GLOBAL WATER PATHOGEN PROJECT
PART FIVE. CASE STUDIES

E. COLI AND ENTEROCOCCI SUBTYPING TO DISCRIMINATE CONTAMINATION SOURCES IN WASTEWATER TREATMENT PONDS

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Summary

Highlights

- The origin of faecal pollution in reclaimed-water open-air ponds was investigated
- *E. coli* and enterococci isolated in the ponds and reclamation plant were phenotyped
- Reclaimed water was not the source of the faecal pollution in the ponds
- Contamination originated in the golf facility, most likely by waterfowl
- Measures to reduce in situ contamination of reclaimed water should be implemented

Management Objective

The objective of this case study was to determine whether the faecal contamination detected in two reclaimed open-air ponds used as a water reservoir for the irrigation of a golf course was related to a regrowth of the reclaimed water bacterial populations or to an input of faecal material related to the golf facility.

Location and Setting

The golf facility was located in Catalonia (North-Eastern Spain), a region with an important affluence of national and international tourism, particularly in the summer. The summer season in this Mediterranean coastal region is generally dry and golf courses require irrigation to maintain the lawn surface quality. However, due to water scarcity in the summer period, the government encourages the use of reclaimed water for uses that do not require a drinking water quality. Thus, the construction of open-air ponds to temporally store reclaimed-water for later use is a common management practice in golf courses.

Figure 1. Location of the golf facility in North-Eastern Spain. The picture shows one of the open-air ponds in the golf course. (photo by A.R. Blanch)

Description of the System

The system studied consisted of two artificial outdoor storage ponds connected in series (pond A and pond B) used to irrigate a golf course. The volume of reclaimed water used for irrigation varied depending on the climatic conditions and the needs of the golf course in the different seasons. In the summer, irrigation was quite intensive, which resulted in a pond water residence time of around 2 weeks. In contrast, the pond water was barely used in the winter. These two ponds were filled with reclaimed water pumped from a local water reclamation plant. The reclamation process involved sand filtration followed by disinfection with a combination of chlorination and ultraviolet light.

Outcome and Recommendations

This study showed that the source of the faecal pollution in the open-air ponds was not related to the reclaimed water, but to an input of faecal material occurring in the golf facility, most likely migrating birds. Physical barriers to reduce the faecal input from birds or additional in situ treatments may be necessary to reduce the contamination levels. Overall, this case-study shows that reclaimed water may be recontaminated after treatment in open-air reservoirs, and thus, the microbial quality should be monitored throughout its use.

Case study description

Introduction

In a global context of water scarcity the use of reclaimed water is expected to increase all around the world, and specifically in heavily populated coastal areas (Angelakis and Durham, 2008). The use of reclaimed water may be a sound alternative for purposes that do not require a drinking water quality such as for crop irrigation, industrial activities, aquifer management, cleaning of streets, and ecological purposes among others. The irrigation of golf courses is one of the sectors where the use of reclaimed water is expected to increase due to government regulations aimed to reduce the use of primary water resources in drought periods. Reclaimed water is generally affordable, abundant and contains nutrients that may be beneficial for the maintenance of the golf course lawn (Mujeriego, 2007).

Water reclamation technologies ensure high quality water effluents that fulfil the microbial regulations for golf course irrigation. However, several studies have reported the occurrence of microbial pathogens such as Cryptosporidium, Giardia, enteroviruses, enteropathogenic Escherichia coli, Legionella, Mycobacterium, Aeromonas, and Pseudomonas and the potential regrowth of some of these in reclaimed water distribution and storage systems (Costán-Longares et al., 2008; Gennaccaro et al., 2003; Jjemba et al., 2010; Ryu et al., 2005). In addition, the recontamination of stored reclaimed water by faecal pathogens from wildlife or domestic animals (Nemec and Massengale, 2010; Vogel et al., 2007), urban runoff (Sauer et al., 2011), or septic system failure (Ahmed et al. 2005a) cannot be ruled out, particularly when water is open-air stored. This case study explores the origin of the low levels of faecal pollution detected in two reclaimed water open-air ponds used as a reservoir for the irrigation of a golf course in North-eastern Spain.

Problem formulation

The purpose of this study was to determine whether the low levels of faecal contamination detected in two reclaimed water ponds used as a reservoir for the irrigation of a golf facility located in Catalonia (North-Eastern Spain) was related to a regrowth of the microbial populations introduced with the reclaimed water or to an input of faecal material occurring in the golf facility during storage.

Description of the System

The system consisted of two artificial outdoor ponds connected in series (pond A and pond B) which were used as reservoirs for the irrigation of a golf course. The capacity of pond A was 21,000 m3 and that of pond B was 13,000 m3. The volume of reclaimed water used for irrigation varied depending on the climatic conditions and the needs of the golf course in the different seasons. In summer, irrigation was quite intensive, which resulted in a pond water residence time of around 2 weeks. In contrast, the pond water was barely used in winter. These two ponds were yearlong filled with reclaimed water pumped from a nearby water reclamation plant. The reclamation process involved sand filtration followed by disinfection with a combination of chlorination and ultraviolet light. These treatments ensured that the concentration of E. coli remained below 200 CFU/100 mL, which is the limit established for water reclamation in Spain (Anonymous, 2007). The historical series obtained from the laboratory of the reclamation plant confirmed that at the end of the treatment the concentrations of E. coli were 20 CFU/100 mL as a 90th percentile of the annual dataset.

Experimental approach

To determine whether the microbial faecal populations in the ponds were similar to those from the treated wastewater, we collected six water samples at three points in the system: the inflow of the reclamation plant, pond A, and pond B. Three of the samples were taken in the summer and another three in the winter. We enumerated faecal coliforms and faecal enterococci by filtering and incubating in selective culture media (mFC agar for faecal coliforms and m-Enterococcus agar followed by confirmation in Bile Esculin agar for faecal enterococci). Then, we randomly selected 25 well-isolated colonies for each sample and bacterial indicator and performed a biochemical phenotyping using the PhP-RE and PhP-RF plates of the Phene-Plate System™ (Bactus AB. Based on the biochemical profiles obtained for each sample, we calculated Simpson's diversity index (Di) and the similarity index (Sp), a similarity coefficient that measures the proportion of isolates that are identical in two compared samples (Kühn, 1985; Kühn et al.,1991; Hunter and Gaston, 1988). Then, the similarity coefficients were clustered using

UPGMA and the populations of both faecal indicators at the three sampling points were compared to a database of populations associated with slaughterhouse wastewater (faecal contamination of animal origin) or human sewage available from previous studies (Blanch et al., 2003; Kühn et al., 2005; Manero et al., 2006; Vilanova et al., 2004). In total, the database consisted of 3,556 faecal coliform isolates and 1,127 enterococci.

In addition, we studied the inactivation of faecal coliforms (FC), total bifidobacteria (TBIF), sorbitol-fermenting bifidobacteria (SBIF), somatic bacteriophages (SOM), and bacteriophages infecting Bacteroides thetaiotaomicron (BACT) in ponds to determine whether they could regrow during storage in the ponds. A water sample from pond B was spiked with a 1:50 dilution of sewage water from the treatment plant. This matrix was used to fill dialysis tubes with a porosity cutoff of 14 kDa (Medicell Dialysis Tubing Visking, London, UK). The dialysis tubes with 50 mL of the water dilution were placed at a depth of 20–25 cm from the surface of pond B. Dialysis tubes were retrieved from the system at different incubation times for a period of 2 weeks in the winter and 1 week in the summer. Independent assays were performed three times during the summer and four times during the winter to enumerate all the microbial indicators. The enumeration results were used to calculate the inactivation kinetics of the culturable populations. The following equations were used to calculate the decay rates (Ks) and the time required for 90 % of the initial population to decay (T90 values (in hour)):

$$\log10(Nt/N0)=-Ks\times t \quad T90=-1/Ks$$

where Nt is the cell concentration per milliliter at time t, and N0 is the initial cell concentration per milliliter (at time t0).

Concentration of faecal indicators in the open-air ponds

The concentrations of faecal coliforms in the reclaimed water did not significantly vary between winter and summer, and were always low (1.25 log10 CFU/ 100 mL) fulfilling the standards established by Spanish regulations for golf course irrigation (Anonymous, 2007). In the winter, the ponds also presented very low numbers of faecal coliforms and enterococci (<0.10 log10 CFU/100 mL), even lower than those in the reclaimed water. In contrast, in summer, the numbers were higher than in winter or in the reclaimed water with average concentrations around 2.75 log10 CFU/100 mL (Table 2). This indicated that the microbial quality of the water deteriorated during storage in the ponds in the summer season.

Determination of faecal origin by biochemical fingerprinting

A total of 308 enterococci (225 in summer and 83 in winter) and 315 faecal coliforms (225 in summer and 90 in winter) were isolated and biochemically phenotyped. We observed high diversity values for both populations in the secondary-treated sewage in summer and in winter (Di > 0.94) in line with previous studies (Blanch et al., 2003; Vilanova et al., 2004). The diversity index was more moderate in the ponds in the summer and was especially low for the enterococci in pond A. In general, low FIB diversities have been related to inputs of faecal pollution associated with few individuals (Kühn et al., 1997). In winter, the diversity indices in the ponds could not be compared to those in summer because of the low number of enterococci and faecal coliforms isolated.

The analysis of the similarity coefficients in summer indicated that faecal coliforms and faecal enterococci populations in pond A and pond B were highly similar (Sp > 0.2), which indicated that they shared the same biochemical profile structure. This result strongly pointed out to a shared source of faecal pollution for both ponds. On the other hand, the populations of faecal coliforms and enterococci in the secondary-treated sewage were different from those isolated in ponds A and B (Sp < 0.2). In contrast, we found no similarity between the faecal coliforms or enterococci populations of the secondary-treated sewage, pond A and pond B in the winter. However, the small number of isolates obtained in this season, reduced the strength of these coefficients.

Finally, the faecal coliform/enterococci ratio (FC/E) was low in the summer, which suggested that the source of the faecal pollution was animal related as it has been previously observed that low FC/E ratios are often related to faecal contamination of animal origin (Feachem, 1975; Geldreich and Kenner, 1969). Although the validity of this ratio has been questioned (Jagals and Grabow, 1996; Sinton et al., 1998), it may serve as a preliminary indicator. The reclamation plant processed exclusively human sewage and presented higher FC/E ratios making it an unlikely source of the pollution in the ponds.

Inactivation of microbial faecal indicators in situ

None of the faecal microbial indicators studied was able to regrow or even persist in the ponds for an extended time. On the contrary, they all presented clear inactivation kinetics, which were significantly different between the microorganisms ($p < 0.0086$ in summer and $p < 0.0032$ in winter). The inactivation rate of the faecal microbial indicators followed the sequence: SBIF > TBIF > FC > BACT > SOM both in summer and in winter, with the lowest and highest T90 for bifidobacteria and somatic coliphages, respectively. These results are consistent with results reported previously (Chung and Sobsey, 1993; Durán et al., 2002; Moce-Llivina et al., 2005; Sinton et al., 1999), in which SOM and BACT were more persistent than faecal coliforms and other indicators in sewage, seawater, and freshwater. Additionally, the low persistence of bifidobacteria confirmed the results previously obtained for river water (Bonjoch et al., 2009). Overall, these results provide evidence that the faecal microbial indicators that entered the ponds through the reclaimed water could not regrow in the natural environment, and consequently, the reclaimed water could not be the source of the increased levels of FIB in the ponds.

Conclusion

Overall, our data suggest that there was a source of faecal matter in the ponds other than the reclaimed water. Indeed, large numbers of birds, particularly Anas platyrhynchos and Anser anser, were observed in and around the ponds in summer. In winter, these birds were hardly ever seen. This suggests that the high pollution levels detected in summer might be related to their droppings. Therefore, although the reclaimed water complied with sanitary regulations, the microbial quality of the water deteriorated during the residence time in the ponds, probably due to wildlife activity. From a general point of view, these results point out an important limitation in the current reclaimed water regulation, which does not foresee the potential recontamination of properly disinfected waters and its consequences (Anonymous, 2007). As a result, the legal interpretation of recontamination is complex, and therefore, its health implications should be studied to gain a better understanding of the associated risks. Nevertheless, our results suggest that a disinfection treatment achieving concentrations of E. coli around 1 log unit at the 90th percentile ensures that ponds will not exceed the regulatory limit of 200 CFU/100 mL considering the in situ input of faecal matter from diffuse pollution, birds or other wildlife.

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Casanovas-Massana, A., and Blanch, A.R. (2013) Determination of faecal contamination origin in reclaimed water open-air ponds using biochemical fingerprinting of enterococci and faecal coliforms. Environmental Science and Pollution Research. 20:3003-3010. doi:10.1007/s11356-012-1197-1.

Summary of quantitative data inputs

The biological hazards presented by faecal contamination in stored reclaimed water were assessed using the enumeration of faecal coliforms and enterococci in several sampling points along the year. Inactivation of faecal microbial indicators in the ponds was analysed on-site. Biochemical fingerprinting of both bacterial indicator populations was performed to determine the faecal source (human or non-human) based on reference biochemical profiles associated to faecal sources. The use of this approach was chosen because the concentration of E.coli when detected in storage ponds was below the detection limit of most of Microbial Source Tracking molecular techniques (<0.10 log10 CFU/100 mL in winter, and 2.7 log10 CFU/100 mL in summer). A total of 308 enterococci and 315 faecal coliforms were isolated and biochemically phenotyped using the PhP-RF and PhP-RE plates.

References

Ahmed, W., Neller, R. and Katouli, M. (2005). Evidence of septic system failure determined by a bacterial biochemical fingerprinting method. Journal of Applied Microbiology. 98, pp. 910–920. doi: 10.1111/j.1365-2672.2004.02522.x.

Angelakis, A.N. and Durham, B. (2008). Water recycling and reuse in EUREAU countries: Trends and challenges. Desalination. 218, Elsevier. pp. 3–12. doi: 10.1016/J.DESAL.2006.07.015.

Blanch, A.R., Caplin, J.L., Iversen, A., Kuhn, I., Manero, A., Taylor, H.D. *et al*. (2003). Comparison of enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions. Journal of Applied Microbiology. 94, pp. 994–1002.

Bonjoch, X., Lucena, F. and Blanch, A.R. (2009). The persistence of bifidobacteria populations in a river measured by molecular and culture techniques. Journal of Applied Microbiology. 107, pp. 1178–1185. doi: 10.1111/j.1365-2672.2009.04297.x.

Chung, H. and Sobsey, M.D. (1993). Comparative Survival of Indicator Viruses and Enteric Viruses in Seawater and Sediment. Water Science and Technology. 27, IWA Publishing. pp. 425–428. doi: 10.2166/wst.1993.0385.

Costán-Longares, A., Montemayor, M., Payán, A., Méndez, J., Jofre, J., Mujeriego, R. *et al*. (2008). Microbial indicators and pathogens: Removal, relationships and predictive capabilities in water reclamation facilities. Water Research. 42, Pergamon. pp. 4439–4448. doi: 10.1016/J.WATRES.2008.07.037.

Durán, A.E., Muniesa, M., Méndez, X., Valero, F., Lucena, F. and Jofre, J. (2002). Removal and inactivation of indicator bacteriophages in fresh waters. Journal of applied microbiology. 92, pp. 338–47.

Feachem, R. (1975). An improved role for faecal coliform to faecal streptococci ratios in the differentiation between human and non-human pollution sources. Water Research. 9, Pergamon. pp. 689–690. doi: 10.1016/0043-1354(75)90178-5.

Geldreich, E.E. and Kenner, B.A. (1969). Concepts of fecal streptococci in stream pollution. Journal - Water Pollution Control Federation. 41, pp. Suppl:R336+.

Gennaccaro, A.L., McLaughlin, M.R., Quintero-Betancourt, W., Huffman, D.E. and Rose, J.B. (2003). Infectious *Cryptosporidium parvum* oocysts in final reclaimed effluent. Applied and Environmental Microbiology. 2003/08/07 ed.69, pp. 4983-4.

Hunter, P.R. and Gaston, M.A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. Journal of clinical microbiology. 26, American Society for Microbiology (ASM). pp. 2465–6.

Jagals, P. and Grabow, W.O.K. (1996). An evaluation of sorbitol-fermenting bifidobacteria as specific indicators of human faecal pollution of environmental water. Water SA. 22, pp. 235–238.

Jjemba, P.K., Weinrich, L.A., Cheng, W., Giraldo, E. and LeChevallier, M.W. (2010). Regrowth of potential opportunistic pathogens and algae in reclaimed-water distribution systems. Applied and environmental microbiology. 76, pp. 4169–4178.

Kühn, I. (1985). Biochemical fingerprinting of *Escherichia coli*: a simple method for epidemiological investigations. Journal of Microbiological Methods. 3, Elsevier. pp. 159–170. doi: 10.1016/0167-7012(85)90043-0.

Kuhn, I., Allestam, G., Engdahl, M. and Stenström, T.A. (1997). Biochemical fingerprinting of coliform bacterial populations — comparisons between polluted river water and factory effluents. Water Science and Technology. 35, No longer published by Elsevier. pp. 343–350. doi: 10.1016/S0273-1223(97)00283-7.

Kuhn, I., Iversen, A., Finn, M., Greko, C., Burman, L.G., Blanch, A.R. *et al*. (2005). Occurrence and Relatedness of Vancomycin-Resistant *Enterococci* in Animals, Humans, and the Environment in Different European Regions. Applied and Environmental Microbiology. 71, pp. 5383–5390. doi: 10.1128/AEM.71.9.5383-5390.2005.

Manero, A., Vilanova, X., Cerda-Cuellar, M. and Blanch, A.R. (2006). Vancomycin- and erythromycin-resistant enterococci in a pig farm and its environment. Environmental Microbiology. 8, pp. 667–674. doi: 10.1111/j.1462-2920.2005.00945.x.

Ministerio de la Presidencia (2007). Real Decreto 1620/2007, de 7 de diciembre, por el que se establece el régimen jurídico de la reutilización de las aguas depuradas. 281:50639–50661. pp. 281:50639–50661.

Moce-Llivina, L., Lucena, F. and Jofre, J. (2005). Enteroviruses and Bacteriophages in Bathing Waters. Applied and Environmental Microbiology. 71, pp. 6838–6844. doi: 10.1128/AEM.71.11.6838-6844.2005.

Mujeriego, R. (2007). Golf course irrigantion with reclaimed water. Water Reuse Studies in Spain. 2, pp. 1–4.

Nemec, M.D. and Massengale, R.D. (2010). The use of carbon-utilization profiling to determine sources of fecal contamination in a central Texas watershed. Lake and Reservoir Management. 26, Taylor and Francis Group. pp. 104–113. doi: 10.1080/07438141.2010.490769.

Ryu, H., Alum, A. and Abbaszadegan, M. (2005). Microbial Characterization and Population Changes in Nonpotable Reclaimed Water Distribution Systems. Environmental science &*amp*; Technology. 39, American Chemical Society. pp. 8600–5. doi: 10.1021/ES050607L.

Sauer, E.P., VandeWalle, J.L., Bootsma, M.J. and McLellan, S.L. (2011). Detection of the human specific *Bacteroides* genetic marker provides evidence of widespread sewage contamination of stormwater in the urban environment. Water Research. 45, Pergamon. pp. 4081–4091. doi: 10.1016/J.WATRES.2011.04.049.

Sinton, L.W., Finlay, R.K. and Lynch, P.A. (1999). Sunlight inactivation of fecal bacteriophages and bacteria in sewage-polluted seawater. Applied and Environmental Microbiology. 65, pp. 3605–13.

Sinton, L.W., Finlay, R.K. and Hannah, D.J. (1998). Distinguishing human from animal faecal contamination in water: A review. New Zealand Journal of Marine and Freshwater Research. 32, Taylor &*amp*; Francis Group. pp. 323–348. doi: 10.1080/00288330.1998.9516828.

Vilanova, X., Manero, A., Cerda-Cuellar, M. and Blanch, A.R. (2004). The composition and persistence of faecal coliforms and enterococcal populations in sewage treatment plants. Journal of Applied Microbiology. 96, pp. 279–88.

Vogel, J.R., Stoeckel, D.M., Lamendella, R., Zelt, R.B., Domingo, J.W.Santo, Walker, S.R. *et al*. (2007). Identifying Fecal Sources in a Selected Catchment Reach Using Multiple Source-Tracking Tools. Journal of Environment Quality. 36, pp. 718. doi: 10.2134/jeq2006.0246.