Dynamics of *Aeromonas* species isolated from wastewater treatment system

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ABSTRACT

Aeromonas are widely distributed in the aquatic environment, and are considered to be emerging organisms that can produce a series of virulence factors. The present study was carried out in a sanitary sewage stabilization pond treatment system, located in Lins, State of São Paulo, Brazil. Most probable number was applied for estimation of the genus *Aeromonas*. Colony isolation was carried out on blood agar ampicillin and confirmed by biochemical characterization. *Aeromonas* species were isolated in 72.4% of influent samples, and in 55.2 and 48.3% of effluent from anaerobic and facultative lagoons, respectively. Thirteen *Aeromonas* species were isolated, representing most of the recognized species of these organisms. Even though it was possible to observe a tendency of decrease, total elimination of these organisms from the studied system was not achieved. Understanding of the pathogenic organism's dynamics in wastewater treatment systems with a reuse potential is especially important because of the risk it represents.

Key words | *Aeromonas*, public health, sanitary surveillance, wastewater

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INTRODUCTION

Organisms from the genus *Aeromonas* are widely distributed in the aquatic environment, and its ability to produce diseases in different animal species is well established and documented in the scientific literature. The potential of the *Aeromonas* species to cause diseases in humans has been studied, but only recently a major number of clinical cases have been confirmed and attributed to these organisms (Clark & Chenoweth 2003; Vila *et al.* 2003; Ouderkirk *et al.* 2004; Pinna *et al.* 2004; Martin-Carnahan & Joseph 2005; Hiransuthikul *et al.* 2005; Daskalov 2006; Hofer *et al.* 2006). According to Ribeiro *et al.* (2010) *Aeromonas* species are the most common organisms reported in infections of burns exposed to contaminated water. However, Lamy *et al.* (2009) reported that studies available are limited to one type of infection, and epidemiological studies are scarce.

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Abiotic factors can influence the growth and survival of aeromonads in the environment, which grow within a temperature range of 22–35°C and tolerate pH ranging from 5.5 to 9.0 (Isonhood & Drake 2002; Sautour *et al.* 2003; Nayak *et al.* 2004). The temperature, pH, sunlight (UV irradiation), available nutrient and presence of suspended solids interact to determine the survival of *Aeromonas* (USEPA 2006).

Polluted aquatic environments can be considered a source of contamination for recreational waters and water used in the irrigation process (Villari *et al.* 2000; Davies *et al.* 2001; Uyttendaele *et al.* 2004; Vally *et al.* 2004; Villarruel-López *et al.* 2005; Daskalov 2006; Dwivedi *et al.* 2008). Besides the determination of indicator bacteria, it is important to understand the distribution of potentially

pathogenic bacteria in a given ecosystem that could affect natural water used for human contact or consumption (Nagvenkar & Ramaiah 2009).

The capability of conventional biological wastewater treatment processes for removing pathogenic microorganisms has been investigated. Efficient removal of pathogens from wastewaters is a difficult task; sewage discharges may increase pathogen contamination of surface waters limiting the recreational and economic use of the resources (Koivunen *et al.* 2003; Gugliandolo *et al.* 2009).

Wastewater reuse is a reality in several countries of Latin America, United States and Israel. Nevertheless, the reuse of treated wastewater needs special attention as pathogenic agents can be present in these waters. Gugliandolo *et al.* (2009) emphasizes that *E. coli* presence is not always enough to determine the quality of the water and suggest other potentially pathogenic organisms to be considered for this determination. Moreover, the United States Environmental Protection Agency observed an association of infectious diseases with the reuse of residual water and reported the presence of pathogenic bacteria, helminth eggs, protozoa and viruses in raw and treated wastewater, demonstrating that wastewater reuse can represent a public health risk, exposing the ecosystem and humans to pathogenic organisms (Piveli *et al.* 2008).

Because *Aeromonas* species are increasing in importance as primary pathogens, and their presence in aquatic environments could pose a risk to public health, the aim of this investigation was to report the dynamics of *Aeromonas* species in a sewage treatment pond in order to better understand the impact of the residuary water from this system on the environment and on public health.

METHODS

The present study was carried out in a sanitary sewage stabilization pond treatment system, operated by the Companhia de Saneamento Básico do Estado de São Paulo (SABESP), in the city of Lins, with population of 65,952 inhabitants (IBGE 2000). The sanitary sewage treatment system in Lins is composed of three serial groups of ponds: an anaerobic pond followed by a facultative one, with a total retention time of 20 days in the system (6 days in the anaerobic pond and 14 days in the facultative one). The ponds' dimensions are 4.1 m in depth and 23,227.03 m^3 of useful volume in the anaerobic tank, and 1.9 m of depth and 55,522.87 m^3 of useful volume in the facultative pond. Samples were collected at the anaerobic pond inflow (site 1), anaerobic pond outflow (site 2) and at the facultative pond outflow (site 3). The same set of ponds were sampled each campaign.

Dynamics of genus *Aeromonas* in the treatment system

Determinations of organisms belonging to the genus *Aeromonas* were carried out by the multiple tubes technique according to Matté *et al.* (1994), with modifications. In brief, serial dilutions of samples (from 10^0 to 10^{-8}) were subjected to enrichment in alkaline peptone water (APW) 1% (pH 8.6), colonies were isolated on blood agar amended with 10 mg ml^{-1} ampicillin (INLAB Diagnóstica, São Paulo, Brazil) (Kelly *et al.* 1988). Fourteen sampling campaigns (from July 2001 to March 2002) were used in the determination of the most probable number of the genus *Aeromonas*, in each collection site (site 1, site 2 and site 3).

Frequency of different *Aeromonas* species in the treatment system

Additionally to the determination of most probable number of the genus *Aeromonas*, the frequency of the different *Aeromonas* species in the system was carried out from April to November 2002. For direct isolation of *Aeromonas*, 100 ml of sample was enriched in 100 ml of two times concentrated alkaline peptone water (APW) 1% (pH 8.6), colonies were isolated on blood agar amended with 10 mg ml^{-1} ampicillin (INLAB Diagnóstica, São Paulo, Brazil). For the final frequency analysis, all the isolates from 29 sampling campaigns (87 samples) recovered from July 2001 to November 2002 were considered.

Identification of *Aeromonas* species

All *Aeromonas* isolates were submitted to biochemical tests in order to determine the affiliation of isolated organisms to the species level. Typical *Aeromonas* colonies–with brownish colour, 2–3 mm diameter, convex, hemolysis

producing or not–were screened in Kligler iron agar (Difco Laboratories, Detroit, Michigan) (35°C for 24 h) for positive reaction for cytochrome oxidase, variable reactions for indole and gas, variable reactions for lactose acidification and positive reaction for glucose fermentation. Additional tests were performed to select isolates presenting positive reaction for nitrate reduction and arginine dihydrolase production; lysine decarboxylase was expected to be positive and ornithine decarboxylase negative; growth in peptone broth without sodium chloride is required for the *Aeromonas* genus but no growth is expected with 6% NaCl (Martin-Carnahan & Joseph 2005).

The strains presenting positive results for the genus *Aeromonas* were submitted to complementary biochemical tests for the determination of the species as follows: acid production from arabinose, mannose, mannitol and sucrose; esculin hydrolysis (48 h); and acetoin production (Voges-Proskauer test) (Forney & Miller 1985). Identification to the species level was carried out by comparison of the biochemical results with Martin-Carnahan & Joseph (2005).

Determination of *Escherichia coli*

The most probable number of *E. coli* was analysed by a chromogenic method (Colilert, (Iddex Laboratories Inc. Westbrook, Maine) at 35°C, according to Standard Methods (1998). *E. coli* counts were used as a quality standard organism in the system to compare its dynamics with the genus *Aeromonas*.

Abiotic factors

The environmental variables such as air and water temperatures, pH, biochemical oxygen demand (BOD), ammoniacal nitrogen ($N\text{-}NH_4^+$), total phosphate ($P\text{-}PO_4^{3-}$) and total suspended solids (TSS) were evaluated according to Standard Methods (1998).

RESULTS

Dynamics of *Aeromonas*

To estimate the number of organisms of the genus *Aeromonas* (MPN/100 ml) in the system, 14 sampling campaigns were carried out from July 2001 to March 2002. The counts (MPN/100 ml) of *Aeromonas* are presented in Table 1. Figure 1 shows the dynamics of *Aeromonas* in the system compared with *E. coli*.

Table 1 | Most probable number of *Aeromonas* spp. at different sampling sites from stabilization ponds of the sewage wastewater treatment system, July 2001 to March 2002

Date	Anaerobic pond inflow (MPN/100 ml)	Anaerobic pond outflow (MPN/100 ml)	Facultative pond outflow (MPN/100 ml)
2 July 2001	4.0×10^7	<3	<3
7 August 2001	4.0×10^3	<3	4.0×10^5
4 September 2001	7.0×10^8	7.0×10^6	3.0×10^3
2 October 2001	1.1×10^9	7.0×10^6	<3
23 October 2001	2.1×10^8	<3	<3
6 November 2001	9.0×10^6	2.0×10^5	9.0×10^5
20 November 2001	3.0×10^7	7.0×10^5	<3
17 December 2001	3.0×10^9	7.0×10^6	<3
7 January 2002	3.0×10^9	<3	<3
21 January 2002	1.1×10^9	3.0×10^9	3.0×10^4
4 February 2002	3.0×10^7	1.1×10^6	9.0×10^5
19 February 2002	3.0×10^5	3.0×10^5	<3
6 March 2002	<3	<3	4.0×10^4
18 March 2002	3.0×10^5	<3	7.0×10^4

Aeromonas species were observed in 72.40% of the samples corresponding to the anaerobic pond inflow, with counts ranging from 0.00 to 9.48 log and mean value 8.82 log; 55.20% of the samples from the anaerobic pond outflow, with counts ranging from 0.00 to 9.48 log and mean value 8.33 log; and 48.30% from the facultative pond outflow with counts ranging from 0.00 to 5.95 log and mean value of 5.22 log.

Frequency of different *Aeromonas* species in the treatment system

From 29 sampling campaigns, 87 wastewater samples were obtained from the stabilization ponds system and were analysed for the frequency of different *Aeromonas* species; 203 isolates were confirmed as belonging to the genus *Aeromonas* and were subjected to identification to the species level.

Biochemical identification of the organisms belonging the genus *Aeromonas* demonstrated the presence of 13 different species of *Aeromonas* in the system as follows: site

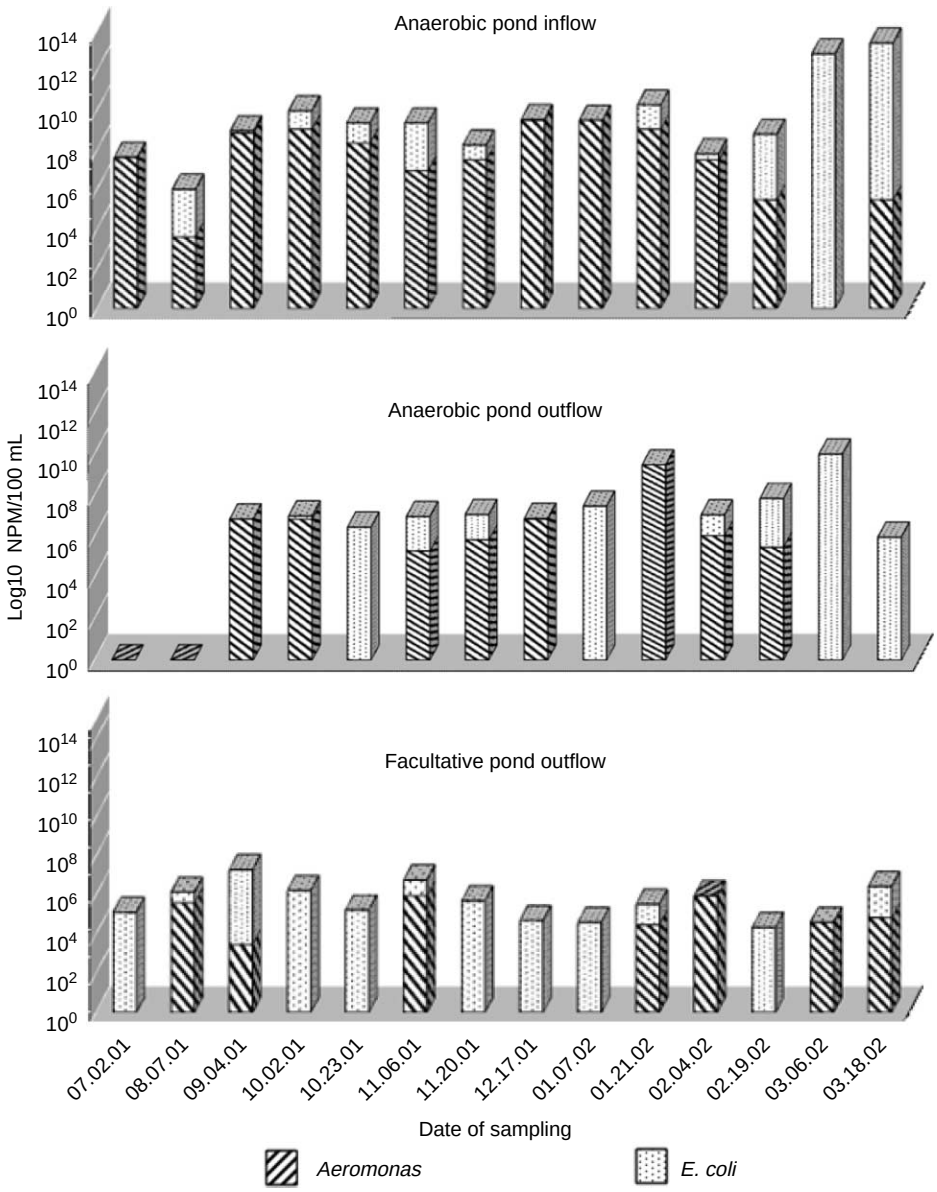

Figure 1 | Dynamic of *Aeromonas* in the stabilization pond system compared with *E. coli*.

1, 11 *Aeromonas* species present; sites 2 and 3, 8 and 9 species present, respectively.

Aeromonas species isolated in this study are presented in Table 2. Organisms that showed biochemical characteristics resembling the genus *Aeromonas*, but had atypical characteristics were classified as *Aeromonas* spp. and resulted in 18.73% (53) of the isolates. *Aeromonas* species isolated from different sampling sites are presented in Table 2.

Abiotic factors

Water temperature varied between 23 and 32°C (average 27°C) during the experiment and pH values observed for the system were between 6.0 and 8.0 (average of 7.0). The BOD values obtained during the experiment, in the facultative pond outflow, ranged between 43 and 123 $mg\,l^{-1}$ (average of $65\,mg\,l^{-1}$); N-NH_4^+ between 4.8 and $40.3\,mg\,l^{-1}$ (average of $18.7\,mg\,l^{-1}$); P-PO_4^{3-} between 4.5

Table 2 | Frequency of *Aeromonas* species isolated at different sampling sites from the stabilization ponds of the wastewater treatment system

	Anaerobic pond inflow		Anaerobic pond outflow		Facultative pond outflow		Total	
Species	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Aeromonas spp.	31	19.38	7	14.58	15	20.00	53	18.73
A. allosaccharophila	28	17.50	6	12.50	35	46.67	69	24.38
A. bestiarum	3	1.88	1	2.08	2	2.67	6	2.12
A. caviae	53	33.13	12	25.00	6	8.00	71	25.09
A. encheleia	1	0.63	0	0.00	0	0.00	1	0.35
A. hydrophila	5	3.13	1	2.08	0	0.00	6	2.12
A. jandaei	2	1.25	0	0.00	0	0.00	2	0.71
A. media	11	6.88	8	16.67	4	5.33	23	8.13
A. popoffii	–	0.00	1	2.08	7	9.33	8	2.83
A. salmonicida	13	8.13	11	22.93	0	0.00	24	8.48
A. schubertii	–	0.00	0	0.00	3	4.00	3	1.06
A. trota	3	1.88	1	2.08	1	1.33	5	1.77
A. veronii sobria	9	5.63	0	0.00	1	1.33	10	3.53
A. veronii veronii	1	0.63	0	0.00	1	1.33	2	0.71
Total	160	100.00	48	100	75	100	283	100

and 8.6 mg l^{-1} (average of 6.6 mg l^{-1}) and total suspended solids between 77 and 270 mg l^{-1} (average of 178 mg l^{-1}).

DISCUSSION

Owing to its capability to adapt to several types of aquatic environments, *Aeromonas* species ubiquity has been one of the reasons for the development of research aiming to assess the real distribution of these organisms, as well as its survival and virulence factors, and to try to elucidate their possible sources and routes of transmission (Pianetti *et al.* 2004; Martin-Carnahan & Joseph 2005).

Like other emerging pathogens, the lack of well-established diagnostic routines for *Aeromonas*, in clinical, food or environmental samples, associated with the lack of familiarity of the technicians involved in the microbiological routine suggest that the available data are still underestimated (Huys *et al.* 2002, 2003; Pidiyar *et al.* 2002; Park *et al.* 2003; Martin-Carnahan & Joseph 2005; Tena *et al.* 2007; Mulholland & Yong-Gee 2008).

Studies on *Aeromonas* in aquatic environments and clinical samples in Brazil are scarce (Falcão *et al.* 1998; Hofer *et al.* 2006; Razzolini *et al.* 2008; Balsalobre *et al.* 2009*a*,*b*). On the other hand, there is growing interest among the international scientific community shown by the increasing number of publications involving organisms from the genus *Aeromonas* (Szabo *et al.* 2000; Villari *et al.* 2000; Ivanova *et al.* 2001; Massa *et al.* 2001; McMahon & Wilson 2001; Benchokroun *et al.* 2003; Pianetti *et al.* 2004; Hiransuthikul *et al.* 2005; Nováková *et al.* 2008).

In sewage treatment systems, a few studies have been published trying to elucidate the role of *Aeromonas* spp. and its importance for public health in those systems (Parodi & Peso 1983; Monfort & Baleux 1990, 1991; Boussaid *et al.* 1991; Hassani *et al.* 1992; Stecchini & Domenis 1994; Bahlaoui *et al.* 1997; Imziln *et al.* 1998; Benchokroun *et al.* 2003; Maalej *et al.* 2003; Pianetti *et al.* 2004).

These organisms were observed in higher numbers in the raw sewage (influent), decreasing throughout the pond system, as observed in Table 2 and Figure 1, although the fluctuation indicates an inconsistency in its removal. Similar results were reported by Parodi & Peso (1983), who investigated the presence of *Aeromonas* in sewage wastewater in Buenos Aires, Argentina. Monfort & Baleux (1990) studied the dynamics of *Aeromonas* in a sewage

treatment system by stabilization ponds located in Mèze, France. The authors observed that *Aeromonas* was present in all phases of the treatment process, with density fluctuations, data similar to those observed in the present study, where detected variations ranged between < 3 and 3.0×10^9 MPN/100 ml.

A study carried out in Marrakech (Hassani *et al.* 1992) revealed that *Aeromonas* was present in abundance at the inflow of the treatment system and a decrease in numbers was observed through the ponds, but there was no total removal after the treatment process with a significant fluctuation in *Aeromonas* density at each sampling campaign. These results strengthen the findings of this study when referring to the dynamics and fluctuation of *Aeromonas* counts in different sampling campaigns. WHO (2002) reports that the values normally found in the treated effluent are 10^3 to 10^5 CFU ml^{-1}. The WHO report confirms previous studies as well as the results of this work, which is the first study on the dynamics of aeromonads in sewage treatment destined for reuse in Brazil.

The study by Boussaid *et al.* (1991) demonstrated a decrease of *Aeromonas* in a wastewater treatment system in Marrakech not exceeding 1.14 log. Although the reduction observed for *Aeromonas* in the present study was 2.57 log, this value does not represent the dynamics of the organism in the system under study, because *Aeromonas* counts vary from <3 to 3×10^9 MPN/100 ml (0.00 to 9.48 log) irrespective of the site of sample collection (anaerobic pond inflow, anaerobic pond outflow and facultative pond outflow; Table 1). Moreover, based only on the reduction, it is not possible to predict the absence of *Aeromonas* at the end of the system.

As for the occurrence of *Aeromonas* and *E. coli*, Figure 1 shows differences in the dynamics between these two groups of organisms. Similar results have been described by other authors (Smoot & Pearson 1997; Bahlaoui *et al.* 1997; Maalej *et al.* 2003; Sharma *et al.* 2005). The absence of correlation can be explained by differences in characteristics of each group of organisms, such as occurrence of enteric organisms, in the case of *E. coli*. On the other hand, the genus *Aeromonas* can get into the stabilization pond system through the faecal pathway, even though they are primarily ubiquitous in the aquatic environment, where they can multiply or tolerate changes in dynamics, association to and/or competition with bacterial flora, and other environmental variations (Hassani *et al.* 1992; Benchokroun *et al.* 2003; Maalej *et al.* 2003; Sautour *et al.* 2003; Blasco *et al.* 2008).

The complex taxonomy of the Aeromonadaceae family can be illustrated in studies developed to classify *Aeromonas* isolates from different sources, into different known species. Several studies showed that biochemical characteristics of *Aeromonas* could vary according to the source or geographic characteristics, favouring the variability of test results and turning the taxonomic classification of the isolates into laborious work (Martin-Carnahan & Joseph 2005; Alperi *et al.* 2010). The results obtained herein for biochemical identification of the isolates allowed us to assign most of the organisms into known *Aeromonas* species, even though 18.7% of isolates were classified as *Aeromonas* spp.

In the present study, most of the known species of *Aeromonas*, a total of 13 species, were found throughout the sewage treatment system. In the raw sewage, *A. caviae* was the dominant species of *Aeromonas* representing 33.13% of isolates, followed by *A. allosaccharophila* (17.50%). Stecchini & Domenis (1994) also observed that *Aeromonas caviae* was predominant in raw sewage in Italy. An inversion was observed in the number of isolates when compared with the final effluents (Table 2), where there was a decrease of *A. caviae* isolates to 8.00% and an increase of *A. allosaccharophila* to 46.6%. Studies carried out elsewhere demonstrated that in fact *A. caviae* is the predominant species in polluted environments (Hassani *et al.* 1992; Imziln *et al.* 1998; Nováková *et al.* 2008).

There are few studies that have identified organisms of the genus *Aeromonas* to species level, isolated from different stages of a sewage treatment system (Monfort & Baleux 1990, 1991; Boussaid *et al.* 1991; Hassani *et al.* 1992; Bahlaoui *et al.* 1997). Irrespective of the characteristics of the treatment system, all studies previously demonstrated the presence of *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria* in the different stages of treatment. Since 1997, new species of *Aeromonas* have been described and validated, and by the time this study was concluded, 17 species of *Aeromonas* were recognized. We have observed a variety of species in the studied system representing 13 known *Aeromonas* species, differing from previous studies.

Although the search for virulence factors was not performed for *Aeromonas* in this study, it was observed that the remaining species in the final effluent (*A. trota*, *A. schubertii*, *A. veronii sobria* and *A. veronii veronii*) were among those considered to belong to the potentially pathogenic group for humans (Martin-Carnahan & Joseph 2005).

As several environmental factors can influence, directly or indirectly, the presence of organisms from the genus *Aeromonas* in the aquatic environment, we observed that values of pH, temperature and BOD were considered suitable for the development of these organisms as demonstrated elsewhere (Boussaid *et al.* 1991; Sautour *et al.* 2003; Maalej *et al.* 2003; Benchokroun *et al.* 2003). Moreover, values of BOD (average of 65 mg l^{-1}) and total solids (average of 178 mg l^{-1}) in the final effluent may be indicative of the high concentration of organic matter in the studied system. Previous studies demonstrated the increment of *Aeromonas* counts during the winter season (Boussaid *et al.* 1991). As reported by Matté *et al.* (1994), the temperature values observed during this study did not show a sufficient change to stimulate seasonal variations of *Aeromonas* counts. The water temperature values obtained ranged from 23 to 32°C with average value of 27°C.

Owing to the pathogenic potential of *Aeromonas* species present in treated wastewater, one can consider that the presence of these organisms may represent a risk to public health because this effluent is disposed of into water bodies which are eventually used for recreation, irrigation and domestic tasks.

CONCLUSIONS

The detection of potentially pathogenic *Aeromonas* strains may represent a potential risk for human health, supporting the idea that further studies on the microbiological quality of water should include specific searches for this bacterium.

This research has shown that *Aeromonas* are present in the effluent of the stabilization ponds treatment system and cannot be estimated by the presence of *E. coli*, which is used in standard tests for the determination of water quality.

Environmental variables studied were shown to be suitable for the occurrence of microorganisms from the genus *Aeromonas* in all phases of the stabilization ponds system. A better comprehension of the dynamics of this pathogen in wastewater with potential for reuse, as demonstrated in this study, could provide useful information to environmental agencies in a future reassessment of criteria for the sanitary quality of water.

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