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Fate of Enrofloxacin in Swine Sewage

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The fate of enrofloxacin present in raw sewage at a swine-breeding facility was investigated by liquid−liquid extraction and reversed-phase liquid chromatography with photodiode array detection. Samples were collected in the storage pits of each pigsty and in a nonaerated lagoon used to stock the sewage. In the pigsties, the sewage was mixed with 10% olive oil groundwater, following a certified procedure (cod. Cer 020301) which reduces the bad odors and produces a better manure. This sewage treatment for 8 months in the lagoon dramatically reduced the amount of enrofloxacin to levels under the detection limit of 0.6 $\mu g \cdot L^{-1}$. The results stress the importance of correct sludge management in limiting the impact of enrofloxacin in the environment.

KEYWORDS: Enrofloxacin; HPLC; swine sewage

INTRODUCTION

Scientific concern about the presence of trace amounts of pharmaceuticals in the environment has been continuously increasing because of their unfavorable ecotoxicity (*1*). The presence of antibiotics in the environment can also lead to resistant bacterial strains (*2*). Because of these problems, risk assessment studies require accurate concentration measurements in different environmental compartments (*3*−*7*).

Penicillins, tetracyclines, sulfonamides, and macrolides have been largely monitored (*8*−*12*). The occurrence of fluoroquinolones (FQs) has not been examined thoroughly (*3*, *4*−*13*). FQs are excreted largely unchanged (<25% metabolized), and they enter the environment via human excretion into wastewater or via animal feces dispersal onto agricultural soils (*3*).

Many studies on this topic were conducted by Golet and coworkers (*4*). They found quinolones to be more persistent than other antibacterial agents (*14*). The concentrations of enrofloxacin in human raw sewage and in final wastewater effluents from activated sludge sewage treatment plants ranged from 255 to 568 ng/L and from 36 to 106 ng/L, respectively (*15*).

Although controversial (*16*), the use of sewage sludge as fertilizer in agricultural soil is widespread. Unfortunately, the anaerobic reactions of putrefaction that occur in reinforced concrete storage pits used to collect sewage lead to the formation of toxic compounds. As a consequence, the manure obtained is often worse than the traditional one. It is contaminated by putrefaction products as well as by pharmaceuticals and integrators, such as heavy metals, largely administered to young animals. The European Union Directive 86/278/EEC was revised to limit the amount of micropollutants that can be released in the environment. Despite this, pharmaceuticals were not included in this directive (*15*).

In the current study, raw sewage from a swine-breeding facility, mixed with 10% olive oil groundwater, was analyzed. Olive oil groundwaters are classified as fertilizer according to the law 748/84 recognized in Reg. CEE No. 2381/94, which modifies enclosure II of Reg. CEE No. 2092/91 certified with cod. Cer. 020301. Samples were collected from the sewage storage pits of each pigsty and from a nonaerated, open-air lagoon after a storage period of 8 months. Treatment with 10% olive oil groundwater seemed to improve the quality of raw sewage as far as bad odors were concerned. The chemical characteristics of manure obtained after the complete treatment (olive oil groundwater addition plus 8 months of storage in the open-air lagoon) have not been examined.

In the current study, the concentration of enrofloxacin prior to and after sewage treatment was examined. Enrofloxacin was chosen for its environmental persistence (*14*).

We developed a simple, rapid, and reliable analytical procedure for preconcentration and quantification of enrofloxacin in sewage samples using liquid chromatography with a reverse phase C18 column and a photodiode array detector. One step of the sample preparation involved the use of liquid−liquid extraction (LLE) to separate enrofloxacin from the matrix. After lagoon storage, enrofloxacin was not found in any sample at levels above our method detection limit (LOD): 0.6 $\mu g \cdot L^{-1}$.

The results obtained with this analytical method outline the importance of sewage management to reduce the presence of pharmaceuticals in the environment.

MATERIALS AND METHODS

Apparatus and Reagents. Solvents, orthophosphoric acid 85%, ammonia solution 25%, trifluoroacetic acid, triethylamine, and enrofloxacin (≥98%) were purchased from Sigma-Aldrich S.r.l. (Milano,

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Table 1. Density and Dry Weight of Swine Liquid Sewage from the Pigsty Storage Pits and from the Lagoon

sample	density (kg/L)	dry wt (g/kg of liquid sewage)
pigsty 1	1.03	45
pigsty 2	0.84	79
pigsty 3	0.99	18
lagoon	1.00	4

Italy). All of the solvents were of HPLC grade. Water used for LLE was purified with a Milli-Q Plus (Millipore Corp., Bedford, MA). A stock solution of enrofloxacin was prepared at a concentration of 1 mg/mL in acetonitrile/methanol 50:50. The pH was measured with a Crison BasiC20 pH-meter (Crison Strumenti S.p.A., Carpi, Italy).

Instrumentation. The HPLC system consisted of a Waters 2690 liquid chromatograph (Waters Corp., Milford, MA) equipped with a quaternary pump, a vacuum degasser, and an autosampler. The system was coupled with a Waters 996 photodiode array detector operating in a range of wavelengths from 210 to 400 nm. All of the measurements were conducted at a wavelength of 278 nm for optimal enrofloxacin detection. The analytical column was a reversed phase Perkin-Elmer Spheri 5 ODS 250 × 4.6 mm packed with spherical particles of 5 μm diameter. The following chromatographic conditions were adopted: eluent A was an orthophosphoric acid solution, 0.02 M, adjusted to pH 2.9 with triethylammine; eluent B was acetonitrile. The separations were obtained with a gradient from 0 to 80% of eluent B in 20 min followed by a washing step of 6 min and equilibration at the initial conditions for 15 min. The flow rate was 1 mL/min, and the injection volume was 10 μL. Identification was performed by comparing UV spectra and chromatographic retention times of the unknown peaks in the experimental samples with the one generated by the injection of enrofloxacin standard solution.

Sampling. All of the experimental samples were collected from a farm (270 ha) located in Sant'Angelo in Vado (PU, Italy).

The farm has three pigsties, each with its own storage pit where the feces, mixed with 10% olive oil groundwater, were collected. Pigsty 1 has a grated floor, which allows for a reduced washing procedure: 4 versus 20 L/day/pig in a nongrated floor. Inside, 150 small pigs, in the first phase of growing, are usually hosted. Pigsty 2 has a partially grated floor and contains 150 pigs in the second phase of growing together with pregnant sows. Pigsty 3 contains 100 pigs in the third phase of growing and does not have a grated floor. This description is crucial to explain the extraction procedure and the experimental data.

The storage pits are aerated and mixed once a month with a pumping system. Therefore, anaerobic reactions are limited. The time necessary to fill the storage pits is ~1 year.

Raw sewage (10 L) was collected from each storage pit just before they were emptied. The material was stored in polypropylene bottles.

At the end of the autumn, the storage pits are emptied and the sewage is carried to the open-air lagoon, where it remains for 8 months. The lagoon, initially empty, was partially filled with the treated sewage.

The lagoon has a maximum depth of 3 m and a capacity of 1447 m^3, and it is not aerated. In the following summer, after the threshing season, the sewage in the lagoon is used to fertilize the agricultural field of the farm (270 ha). To obtain more representative results, experimental samples (4 L each) were collected from seven different points of the lagoon just before the end of the threshing season. All of the samples were examined following LLE using separatory funnels and with the analysis procedure described below.

The physical properties, density and dry weight, were calculated as follows: aliquots of 500 mL of each sewage sample were accurately shacked and weighed. The ratio between weight and volume (20 °C) represents the empiric density value. Then, to measure the dry weight, samples were dried to constant weight by an infrared (IR) lamp. The values obtained are reported in **Table 1**.

Extraction Procedure. LLE was performed by shaking each raw sewage sample with 15 mL × 4 of dichloromethane and assembling the extracts in a Teflon beaker. Before the extraction, the pH was adjusted to 6.8 with trifluoroacetic acid. The sewage volumes used for LLE were 20 mL for samples coming from pigsty 3, 100 mL for samples coming from the lagoon, and 5 mL (added to 15 mL of water) for those coming from pigsties 1 and 2. This procedure was necessary because of the different content of suspended solids in the samples as explained under Results and Discussion.

Table 2. Percentage Recovery and Relative Standard Deviation from Water and Liquid Sewage Spiked with 100 μg of Enrofloxacin (Each Value Is the Mean of 10 Analyses)

	$\bar{x} \pm$ RSD%				
compound	lagoon	pigsty 1	pigsty 2	pigsty 3	water
enrofloxacin	87 ± 6	92 ± 6	93 ± 8	94 ± 7	96 ± 6

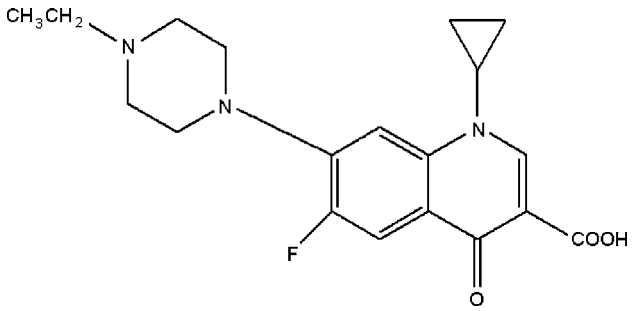

Figure 1. Chemical structure of enrofloxacin.

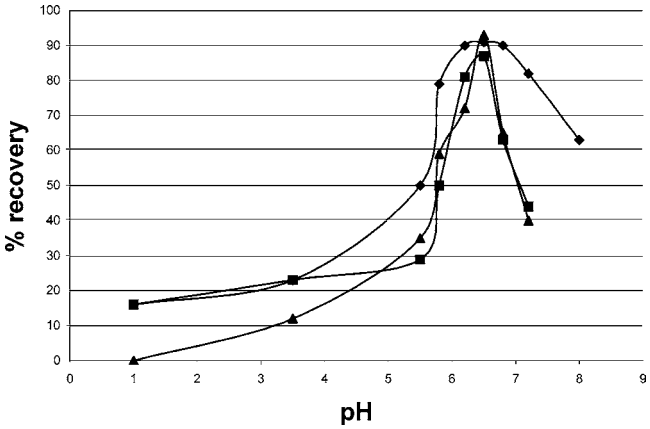

Figure 2. Percentage recovery from different matrices [(◆) Milli-Q water; (■) raw sewage from lagoon; (▲) raw sewage from pigsty 3] spiked with 100 μg of enrofloxacin at different pH values.

The extracts were evaporated to dryness under a gentle flow of nitrogen at room temperature. Recovery data were obtained by extracting each sample spiked with 100 μg of enrofloxacin. Blank experiments were carried out with unspiked samples. All of the extracts, evaporated to dryness, were dissolved in 1 mL of acetonitrile/methanol 50:50 and analyzed. The area of enrofloxacin in each blank sample was subtracted from the area of enrofloxacin found in the corresponding spiked sample and compared with the calibration curve. The results are reported in **Table 2**.

Experimental samples from the lagoon extracts were dissolved in 200 μL of acetonitrile/methanol 50:50 to achieve greater sensitivity.

Calibration Curve and Limit of Detection. A calibration curve was obtained by injecting 10 μL of standard solutions containing 200, 100, 50, 20, 10, 5, or 1 $\mu g \cdot mL^{-1}$ (corresponding to 2, 1, 0.5, 0.2, 0.1, 0.05, or 0.01 μg). Good linearity was observed over 2 orders of magnitude with a correlation factor (r^2) of 0.9998 and a relative standard deviation (RSD) of ~1% calculated on 10 replicates for each point.

The straight line equation is $y = -2115.6 + 21117.0x$, with errors of 7846 (σA) and 262 (σB), respectively. The instrumental quantification limit (LOQ) was the lowest point of the curve, whereas the instrumental detection limit (LOD) was found at 3 ng (S/N 3:1). The detection limits of the method, calculated under the same conditions

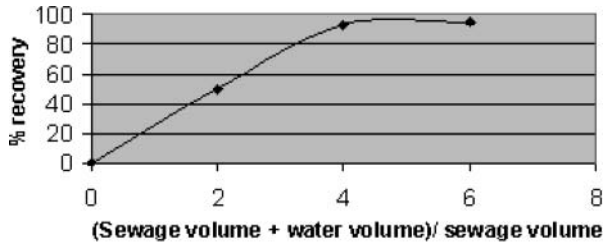

Figure 3. Variation of percentage recovery from sewage of pigsty 2 (20 mL), spiked with 100 μg of enrofloxacin, with dilution.

reported above, were 0.6 $\mu g \cdot L^{-1}$ for lagoon sewage, 12 $\mu g \cdot L^{-1}$ for pigsties 1 and 2, and 3 $\mu g \cdot L^{-1}$ for pigsty 3.

RESULTS AND DISCUSSION

The colloidal nature of raw sewage samples and the high content of solid matter (**Table 1**) complicated the separation of liquid from solid matter. Centrifugation and filtration were not useful. In fact, a centrifuge that works at 8000 rpm is not adequate and filters of 0.45 μm diameter were soon clogged. Therefore, a LLE method was adopted allowing a global evaluation of enrofloxacin concentration in the nonseparated raw sewage.

The efficiency of LLE is strongly dependent on pH. This is due to the chemical structure of enrofloxacin (**Figure 1**), which contains both acidic and basic functional groups, and to the high content of polyphenols, due to the addition of olive oil groundwater.

For this reason, an investigation of the range of pH values that could be used for the extraction of enrofloxacin was necessary. Milli-Q water samples (100 mL) were spiked with 100 μL of stock solution (corresponding to 100 μg of enrofloxacin) to a concentration of 1 $ng \cdot mL^{-1}$. The pH of the samples varied from 1 to 8, and they were all extracted. The results are reported in **Figure 2**. The best recoveries were obtained in a small range of pH. In fact, the molecule of the analyte has to be neutral to obtain the lowest solubility in water and the highest in dichloromethane. These water extraction experiments allowed us to verify that the method worked best under the simplest conditions.

The second step was to test the method on lagoon sewage samples, which have the lowest dry weight but represent a much more complex matrix than water (**Table 1**). In this case, the range of pH values useful for the extraction is narrower. This is due to the presence of polyphenols coming from the olive oil groundwater. These molecules are similar to humic acids, which are naturally present in soil. These compounds are capable of masking several pollutants, and they interfere with their extraction (*19*−*29*). Polyphenols, as well as humic acids, have a complex chemical structure characterized by a large number of phenolic hydroxyls. The pH influences the interactions of those groups with the analyte, regulating their dissociation. However, even though the fertilizer makes the LLE procedure more complex, it is still possible to get complete recovery by careful regulation of the pH to 6.8.

The pigsty sewage samples represent the most complex matrix, having the highest amount of suspended solids (**Table 1**). However, there is a great variation among pigsties. The dry weight of the sewage is greater if a lower quantity of water is used in the pigsty cleaning procedure. This happens in pigsties

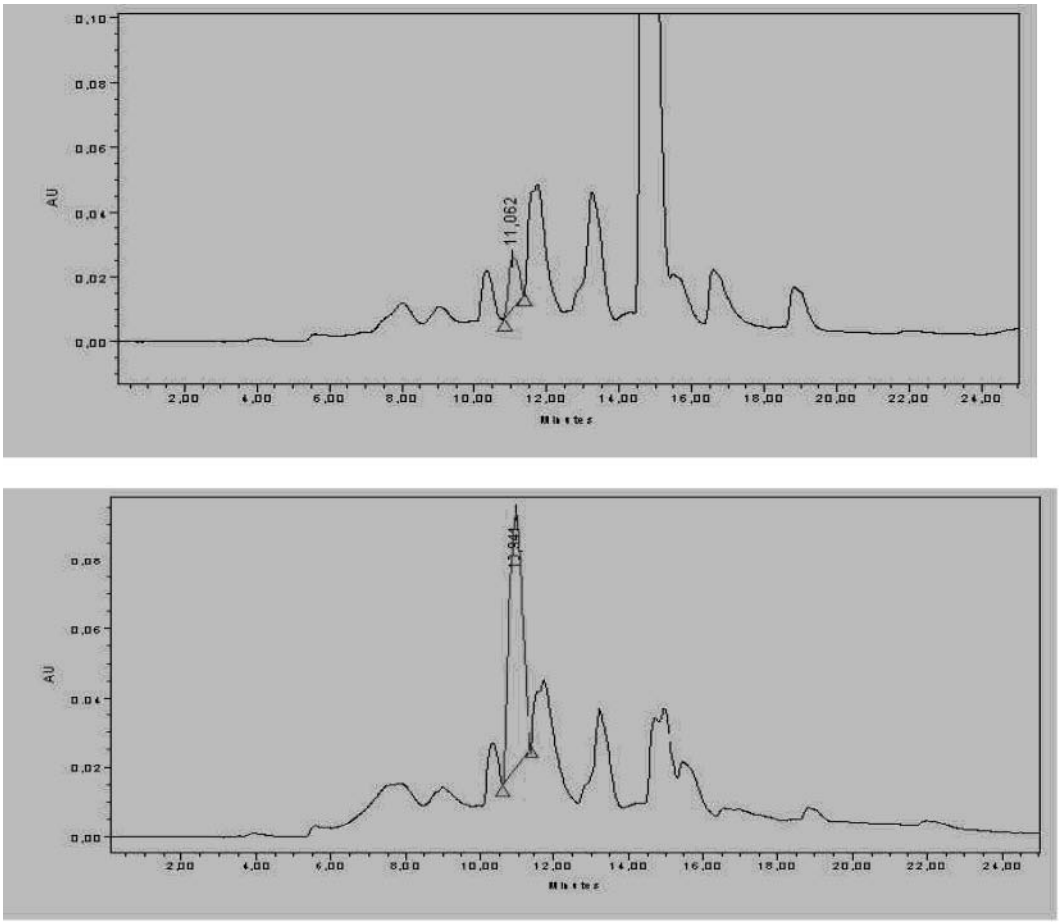

Figure 4. Chromatogram of LLE extract from pigsty 3 sewage (20 mL): (a, top) evaporated and redissolved in 1 mL of a mixture of acetonitrile/methanol 50:50; (b, bottom) spiked with 100 μg of enrofloxacin, evaporated, and redissolved in 1 mL of a mixture of acetonitrile/methanol 50:50.

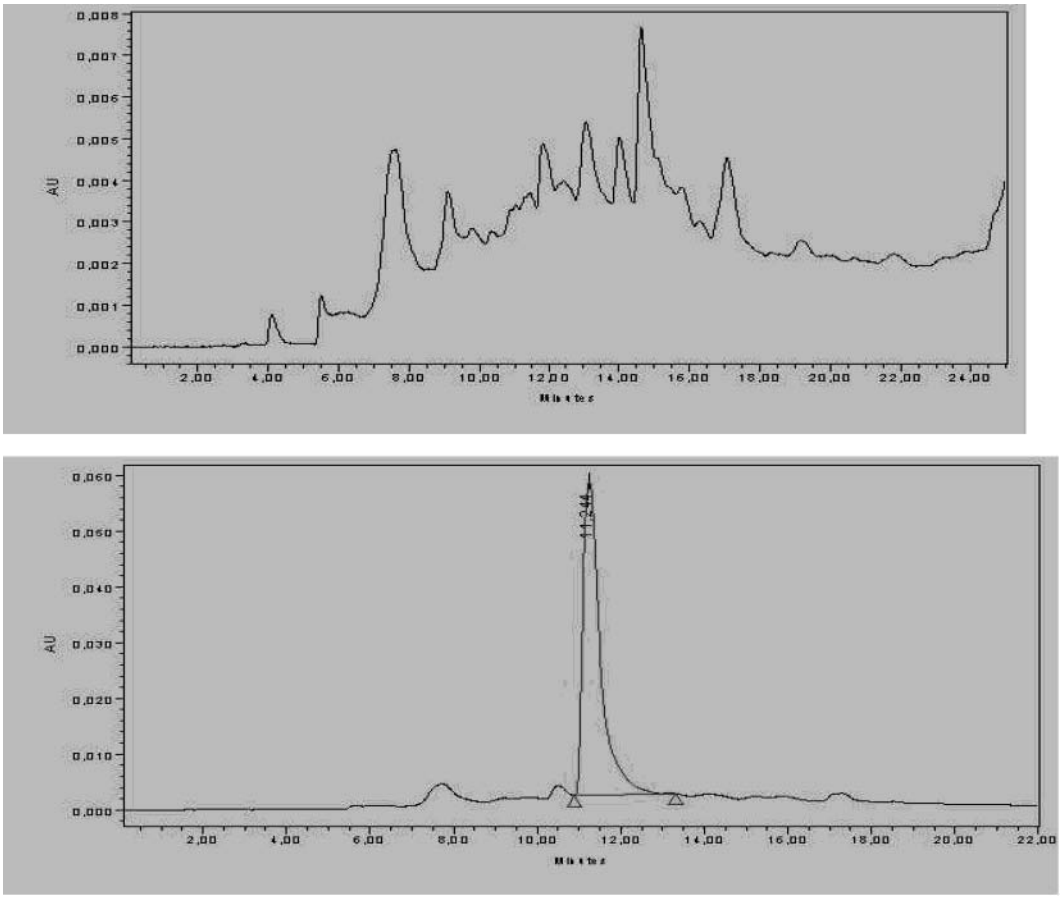

Figure 5. Chromatogram of LLE extract from lagoon sewage (100 mL): (a, top) evaporated and redissolved in 0.2 mL of a mixture of acetonitrile/methanol 50:50; (b, bottom) spiked with 100 μg of enrofloxacin, evaporated, and redissolved in 1 mL of a mixture of acetonitrile/methanol 50:50.

1 and 2, due to their grated floors. The large difference between pigsties 1 and 2 could be explained by the presence of still nursing pigs in pigsty 1. A more liquid diet is administered to these pigs.

The amount of suspended solids present in the raw sewage plays a central role in the extraction efficiency. The variation of recovery data with pH, for pigsty 3, is very similar to the profile obtained for the lagoon samples (**Figure 2**). The same procedure applied to the two other pigsties with a higher solid content gave no recovery.

In fact, as reported in the literature, enrofloxacin is strongly adsorbed on sediments (*14*), and this phenomenon could explain the low recoveries obtained. To get better results, the LLE procedure was modified to work with lower amounts of sewage. Four different samples from pigsty 2 were prepared, respectively, at 0-, 2-, 4-, and 6-fold dilutions with Milli-Q water expressed as (sewage + water)/sewage volume. Then, 20 mL of the diluted samples was spiked with 100 μL of the stock solution, corresponding to 100 μg of enrofloxacin, extracted, and analyzed. The results are reported in **Figure 3**.

The data in **Figure 3** and **Table 1** suggest that, to obtain complete recoveries, the dry weight of the sewage samples used in the extraction procedure should span from 360 to 400 mg.

The optimal conditions found for pigsty 2 were applied to pigsty 1 as well. **Table 2** reports the good recovery data and their relative standard deviations obtained from liquid sewage spiked with 100 μg of enrofloxacin, at the best conditions. Results from unspiked samples are reported in **Table 3**.

Typical chromatograms from spiked and unspiked sewage samples are presented in **Figures 4** and **5**.

Table 3. Concentration of Enrofloxacin in Pigsty Sewage (Each Value Is the Mean of Five Analyses)

sample	concn (mg/L ± RSD%)	concn (mg/kg ds)
pigsty 1	0.51 ± 23	11.3
pigsty 2	0.27 ± 20	3.4
pigsty 3	0.27 ± 13	15.0

The concentrations found in the pigsty sewages are higher than values reported in the literature. In fact, Golet reports concentrations of enrofloxacin in human raw sewage in the range of 0.2−0.6 $\mu g \cdot L^{-1}$ (*4*, *13*, *15*). This could be explained by a massive drug treatment of growing animals in order to prevent infections and also by the usually lower dry weight of human sewage (*15*).

According to these considerations, only slightly higher concentrations, with respect to human sewage, were found. The concentration of enrofloxacin was below our method LOD in all lagoon samples. These results are not to be ascribed only to rain dilution or to solid deposition onto the bottom of the lagoon. In fact, LLE was performed on different volumes so that the amount of solid matter extracted was comparable (see **Table 1**). Furthermore, the final volume of the preconcentrated extracts from lagoon sewage samples is 0.2 mL with respect to 1 mL of the extracts from the pigsty sewages. In this paper, a degradation of enrofloxacin due to bacterial strains or chemical reactions could be hypothesized, which would require further investigation.

Although not exhaustive, the method proposed is a simple and rapid procedure to monitor enrofloxacin in residual sewages of swine breeding. The treatment realized on this farm (addition

of 10% of olive oil groundwater and 8 months of storage in the lagoon) resulted in the reduction of dry weight, pH value (to ~6.5), and bad odors. Furthermore, the long storage period in the open-air lagoon results in growth of bacterial strains and chemical reactions that decompose organic compounds such as enrofloxacin. In fact, all lagoon samples were found to be negative. This result is particularly encouraging in the attempt to reduce the environmental impact of raw sewages largely utilized as fertilizer in agriculture.

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