TW) and open circles Mallards. The dotted vertical line indicates the ID-level (0.965) determined by the reproducibility of the typing method [37]. Isolates showing similarity to each other higher than this level were assigned to the same BP. 11 BP clusters. n=84

Other enterococci exhibiting a corresponding biochemical profile in the reference database (PhP™) included *E. hirae*, *E. durans*, the Cgf-group (*E. casseliflavus*, *E. gallinarum*, *E. flavescens*), *E. raffinosum* and *E. cecorum*. Among these species, few BPs included Mallard and wastewater isolates (Fig. 3). It is notable that *E. durans* and *E. hirae*, both isolated in this study, seem to be more biochemically homogeneous than *E. faecalis* and *E. faecium*.

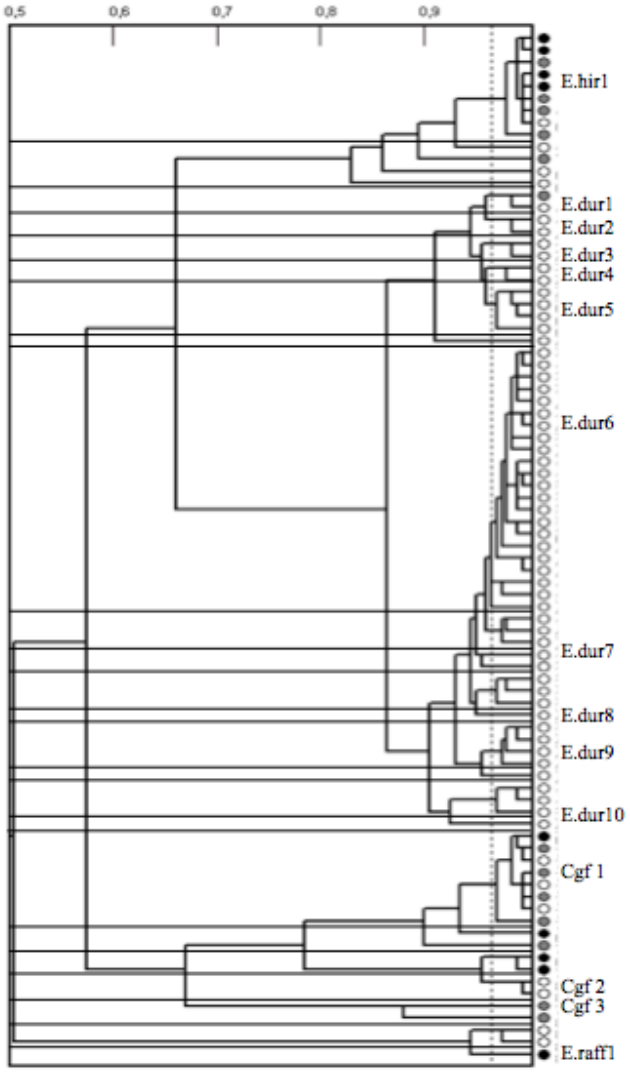

Fig. 3 Dendrogram showing UPGMA clustering of PhP-FS data based on the biochemical profile (BP) of *E. hirae*, *E. durans*, Cgf-group and *E. raffinosus* isolated from Mallards and Swedish wastewater. Solid black circles indicate raw wastewater (RW), grey circles treated wastewater (TW) and open circles Mallards. The dotted vertical line indicates the ID-level (0.965) determined by the reproducibility of the typing method [37]. Isolates showing similarity to each other higher than this level were assigned to the same BP. 13 BP clusters. n=85

Isolates with no corresponding profile (NT) in the reference database (PhP™) were 75% Mallard isolates. When clustered, 11 different BPs were recovered. One of these included isolates from wastewater and Mallard faeces (data not shown).

C. Antibiotic Resistance and Isolates from Supplemented Agar and Enrichment Culture

Among the Mallard normal isolates, 5.3% (7 isolates) showed resistance to at least one of the antibiotics tested, with 5 of these collected on the same sampling occasion. An additional 6 resistant isolates were recovered from enrichment cultures (IMF) and selected plates.

28% of RW isolates (13) and 9% of TW isolates (4) showed resistance to at least one of the antibiotics tested. However, this difference in the prevalence of resistant enterococci in RW and TW may be biased due to the fact that samples were taken using different sampling methods (grab and continuous sampling, respectively) during a 24 hour period, and that all RW samples and two TW samples were collected after the experimental period had finished.

Ampicillin and Ciprofloxacin resistance were the most ubiquitous of all resistances in this study, among enterococci isolates showing any resistance. More wastewater than Mallard isolates with multiple resistances were recovered

Two Mallard and four wastewater isolates were confirmed as Vancomycin resistant when tested via the MIC and disc diffusion methods. Two of the wastewater isolates belonged to the same phenotypical group (E.fcm1), and the rest to other groups and species. The isolates had an inhibition zone of $\leq$ 11 mm, corresponding to an MIC value of 4 mg L^{-1}.

Few of the isolates grown on MEA-plates with antibiotic supplement exhibited resistance. This was particularly obvious for Mallard isolates as only one out of the 50 isolates obtained from supplemented plates was confirmed resistant by the MIC and zone breakpoint test. Of the 22 wastewater isolates, 17 were confirmed resistant.

The antibiotic supplement on each MEA plate had no relationship with species occurrence, since the same species-pattern was observed as for normal isolates. However, a different pattern was observed after enrichment in broth, i.e. in the individual faecal samples, where *E. durans* disappeared and strains with no corresponding profile in the reference database dominated together with *E. faecium* and *E. faecalis*.

IV. DISCUSSION

Hässleholm wastewater wetland is an important site for waterfowl [44], with up to 34 species breeding in the area every spring. The average number of Mallards typically fluctuates between 100 individuals in spring and summer to 200 individuals in autumn and winter, although 896 individuals were recorded in January 2005 [44].

The present study exposed waterfowl to urban wastewater during a prolonged time period, specifically to human faecal bacteria in an artificial wastewater wetland. Levels of enterococci in the treated wastewater are within the realistic range both for annual data published by the Municipality of Hässleholm [45] and results obtained in other studies [22, 46]. We found no evidence for the transmission of enterococci from wastewater to adult Mallards during the exposure period. However, the Mallards had already been colonized by enterococci strains biochemically compatible with wastewater enterococci, indicating the presence of strain overlap among Mallards and humans (Table 2). The latter is of great importance as there is growing evidence to suggest that vectors such as birds may carry pathogenic faecal bacteria identical to human strains [11, 47]. As a consequence, birds may act as reservoirs and in the worst case wetlands may serve as breeding grounds not only for birds but also as amplification sites for zoonotic microorganisms.

The pattern of *Enterococcus* spp found in wastewater in the present paper corresponds to that obtained by other studies, with *E. faecalis* and *E. faecium* the most common [8, 24, 48, 49]. The amount and frequency of *E. faecium*, *E. faecalis*, *E. hirae* and *E. durans* in other wild birds has been reported to vary on both a geographical and a temporal scale [7, 50, 51]. However, based on our results it seems that exposure to wastewater enterococci for 3 months does not induce any major change in adult Mallard enterococcal biota. We thus speculate that other factors may have overridden the effect of Mallard exposure to enterococci. For instance, healthy adult Mallards with an intact and developed biota may have partial protection against infection from new enterococci, while young ducklings with a less developed immune defence and an undeveloped intestinal biota may be more prone to infections from other sources than Mallards. It must also be considered that the frequency of any infection found in this study may have been below the level detectable by the methods used.

Our study has shown that Mallards not yet exposed to wastewater carried enterococci whose biochemical profile corresponded to isolates found in wastewater. The Mallard has adapted to an extremely wide range of habitats [52, 53], including very small wetlands, especially during the breeding season. Mallards are also commonly seen in areas close to human activity. Given that treatment plants are permanent suppliers of enterococci to the environment [1, 2], it seems likely that human enterococci may have reached susceptible waterfowl individuals during decades of exposure. It has been speculated that the extent of microorganism transmission between species is directly proportional to the duration of exposure [54], while other human faecal bacteria strains have previously been found in birds living close to human waste [55-59]. As can be seen in Figures 1, 2 and 3, many of the isolates are typical for either birds or sewage, i.e. the biochemical groups consist solely of Mallard or sewage isolates. However, there are a few biochemical groups in which both Mallard and sewage isolates are represented. Studies have shown that enterococci found in different hosts and places may share genetic clonal complexes [11, 60]. Vancanneyt [60] demonstrated that *E. faecium* can be divided into two major groups, one including human isolates and the other both human and clinical strains isolated from birds, a pattern which very much resembles the distribution of isolates displayed in Figure 2.

We do not, per se, consider transmission and adaption of enterococci to wildlife as a problem, either for the Mallards or humans, but rather from the aspect that liberal use of antibiotics in medicine and animal husbandry has promoted the rapid development of resistant bacteria [61]. Enterococci are of particular concern since not only are they ubiquitous in human and animal digestive systems, they also easily acquire resistance which they readily transmit to both other enterococci and other genera of bacteria. Resistant human enterococci may infect birds, or pathogenic genetic elements may be transmitted to the enterococcal biota of waterfowl. Male dabbling ducks show a tendency for abmigration - the switching of breeding grounds between years - which is in part due to mate choice as the females are philopatric [62]. This behaviour could increase the risk of microorganism transfer between different Mallard subpopulations or places [63].

The amount of resistant faecal bacteria in wastewater mirrors the frequency of human bacteria or those from other sources connected to wastewater treatment plants [8]. Previous studies have found that numbers of antibiotic-resistant bacteria may

decrease [64, 65], remain unchanged, or even increase depending on the substance, during the treatment process [49]. Ferreira da Silva et al. [49] have proposed that the increase observed within treatment plants could be due to either horizontal transfer of genes, or that a larger proportion of resistant enterococci survive, with some strains more fit for survival in treatment plants than others [42]. However, the factors responsible for the selection of horizontal gene transfer in natural microbial habitats remain unclear [66]. Nevertheless, a selective environment with high levels of antimicrobial agents in effluent wastewater has been shown to increase the abundance of resistant bacteria in receiving environments [67]. Our study indicated that the prevalence of resistant enterococci was lower in treated wastewater, to which the ducks were exposed, compared to raw wastewater. This indicates that the Hässleholm treatment plant did not mediate the survival of resistant enterococci and/or the transfer of antibiotic genes during the time of sampling, at least not to a detectable level. Six isolates with resistance to Vancomycin (VRE) were found, two in Mallards and four in sewage. Indeed, previous studies have shown that Vancomycin-resistant enterococci are commonly found in Swedish sewage [68]. Despite the early ban of antibiotics within animal husbandry in Sweden (1986), VRE still can be found among broilers due to the spread of VRE-clones between different breedings [69]. The spread of VRE is particularly undesirable because they cause serious infections, mainly in hospitalised immunocompromised patients [70]. Significantly, the release of human pathogenic enterococci into the environment may in the long run counteract any containment measure undertaken at other levels in the community. It remains unknown as to when and to what extent certain strains or genetic elements are transmitted from wastewater to waterfowl, a question that is difficult to answer without more extensive analysis, including genotyping.

V. CONCLUSION

Our study examined the relationship between Mallard enterococcal biota and enterococci isolated from wastewater. We found no evidence for transmission of enterococci from wastewater to adult Mallards during the exposure period. However, Mallard isolates were biochemically consistent with wastewater enterococci, indicating the presence of overlapping strains among Mallard as well as human enterococci. We believe our study to be the first examining waterfowl exposed to wastewater during a prolonged period and under controlled conditions. Even though this study is limited to phenotypical analyses, we argue that its findings indicate the possible infection of waterfowl and humans by the same strain of enterococci. The findings also highlight the necessity of carrying out more large-scale studies in which the human impact on wildlife can be shown in situ. This will lead to a better understanding not only of the effect of wastewater contaminants and diseases on waterfowl, but also of the role of waterfowl as conservation and/or propagation hubs of zoonotic disease. Wastewater wetlands are and will remain an important and cost-effective tool with which to prevent overload of nutrients in recipients, but are also important areas where waterfowl can breed and survive successfully.

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