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Human norovirus in untreated sewage and effluents from primary, secondary and tertiary treatment processes

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ABSTRACT

Wastewater treatments are considered important means to control the environmental transmission of human norovirus (NoV). Information about NoV concentrations in untreated and treated effluents, their seasonality and typical removal rates achieved by different treatment processes is required to assess the effectiveness of sewage treatment processes in reducing human exposure to NoV. This paper reports on a characterisation of concentrations of NoV (genogroups I and II) in untreated sewage (screened influent) and treated effluents from five full scale wastewater treatment works (WwTW) in England. Results are shown for effluent samples characteristic of primary- (primary settlement, storm tank overflows), secondary- (activated sludge, trickling filters, humus tanks) and tertiary (UV disinfection) treatments. NoV occurrence in untreated sewage varied between years. This variation was consistent with the annual variation of the virus in the community as indicated by outbreak laboratory reports. Significant differences were found between mean NoV concentrations in effluents subject to different levels of treatment. Primary settlement achieved approximately 1 $\log_{10}$ removal for both genogroups. Concentrations of NoV and *Escherichia coli* in untreated sewage were of the same order of magnitude of those in storm tank overflows. Of the secondary treatments studied, activated sludge was the most effective in removing NoV with mean $\log_{10}$ removals of 3.11 and 2.34 for GI and GII, respectively. The results of this study provide evidence that monitoring of NoV in raw sewage or treated effluents could provide early warning of an elevated risk for NoV and potentially help prevent outbreaks through environmental exposure. They also provide evidence that elimination of stormwater discharges and improvement of the efficiency of activated sludge for NoV removal would be effective for reducing the risk of environmental transmission.

1. Introduction

Human noroviruses (NoV) have been responsible for millions of cases of gastrointestinal disease in the developed world. These viruses are excreted in high numbers in the faeces of infected individuals and are commonly detected in raw sewage (Cantalupo et al., 2011). Norovirus is extremely contagious and transmission occurs via several routes (direct person-to-person, foodborne, waterborne or through environmental fomites). Wastewater treatments are important means to reduce NoV transmission in the environment outside the host and to prevent new cycles of human infection. Therefore, it is important to evaluate the effectiveness of wastewater treatments in removing NoV from sewage. Full characterisation of microbiological concentrations in effluent discharges has been undertaken for faecal indicator organisms (FIO) (Kay et al., 2008) but not for NoV. Where concentrations and removal rates of NoV and FIO have been compared in multiple wastewater treatment works (WwTW) the studies were limited to winter periods which precluded the investigation of seasonal variability (La Rosa et al., 2010; Palfrey et al., 2011). Furthermore, few studies have quantified typical NoV concentrations in primary settlement and certain types of biological treatment such as trickling filters (Nordgren et al., 2009). In communities served by combined sewerage systems, sampling has not been undertaken during wet weather periods (Katayama et al., 2008) and data are lacking on how NoV concentrations in stormwater discharges compare with those in untreated sewage and treated effluents. Comparisons of NoV removal rates between multiple WwTW provide an opportunity to evaluate how different treatment technologies perform in relation to different levels of virus prevalence

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in infected populations.

In this study, five full scale WwTW operating different types of treatments characteristic of primary-, secondary- and tertiary-treated effluents were monitored for NoV genogroups I and II during different periods of virus prevalence in the human populations. Comparative data on *Escherichia coli* concentrations were obtained because this bacterium is the faecal indicator organism of interest in relation to regulatory monitoring of protected areas under the Water Framework Directive (bathing waters and shellfish waters) in the UK. *E. coli* can also be used to provide information on WwTW treatment process performance. The main focus of the study was to help inform NoV risk assessments and also pollution reduction plans by water companies and regulators.

2. Sampling sites and approach

Concentrations of NoV (GI and GII) and *E. coli* were monitored in five full scale municipal WwTW discharging into bathing waters and shellfish waters in England. These two genogroups are the most commonly implicated in outbreaks of NoV gastroenteritis in humans (Hall et al., 2014; Tao et al., 2015). The characteristics of the study WwTW are summarised in Table 1. In four of these treatment works (WwTW A, WwTW B, WwTW C and WwTW D), samples were taken at post-preliminary, post-primary, post-secondary (biological) (more than one stage, if applicable) and post-tertiary (where applicable) stages of the treatment processes (intensive sampling programme). In the WwTW A, samples of storm tank overflows (STO) were also taken. In the WwTW E, samples were only collected at post-preliminary and final effluent stages and no *E. coli* testing was undertaken. Some advanced forms of treatment (e.g. disinfection by chlorination, membrane bioreactors) and constructed wetlands were not considered in this study because they are not commonly used in the UK.

3. Methods

3.1. Sewage sample collection

Single grab samples were collected manually directly into 250 ml sterile polystyrene containers (Sterilin™) using a telescopic sampling pole. The sample containers were wiped with alcohol impregnated towel, placed in labelled plastic bags and stored in the dark inside coolboxes containing freezer packs and transported to Cefas Weymouth Laboratory for microbiological testing. The time lapse between sample collection and beginning of microbiological testing did not exceed 24 h. The same sampling protocol was utilised in all WwTW.

3.2. Microbiological testing

3.2.1. Detection and quantification of E. coli

Concentrations of *E. coli* were quantified in sewage samples following standard UK methods based on membrane filtration (Standing Committee of Analysts, 2009). Coliform bacteria were isolated by incubation on membrane lauryl sulphate broth for 4 h at 30 °C followed by 14 h at 44 °C (±0.5 °C). *E. coli* were isolated by sub-culture of up to 10 colonies from each membrane on nutrient agar at 37 °C for 24 h. The pure cultures were tested for oxidase then inoculated onto MacConkey agar and incubated at 44 °C for 24 h to confirm lactose fermentation. Cultures were also inoculated onto tryptone nutrient agar and incubated at 44 °C for 24 h. Indole formation was demonstrated by adding two or three drops of Kovacs' reagent to each plate and the development of a pink-red colour in the agar. Colonies that were oxidase negative and positive for lactose and indole were recorded as confirmed *E. coli*. The proportion of *E. coli* from each membrane was then used to calculate the *E. coli* count on the corresponding coliform plate. The results were expressed by the laboratory as cfu/100 ml.

3.2.2. Detection and quantification of NoV

The preparation of wastewater concentrates followed the procedure developed by Cross (2004) as modified from Puig et al. (1994). The following provides a summary of the methods used. Each sewage sample was shaken by hand to mix. Separate 20 ml volumes were added to each of two polycarbonate centrifuge bottles and 10 μl of Mengo virus strain vMC0 were added to each bottle as a process control (Costafreda et al., 2006). The samples were ultracentrifuged at >150,000*g* and 4 °C for 1 h using a Beckman LE-80K ultracentrifuge. The supernatants were discarded and the two pellets for each sample were combined by stepwise resuspension in a single 2 ml volume of glycine buffer (0.25 M, pH = 9.5). The bottle containing the resuspended pellet was incubated on ice for 20 min to enable the viruses to elute and 2 ml of cold 2 x Phosphate Buffered Saline (PBS) were added. The sample was centrifuged at 12,000*g* and 4 °C for 20 min to pellet particulate matter. The supernatant was transferred to a clean bottle and the pellet discarded. 18 ml 1 x PBS were added to the tube which was ultracentrifuged at >150,000*g* and 4 °C for 1 h to pellet viruses. Finally, the supernatant was discarded then the pellet resuspended in 1 ml 1 x PBS. This was transferred to a clean tube and retained at 4 °C for RNA purification and RT-PCR.

Viral RNA extraction was carried out using NucliSENS magnetic extraction technology (bioMérieux) as described in Lowther et al. (2012) and broadly compliant with the international standard method for determination of viruses in foods ISO/TS 15216-1 (ISO,

Table 1
Characteristics of sewage treatment works monitored during the study.

	WwTW A	WwTW B	WwTW C	WwTW D	WwTW E
Dry weather flow of discharge (m^3/day)	14,458	1221	6565	8414	29,284
Population equivalent	34,832[a]	3,847[b]	20,381	22,140[c]	115,299
Preliminary treatment	Screens and grit removal	Screens and grit removal	Screens and grit removal	Screens and grit removal	Screens and grit removal
Primary treatment	Primary settlement	Primary settlement	Primary settlement	Primary settlement	Primary settlement
Secondary (biological) treatment	Optimised activated sludge (modified Ludzack-Ettinger)	Trickling filters; humus tanks	Trickling filters; humus tanks	Trickling filters (biotower); biological-aerated filters; humus tanks	Activated sludge
Tertiary treatment	UV disinfection	UV disinfection	None	None	None
Sampling period	October 2012–January 2015	October 2012–January 2015	October 2012–January 2015	February 2014–July 2015	June 2009–May 2011

[a] Estimated for 2013.
[b] As per discharge consent.
[c] Estimated for 2011.