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# Assessment of airborne virus contamination in wastewater treatment plants

Frédéric G. Masclaux [a,1], Philipp Hotz [b], Drita Gashi [b], Dessislava Savova-Bianchi [a], Anne Oppliger [a,*]

[a] Institute for Work and Health (IST), University of Lausanne and University of Geneva, Route de la Corniche 2, 1066 Epalinges-Lausanne, Switzerland
[b] Occupational and Environmental Medicine Unit, Med. Poliklinik USZ, Rämistrasse 100, 8091 Zürich, Switzerland

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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 WWTPs 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 $2.27 \times 10^6$ 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 are non-negligible and could partly explain the work-related gastrointestinal symptoms often reported in employees in this sector.



## 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 fecal-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., 2011). 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

* Corresponding author. Fax: +41 21 314 74 20.
*E-mail addresses:* Frederic.Masclaux@unil.ch (F.G. Masclaux), Philipp.Hotz@usz.ch (P. Hotz), Anne.Oppliger@hospvd.ch (A. Oppliger).
[1] Present address: Department of Ecology and Evolution, University of Lausanne, Switzerland.

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primarily via respiratory droplets, however, they can also spread along fecal routes and their presence in wastewater (Carducci et al., 2008; Jiang, 2006) and air has been demonstrated (Echavarria et al., 2000; Carducci et al., 2011; Wan et al., 2012). Another virus which deserves particular attention, because of its emerging status, is the hepatitis E virus (HEV) (Dalton et al., 2008). It is responsible for enterically transmitted viral hepatitis around the world. In industrialized countries, the endemic genotype 3 HEV appears to be more common than previously thought, even if it is rarely virulent. One main route of human transmission of the genotype 3 has been suggested to be via consumption of contaminated pork, but waterborne transmission cannot be excluded in these countries (Renou et al., 2008). HEV has been detected in wastewater in France (Clemente-Casares et al., 2003), Italy (La Rosa et al., 2010), Spain (Clemente-Casares et al., 2009; Rodriguez-Manzano et al., 2012) and, very recently, the presence of HEV (genotype 3) was reported in 50% of water samples from 31 WWTPs in Switzerland (Masclaux et al., 2013).

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

## 2. Materials and methods

### 2.1. Description of study sites

Thirty-one out of the 79 WWTPs in the Canton of Zurich, Switzerland (about 1.39 million inhabitants; 1729 $km^2$), 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 (2000–10,000 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, WWTPs 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 h 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, USA, or SKC pocket pump 210-1002, SKC Inc., PA, USA) calibrated at 4 L $min^{-1}$. Airflow was calibrated using a pocket calibrator (DryCal DCLite, Bios International, Pompton Plains, NJ, USA), both before and after field sampling.

After sampling, filters were immediately immersed in 1 mL of RNAlater (Ambion, Life Technologies, Europe) stabilizing solution. Samples were kept at 4 °C until return to the laboratory, where they were stored at −20 °C.

Given that the 3 investigated viruses are potentially pathogenic, we do not expect to find them in non-contaminated ambient air. That is why we did not collect control air samples in other environment.

### 2.3. Virus recovery and nucleic acid extraction

Samples were allowed to liquefy briefly at room temperature and were then kept on ice. An amount of $2 \times 10^6$ genome equivalent (GE) copies of the control virus (RYMV, rice yellow mottle virus) (Kouassi et al., 2005) was added to each sample. After brief swirling, and 10 min incubation, samples were centrifuged at 1500 g in a swinging rotor for 5 min (4 °C). The supernatant was carefully recovered in a 2 mL centrifuge tube, and centrifuged for 10 min at 16,000 g to pellet precipitated materials. The pellet was treated with 560 μL of AVL (viral lysis buffer) from the QIAamp Viral RNA Mini Kit (Qiagen AG, Hombrechtikon Switzerland) as per the manufacturer's protocol. The gelatine filter was treated immediately with 560 μL of AVL buffer. During 10 min incubation, both preparations were combined in the same tube and a volume of 120 μL of ethanol was added to the sample. After the incubation period, the samples were extracted as per the manufacturer's protocol. After final elution, an additive ethanol precipitation step was carried out on the samples, using Glycoblue (Ambion, Life Technologies, Europe) as a co-precipitant. Lastly, the nucleic acids were suspended in a final volume of 60 μl of AVE buffer and stored at −20 °C until used.

### 2.4. Reverse transcription

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

### 2.5. qPCR assay

The qPCR reaction was performed on 5 μL of nucleic acid solution (either DNA or cDNA) using the qPCR Core kit (No ROX, with dUTP) from Eurogentec (www.eurogentec.com).

Three duplex qPCR assays were developed to allow simultaneous detection of viruses: NoV-GGII/RYMV and HEV/RYMV for RNA viruses, and AdV-40/AdV-E/D for DNA viruses. The reaction efficiencies (Table 2) were measured on serial 10-fold dilution mixtures of 2 virus amplicons cloned in pGEM-T as described for the monoplex assays. Cross-reactivity between the assays in duplex was evaluated by comparing the amplification of the target in single-plasmid solution and in multiple plasmid solution. The primers used are detailed in Table 1. RYMV was used as a control for the quality of each sample. The sample validation threshold was $4 \times 10^5$ GE copies of RYMV. Samples with an amplification of spiked RYMV under the threshold were reanalyzed or not considered, as described in Masclaux et al. (2013).

The reactions were run in triplicate on a RotorGene-3000 (Qiagen AG, Hombrechtikon Switzerland) using the following profile: digestion with uracil-N-glycosylase at 50 °C for 2 min, initial denaturation at 95 °C for 10 min, 45 cycles of 15 s denaturation at 95 °C, and 30 s annealing at 60 °C. No template controls were included in the runs. Good laboratory practices were followed strictly to prevent PCR contamination (separated working areas and different materials for each extraction, preparation and amplification of samples). Quantitative data were obtained using RotorGene software, version 6.1.93, and were subsequently analyzed with custom-designed Excel spreadsheets using the standard curve equation as a reference for the quantification. All signals (Cq values) above the limit of detection (LOD) were considered as positive for the detection (Table 2). A standard curve was generated for each virus using ten-fold dilutions of plasmid DNA containing the corresponding PCR product (see details in Masclaux et al. (2013)). Concentrations were expressed in genome equivalent (GE) copies/μl and converted to GE copies/$m^3$ of air on the basis of the volume of air sampled.

## 3. Results

Airborne AdV-F was detected in all the WWTPs (either inside or outside or both) in summer and 97% of WWTPs (30/31) in winter. In total, 84% (104/123) of samples were positive (Table 3).