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Settling and survival profile of enteric pathogens in the swine effluent for water reuse purpose

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ABSTRACT

The present study evaluated the pathogens persistence and settling profile in swine effluent. We determined the enteric pathogens settling characteristics, their survival and inactivation profile in swine effluent (for water reuse purpose) and in sludge (generated after aerobic treatment – during secondary settling process). The study was performed in laboratorial-scale and in full-scale (manure treatment plant). Enteric viruses and enteric bacteria were used as biomarkers. Results showed that these enteric pathogens were significantly reduced from swine effluent during secondary settling process, and enteric viruses removal was correlated with the suspended solids decantation. The design of secondary settlers can be adapted to improve pathogens removal, by diminishing the solids loading rate per area and time, ending in higher hydraulic retention times.

1. Introduction

Swine manure is mainly consisted of urine, feces and water, characterizing high content of solids, organic matter, phosphorus and nitrogen. Good management and treatment are necessary for reducing its environmental impact (Kunz et al., 2012).

When an aerobic treatment is used, such as activated sludge, bacteria take organic material to produce energy and cell biomass (sludge). After the biomass stabilization in an aerobic reactor, the solid and liquid fractions can be separated by natural or chemical settling (Tchobanoglous et al., 2003). The major advantage of the solid liquid separation is that it enables better management and utilization of both solid and liquid fractions (Worley and Das, 2000). The nutrient rich solid fraction can be used to fertilize crops, to produce compost, or to generate energy, while the less odorous liquid portion after treatment can be applied on farmlands or be reused as water reuse in the animal barns for cleaning, depending on the treated liquid manure quality (Worley and Das, 2000). "More than 10% of the world's population consumes food irrigated with reused water derived from domestic and industrial uses (WHO, 2006). For the WHO (2006), water reuse is allowed to irrigate food crops through drip irrigation, only if the water contains less than 10^4/100 mL of *Escherichia coli*. For Environmental Protection Agency (EPA/USA) the regulation for reuse water advocates the level less than 10^2/100 mL of *E. coli*."

Wastewater treatments are normally addressed for solids and organic matter reduction and it is rarely considered for removing pathogens, such as viruses, protozoa, and bacteria, that can cause important economic losses on the swine production and impact human health (Hundesa et al., 2009; Viancelli et al., 2011). As an example, the settling method is employed, and this method depends mainly on the size and density of suspended matter, and generates liquid and solid fractions. The pathogens removal during settling processes depends on the characteristics of each type of pathogen, such as size, surface charge and hydrophobic interactions (Wong and Xagoraraki, 2012).

Swine wastewater recycle depends particularly on the presence of pathogenic microorganisms that can contaminate derived food items, soil, water and re-infect humans and animals (Topp et al., 2009), representing a potential "One Health" risk, i.e. to human, animal and environmental health (Nguyen-Viet et al., 2015). DNA viruses, such as Porcine Circovirus-2 (PCV2) and Porcine Aden-

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ovirus (PAdV), are generally very resistant in the environment and to regular disinfection procedures (chlorination, UV, temperature storage), and so they can be used as biomarkers for sanitization purposes (Viancelli et al., 2013). Rotavirus-A (RV-A), a RNA virus, is the major pathogen associated with acute zoonotic gastroenteritis, being considered, in environmental samples, one of the most resistant RNA enteric virus (Estes and Kapikian, 2007). Bacteriophages, as PhiX-174, f-specific RNA phages, MS2 and phages that infect *Bacteroides fragilis,* are considered surrogates for enteric viruses in spiking tests (Langlet et al., 2008; Boudaud et al., 2012). Bacteria are widely used as biomarkers of fecal contamination, especially *Salmonella* spp., which is one of the most prevalent bacterial zoonotic pathogen. It is remarkable that one of main features of the epidemiology of Salmonella is its presence in swine and avian herds as main reservoirs of human infections and the high survival rates in the environment (Griffith et al., 2006).

In this context the present study aimed to evaluate the enteric pathogens survival and settling profile from swine wastewater in a full-scale and in a laboratorial-scale settling system.

2. Materials and methods

2.1. Swine manure treatment

Laboratory-scale (LS) and Full-scale (FS) studies were performed using samples collected from a swine manure treatment system (SMTS) located at Embrapa Swine and Poultry, Concórdia, Santa Catarina State, Brazil (27°18′ S, 51°59′ W). SMTS receives liquid swine manure from Embrapa's swine production experimental facilities. The plant is based on physico-chemical and biological processes (Kunz et al., 2012), composed by a preliminary treatment (screening), primary treatment (coagulation/primary settling), and secondary/tertiary treatment (Up flow Anaerobic Sludge Blanket Reactor – UASB + Activated Sludge, this last one composed by an aerobic tank + secondary settling tank).

Samples for FS studies were collected after aerobic tank (FS-AT) and after settling tank (FS-ST). Samples from the aerobic tank were collected for the LS studies (LS-AT) (Fig. 1).

2.2. Physical-chemical analysis

Samples were submitted to analysis of solids (fixed, volatile, and suspended), chemical oxygen demand (COD), total ammonia nitrogen (TAN), pH and temperature according to APHA, 2012.

2.3. Laboratory scale experimental set-up

In order to study the pathogens survival and settling profiles, in a controlled experiment at lab scale, the swine effluent samples were collected from aerobic tank (LS-AT) and inoculated (artificial contamination) with 1% (v/v) known amounts of bacteriophage PhiX-174, HAdV-2, *Salmonella enteric* – serovar Typhimurium (ATCC 14028) as models for pathogens. The final concentrations of the respective microorganisms in the effluent used in the experiment were 5×10^5 PFU mL^{-1} for PhiX-174, 6×10^6 PFU mL^{-1} for HAdV, and 5×10^4 UFC mL^{-1} for Salmonella.

Imhoff cones (v. 1 L – in triplicate) were filled with the AT samples and the settling experiment was performed. Samples from the top 5 cm of the cones were collected at ten different times (n = 3 per time): 0, 0.08, 0.16, 0.33, 0.75, 2.5, 5, 10, 24, 48, 72 and 120 h, totalizing 36 samples. The solid-fractions (sludge) were collected at 24, 48, 72 and 120 h of settling (n = 3 per time), totalizing 12 samples.

2.3.1. Methods for pathogens quantification in the LS experiment

For *S.* Typhimurium analysis, 1 mL of sample was diluted with 9 mL buffered NaCl-peptone solution with Tween, pH 7.0, followed by a 10-fold dilution series using the same buffer solution. Quantification was performed using xylose-lysine-desoxycholate (XLD) agar containing novabiocin and cultivated at 37 °C for up to 48 h according to described by Magri et al., 2013.

PhiX-174 was propagated in agar with the host *Escherichia coli* (ATCC13706), and the viable virus quantification from samples was measured by the double agar layer method according to ISO 10705-2:2000 (2000), using 1 mL of sample diluted with 9 mL peptone saline solution, pH 7.0, followed by a 10-fold dilution series using the same solution.

HAdV-2 was propagated in a continuous line of A549 cells (permissive cells derived from human lung carcinoma cells, European Collection of Cell Cultures) and enumerated by integrated cell culture assay – preceded by reverse transcriptase and followed by qPCR (Ko et al., 2003; Fongaro et al., 2013).

Samples, in a non-cytotoxic dilution (1:100), were inoculated in triplicate in A549 and incubated at 37 °C with rotation every 15 min. The inoculum was removed and the cell layers were overlaid with high-glucose Dulbecco's Modified Eagle's Medium (DMEM) before being incubated at 37 °C for 24 h. The supernatant was recovered and 0.2 mL was used for nucleic acids extraction, which was performed using a QIAmpMinElute® Virus Spin Kit (Qiagen®) following the manufacturer's instructions. Immediately after the extraction, enzymatic treatment, with DNase I, and reverse transcription reaction were used to generate cDNA, followed by Real-time quantitative PCR (qPCR), according to the protocol described by Hernroth et al. (2002). The reactions were performed in triplicate, as described by using the TaqMan assay in StepOne Plus® Real-Time PCR System (Applied Biosystems). HAdV-2 genome (fragment cloned in a commercial vector) was used as control to generate the standard curves and ultrapure water was used as the non-template control for each assay.

2.4. Full scale assessment

In order to meet the pathogens reduction in swine effluent after the settling process in FS, samples were collected from aerobic tank (FS-AT) and from the secondary settling tank outlet (FS-ST) (Fig. 1).

Samples were collected (2 L) weekly during two months. Briefly, a total of 64 samples were collected, n = 32 during winter and n = 32 during summer. For analyses were performed sampling pools, totalizing 8 sampling campaigns evaluated (4 during winter and 4 during summer of 2013, in February and August, respectively).

2.4.1. Methods for pathogens quantification in the FS system

For *Salmonella* spp. quantification, 25 mL of sample was added to 225 mL buffered peptone-NaCl solution and incubated at 37° C for 24 h. The solution was then added to Rappaport-Vassiliadis broth and Tetrathionate and incubated at 42° C for 24 h, followed by plating in xylose-lysine-tergitol-4 (XLT4), according to ISO 6579 (2002).

For PCV2, PAdV and RV-A analysis, 25 mL of sample was clarified and concentrated using the glycine buffer method coupled with polyethylene glycol precipitation. Viral particles were eluted from the precipitated sample using glycine buffer (pH 9.5) and concentrated by PEG 6000 precipitation. After centrifugation (8000 rpm during 90 min), the supernatant was discarded, and the pellet was suspended in 5.0 mL of 0.1 mol L^{-1} phosphate buffer (pH 7.2) (Viancelli et al., 2011). The nucleic acid extraction was performed using a QIAmpMinElute® Virus Spin Kit (Qiagen®) following the manufacturer's instructions.

PCV2, PAdV and RV-A genomes were quantified by Real-time quantitative PCR (qPCR). The reactions were performed in triplicate, as described by Hundesa et al. (2009), Opriessnig et al. (2003) and Zeng et al. (2008) for PAdV, PCV2 and RV-A, respectively, using the TaqMan assay in StepOne Plus® Real-Time PCR System (Applied