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Assessment of airborne virus contamination in wastewater treatment plants

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ABSTRACT

Introduction: Occupational exposure to bioaerosols in wastewater treatment plants (WWTP) and its consequence on workers' health are well documented. Most studies were devoted to enumerating and identifying cultivable bacteria and fungi, as well as measuring concentrations of airborne endotoxins, as these are the main health-related factors found in WWTP. Surprisingly, very few studies have investigated the presence and concentrations of airborne virus in WWTP. However, many enteric viruses are present in wastewater and, due to their small size, they should become aerosolized. Two in particular, the norovirus and the adenovirus, are extremely widespread and are the major causes of infectious gastrointestinal diseases reported around the world. The third one, hepatitis E virus, has an emerging status. **Goal and methods:** This study's objectives were to detect and quantify the presence and concentrations of 3 different viruses (adenovirus, norovirus and the hepatitis E virus) in air samples from 31 WWTP by using quantitative polymerase chain reaction (qPCR) during two different seasons and two consecutive years. **Results**: Adenovirus was present in 100% of summer WWTP samples and 97% of winter samples. The highest airborne concentration measured was 2.27×10^6 genome equivalent/m^3 and, on average, these were higher in summer than in winter. Norovirus was detected in only 3 of the 123 air samples, and the hepatitis E virus was not detected. **Conclusions:** Concentrations of potentially pathogenic viral particles in WWTP air is non-negligible and could partly explain the work-related gastrointestinal symptoms often reported in employees in this sector.

Keywords: adenovirus; bioaerosol; norovirus; occupational health; sewage; hepatitis E virus

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1. **Introduction**

Wastewater is a significant transmission route for several viral and bacterial pathogens which are reflected in both the clinical and subclinical infections currently present in the human population (Vaidya et al., 2002). Therefore, wastewater treatment workers are exposed to a lot of biological risks and have been shown to be at a higher risk of developing a large variety of work-related symptoms compared with the general population, including respiratory and gastrointestinal (e.g. diarrhoea) effects (Douwes et al., 2001; Rylander, 1999; Thorn et al., 2002; Thorn and Beijer, 2004). Viruses could be responsible from some of these work-related symptoms observed in WWTP workers. Numerous studies have investigated the presence of airborne cultivable bacteria in WWTP (Oppliger et al., 2005; Rinsoz et al., 2009; Heinonen-Tanski et al. , 2009; Haas et al., 2010; Kaarakainen et al., 2011; Han et al., 2013). However, very few studies have investigated the airborne concentrations of virus particles in WWTP although the health risk for workers is present (Divizia et al., 2008).

Among the most common human viral infections found in temperate regions and transmitted by the faecal-oral route, norovirus (NoV) and human adenovirus (AdV) are good candidates for being responsible for the observed symptoms reported in WWTP workers. NoV is recognized as the major source of gastroenteritis outbreaks in adults worldwide. It is presumed that infection mainly occurs by person-to-person transmission and consumption of contaminated food or water, but airborne transmission is likely to occur too (Friesema et al., 2009; Kimura et al., 2011; Kirking et al., 2010; Wikswo et al., 2011; Uhrbrand et al., 2012). AdV is made up of seven groups (A–G), with a total of 57 serotypes associated with a number of clinical syndromes such as gastroenteritis, respiratory diseases and conjunctivitis. They spread primarily via respiratory droplets, however, they can also spread along faecal routes and their presence in wastewater (Carducci et al., 2008; Jiang, 2006) and air has been demonstrated (Echavaria et al., 2000; Carducci et al., 2011; Wan et al., 2012). Another virus

89 which deserves particular attention, because of its emerging status, is the hepatitis E virus
90 (HEV) (Dalton et al., 2008). It is responsible for enterically transmitted viral hepatitis around
91 the world. In industrialized countries, the endemic genotype 3 HEV appears to be more
92 common than previously thought, even if it is rarely virulent. One main route of human
93 transmission of the genotype 3 has been suggested to be via consumption of contaminated
94 pork, but waterborne transmission cannot be excluded in these countries (Renou et al. 2008).
95 HEV has been detected in wastewater in France (Clemente-Casares et al., 2003), Italy (La
96 Rosa et al., 2010), Spain (Clemente-Casares et al., 2009; Rodriguez-Manzano et al., 2010)
97 and, very recently, the presence of HEV (genotype 3) was reported in 50% of water samples
98 from 31 WWTPs in Switzerland (Masclaux et al., 2013).

99 Treatment of wastewater generates aerosols of different sizes and all microorganisms
100 present in wastewater can consequently be aerosolized and deposited on surfaces (Han et al
101 2013). Thus, WWTP workers can be exposed to viral particles either via aerosol
102 inhalation/deglutition or via contact with contaminated surfaces, clothes or tools. Both regular
103 monitoring and accurate estimations of exposure to airborne viral particles are therefore
104 important in the assessment of occupational exposure risks and their prevention. Using
105 quantitative polymerase chain reaction (qPCR), this study aimed to investigate the presence of
106 3 different viruses (AdV groups F and E/D, NoV and HEV) in air samples from 31 WWTPs
107 located in the same area as a previous cohort study (Jeggli et al., 2004; Tschopp et al., 2009)
108 during two different seasons.

109

110 **2. Materials and methods**

111

112 2.1. Description of study sites

113 Thirty-one out of the 79 WWTP in the Canton of Zurich, Switzerland (about 1.39 million
114 inhabitants; 1,729 km^2), were selected for study in order to represent a broad array of plant

sizes (2–117 workers). These included 6 very large (> 50,000 inhabitants or inhabitant-equivalents), 12 large (10,000–50,000 inhabitants or inhabitant-equivalents) and 13 small WWTPs (2,000–10,000 inhabitants or inhabitant-equivalents). The selection was made using the following criteria. First, WWTPs where a seroconversion in workers had been positively ascertained in a recent cohort study on hepatitis E incidence (Tschopp et al., 2009) were included. Second, the WWTP servicing Zurich's international airport was included because international travel increases the probability of the occurrence of HEV. Third, WWTP where occupational hygiene measurements had been taken in a previous study (Oppliger et al., 2005; Daneshzadeh Tabrizi et al., 2010) were also included. Finally, further WWTPs were selected to represent a well-balanced sample of sizes across the whole canton. All the plants were municipal plants treating household wastewater only, and comprised a cleaning and an activated sludge step (Zurich WWTP website, 2013). The presence of the 3 viruses under investigation (NoV, AdV and HEV) in raw wastewater was known from a previous study (Masclaux et al., 2013).

2.2 Air sampling

Each WWTP was visited once in winter (mean temperature 4°C) and once in summer (mean temperature 21°C). At each visit, samples were collected at stationary points, continuously for at least 1 hour during the working day in two different workstations: (i) one sample in the enclosed area, at the water inlet, near the rake that removes big particles from incoming water (termed 'inside'), and (ii) one sample in the unenclosed area, above the bubbling aeration basin (termed 'outside'). All samples were collected 1.5 m above the floor or the basins. In each WWTP, the inside and outside samples were taken in parallel during the same day.

In total, 123 airborne virus samples were collected onto 3 μm pore size, 25 mm gelatine filters embedded in standard cassettes (SKC, Inc. Eighty Four, USA). Sampling was carried out using a pocket pump (MSA Escort Elf, Mine Safety Appliance Company, Pittsburgh, PA,

141 USA, or SKC pocket pump 210-1002, SKC Inc., PA, USA) calibrated at 4 L min^{-1}. Airflow
142 was calibrated using a pocket calibrator (DryCal DCLite, Bios International, Pompton Plains,
143 NJ, USA), both before and after field sampling.
144 After sampling, filters were immediately immersed in 1 mL of RNAlater (Ambion, Life
145 Technologies, Europe) stabilizing solution. Samples were kept at 4°C until return to the
146 laboratory, where they were stored at -20°C.
147 Given that the 3 investigated viruses are potentially pathogenic, we do not expect to find them
148 in non contaminated ambient air. That is why we did not collect control air samples in other
149 environment.
150
151 *2.3. Virus recovery and nucleic acid extraction*
152 Samples were allowed to liquefy briefly at room temperature and were then kept on ice. An
153 amount of 2×10^6 genome equivalent (GE) copies of the control virus (RYMV, rice yellow
154 mottle virus) was added to each sample. After brief swirling, and 10 min incubation, samples
155 were centrifuged at 1,500 g in a swinging rotor for 5 min (4°C). The supernatant was carefully
156 recovered in a 2 mL centrifuge tube, and centrifuged for 10 min at 16,000 g to pellet
157 precipitated materials. The pellet was treated with 560 µL of AVL (viral lysis buffer) from the
158 QIAamp Viral RNA Mini Kit (Qiagen AG, Hombrechtikon Switzerland) as per the
159 manufacturer's protocol. The gelatine filter was treated immediately with 560 µL of AVL
160 buffer. During 10 min incubation, both preparations were combined in the same tube and a
161 volume of 120 µL of ethanol was added to the sample. After the incubation period, the
162 samples were extracted as per the manufacturer's protocol. After final elution, an additive
163 ethanol precipitation step was carried out on the samples, using Glycoblue (Ambion, Life
164 Technologies, Europe) as a co-precipitant. Lastly, the nucleic acids were suspended in a final
165 volume of 60 µl of AVE buffer and stored at - 20°C until use.

166

167 *2.4. Reverse transcription*

168 RNA viruses (NoV and VHE) were reverse-transcribed using the SuperScript III First-Strand
169 Synthesis System for RT-PCR (Life Technologies, www.lifetechnologies.com) and a mixture
170 of reverse primers priming toward the particular RNA viruses to be detected (Table 1). A 20
171 µL reaction mixture was prepared as per the manufacturer's protocol, including RNasin
172 (Promega AG, Wisconsin, USA). The reaction was incubated for 60 min at 50°C, followed by
173 heat-inactivation at 70°C for 15 min. The volume was finally adjusted to 100 µL with TE
174 (Tris EDTA buffer) 0.1X.

175

176 *2.5. qPCR assay*

177 The qPCR reaction was performed on 5 µL of nucleic acid solution (either DNA or cDNA)
178 using the qPCR Core kit (No ROX, with dUTP) from Eurogentec (www.eurogentec.com).
179 Three duplex qPCR assays were developed to allow simultaneous detection of viruses: NoV-
180 GGII/RYMV and HEV/RYMV for RNA viruses, and AdV-40/AdV-E/D for DNA viruses.
181 The reaction efficiencies (Table 2) were measured on serial 10-fold dilution mixtures of 2
182 virus amplicons cloned in pGEM-T as described for the monoplex assays. Cross-reactivity
183 between the assays in duplex was evaluated by comparing the amplification of the target in
184 single-plasmid solution and in multiple plasmid solution. The primers used are detailed in
185 Table 1. RYMV was used as a control for the quality of each sample. The sample validation
186 threshold was 4×10^5 GE copies of RYMV. Samples with an amplification of spiked RYMV
187 under the threshold were reanalyzed or not considered, as described in Masclaux et al. (2013).
188 The reactions were run in triplicate on a RotorGene-3000 (Qiagen AG, Hombrechtikon
189 Switzerland) using the following profile: digestion with uracil-N-glycosylase at 50°C for 2
190 min, initial denaturation at 95°C for 10 min, 45 cycles of 15 s denaturation at 95°C, and 30 s

191 annealing at 60°C. No template controls were included in the runs. Good laboratory practices
192 were followed strictly to prevent PCR contamination (separated working areas and different
193 material for each extraction, preparation and amplification of samples). Quantitative data were
194 obtained using RotorGene software, version 6.1.93, and were subsequently analyzed with
195 custom-designed Excel spreadsheets using the standard curve equation as a reference for the
196 quantification. All signals (Cq values) above the limit of detection (LOD) were considered as
197 positive for the detection (Table 2). A standard curve was generated for each virus using ten-
198 fold dilutions of plasmid DNA containing the corresponding PCR product (see details in
199 Masclaux *et al.,* 2013). Concentrations were expressed in genome equivalent (GE) copies/µl
200 and converted to GE copies/m^3 of air on the basis of the volume of air sampled.
201
202 **3. Results**
203
204 Airborne AdV-F was detected in all the WWTP (either inside or outside or both) in summer
205 and 97% of WWTPs (30/31) in winter. In total, 84% (104/123) of samples were positive
206 (Table 3). Among these positive samples, 61.5% (64/104) were above the limit of
207 quantification (LOQ) (i.e. 2.72×10^3 GE copies/m^3) with a maximum concentration of $2.27 \times$
208 10^6 GE copies/m^3. In summer, 93% (58/62) of samples were positive compared to 75%
209 (46/61) in winter. This difference was statistically significant (Pearson Chi-square = 7.74, df
210 =1, p = 0.005). In summer, 75% (44/58) of positive samples were above the LOQ compared
211 to only 20% (20/46) in winter (Pearson Chi-square = 11.36, df =1, p = 0.001), and the mean
212 concentration of airborne AdV of positive samples (samples above the LOQ) was higher in
213 summer than in winter (mean ± SE, *median*: 95,179 ± 54,066 GE copies/m^3, *12,492 GE*
214 *copies/m^3*, N = 44; versus 31,121 ± 10,594 GE copies/m^3, *13,884 GE copies/m^3*, N = 20,
215 respectively; Mann-Whitney U test = 2,652, $p < 0.001$, N = 64) (Fig 1, and Table 4). There
216 were no differences in AdV occurrence between each WWTP's two sampling sites, with

85.4% (53/62) of positive outside samples compared to 83.6% (51/61) of positive inside samples (Pearson Chi-square = 0.08, df = 1, p = 0.7). Median concentrations were slightly lower outside than inside (Fig 1), but these differences were not significant (Mann-Whitney U test, U = 2001.5, p = 0.569). The size of the WWTPs (small, large or very large) had no significant influence on airborne concentrations of AdV (Kruskal-Wallis = 2.474, p = 0.29, N = 31). Group E/D adenoviruses were only detected in winter in 6 air samples (2 inside and 4 outside).

NoV was detected in 3 of the 123 air samples, and these results were below the LOQ (i.e. $< 6.55 \times 10^2$ GE copies/m^3). HEV was not detected in any of the 123 air samples (LOQ = 3.34×10^3 GE copies/m^3).

4. Discussion

Of the 3 different pathogenic virus species previously found in influent water at WWTP in the Canton of Zurich, only AdV was very frequently present (84% of 124 samples) in relatively high concentrations (up to 22.76×10^5 viral particles/m^3) in WWTP air samples. The HEV was not observed at all, and the NoV was detected in only 2.4% of samples. These results therefore demonstrated that the working environments of WWTP can expose workers to airborne pathogenic viruses. The AdV are known to be more resistant to the effects of UV light than other enteric viruses (Thurston-Enriquez et al., 2003). Moreover, a lot of non-enveloped viruses tend to be more stable in environments with relatively high relative humidity (Yang and Marr, 2012), as is the case in WWTP and they could therefore remain infectious in aerosol for a long time. Once in suspension in the air, AdV can be deposited onto any WWTP surface, and contamination of workers can occur not only via inhalation but also via contaminated hands, objects or surfaces. When AdV are inhaled, respiratory symptoms (fever, pharyngitis, tonsillitis, cough, and sore throat) can occur. When ingested, some

243 serotypes (group F) are known to infect the gastrointestinal tract primarily, with symptoms of
244 diarrhea and gastroenteritis.

245 Numerous studies have reported that WWTP workers have more frequent
246 gastrointestinal symptoms than the general population, though without highlighting clear
247 relationships with occupational exposure to one specific biological agent (Thorn and Kerekes,
248 2001; Khuder et al., 1998; Thorn et al., 2002; Douwes et al., 2001). Rylander (1999) reported
249 prevalence of diarrhea of nearly 45% in Swedish sewage workers (versus 3% in the control
250 population), and assumed that endotoxins are responsible of these symptoms. Exposure to
251 airborne endotoxins was also suspected to be related to the diarrheal symptoms observed in
252 workers from the WWTP investigated in this study (Jeggli et al., 2004), but the authors
253 noticed that other components could also be involved. Therefore, the presence of high
254 airborne quantities of AdV in almost all WWTP, sometimes in association with airborne NoV,
255 could explain the gastrointestinal symptoms often reported in WWTP, yet never elucidated.
256 To the best of our knowledge, this was the first time that the presence of airborne AdV was
257 investigated in WWTP, and such high airborne concentrations were previously reported only
258 in solid-waste processing facilities in Italy, where AdV was found in 25% (8/40) of air
259 samples, with concentrations ranging between 10×10^5 to 98×10^6 viral particles/m^3
260 (Carducci et al., 2013). As point of comparison, in Taiwan, a survey in a paediatric
261 emergency room showed that airborne concentrations of AdV were only between < 10 and
262 104 copies/m^3 (Wan et al., 2012). The major limit of our study is that the infectivity of these
263 viral particles is unknown, since PCR methods of quantification take into account both
264 infectious and non-infectious viral particles. However, AdV is known to be very resistant
265 (Gordon et al., 1993; Thurston-Enriquez et al., 2003; Enriquez et al., 1995), and it is likely to
266 remain infectious in the environment for a long time. In a recent study (Carducci et al., 2013)
267 in solid-waste processing facilities in Italy, 75% of the surface samples which were positive

for AdV using PCR were confirmed as infectious using cell culture. The infectious dose of AdV is estimated at 30 – 1,000 viral particles, thus, with regard to the high airborne virus concentrations found in this study, potential infection via aerosols cannot be excluded. , even if it was not found in the cross-sectional examination of these workers (Steiner et al., 2005).

Due to the specificity of the primers used, it can be surmised that airborne AdV isolated in this study belong to group F (type 40-41), i.e. one of those involved in gastrointestinal tropism. The presence of AdVs from the groups E/D, which can be more specifically involved in respiratory tropism, was also identified in 6 samples. The present study also observed that concentrations of AdV viruses were higher in summer than in winter. This is also the case for other microbial aerosols in WWTP (Grisoli et al. 2009; Oppliger et al. 2005) and certainly due to the higher temperature measured in summer time,

NoV was only detected in 3 air samples and at concentrations too low to be quantifiable. The presence of airborne NoV in WWTP was also detected at a concentration of 1,420 viral particle copies/m^3 in an air sample in Denmark (Uhrbrand et al., 2011). These two results show that the risk of airborne NoV exposure does indeed exist for wastewater workers. Even though the levels and frequencies of exposure are lower than those to AdV, because the infectious dose of NoV is at an extremely low level (18 viral particles) (Teunis et al., 2008), and because NoV is known to survive very well in adverse environments (Rodriguez-Lazaro et al., 2012), the risk of contracting an infection is non-negligible, and the risk is even greater for immunocompromised patients since more severe symptoms can occur.

HEV was not detected in air samples despite its presence in 50% of wastewater samples. This can be explained by the fact that HEV was found in very low concentrations (detectable, but not quantifiable) in wastewater compared to the waterborne concentrations of AdV and NoV, and therefore aerosolisation was less probable. This can also be explained by the fact that we have collected small volume of air which limits the probability of detecting

low airborne virus concentrations. Due to the high concentrations of AdV and NoV previously found in the effluent of the WWTP investigated (Masclaux et al., 2013), it is highly likely that influent water is the source of airborne contamination in WWTP. Investigated WWTP are geographically isolated from urban centres and no other potential sources of AdV or NoV were present in the near environment of the WWTP. However, the lack of control air samples collected in other outdoor environments is a limit of our study. Once aerosolised, certain viruses can be disseminated over long distances and several examples of airborne virus transmission exist. For instance, the foot and mouth animal virus, SRAS and the influenza virus have all been reported to travel over very long distances (Gloster et al., 2010; Booth et al., 2005; Ssematimba et al., 2012). The dissemination of viral particles from WWTP into the environment disserves to be further evaluated to assess possible public health risks.

5. Conclusion

Pathogenic viral particles responsible of gastrointestinal symptoms are frequently present in WWTP air. Occupational exposure to these airborne micro-organisms could explain part of the work-related gastrointestinal symptoms often reported in this occupational sector, especially during the first months of employment in WWTP. We can infer that workers' repeated exposure to these viruses triggers off a good immune response and consequently a decrease in symptoms after the first year of work. However, because more and more workers (included workers of WWTP) use immunosuppressive drugs (transplant patients, HIV patients, etc.), some can develop severe complications when infected by these viruses. Objective data useful for an adequate risk assessment for these workers are largely lacking, and this subject requires further investigation.

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