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Ciliated Peritrichous Protozoa in a Tezontle-Packed Sequencing Batch Reactor as Potential Indicators of Water Quality

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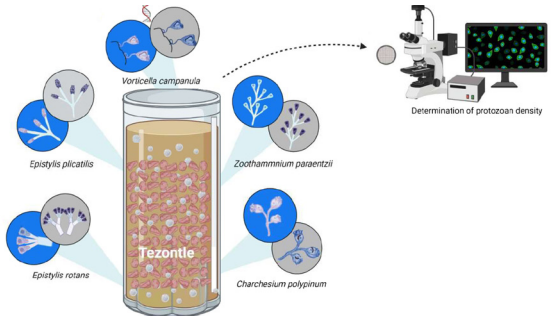
Abstract

The presence of colonial and solitary ciliated peritrichous protozoa was determined in a Sequencing Batch Reactor system filled with tezontle, a volcanic rock, economic, and abundant material that can be found in some parts of the world, like Mexico. The presence of these protozoa was related to the removal efficiencies of organic matter. Also, two novel staining techniques are proposed for staining both colonial and solitary peritrichous protozoa. The results show that tezontle promotes the growth of solitary and colonial ciliated peritrichous protozoa, which, once identified, could be used as indicators of the efficiency of the wastewater treatment process. Additionally, the staining techniques established in the current study allowed the precise observation of protozoan nuclei. They can represent a useful complementary methodology for identifying protozoan species present in water treatment processes, along with the already existing identification techniques. The number and variety of protozoa found in the system may be considered potential bioindicators of water quality during biological treatments.



K e y w o r d s: peritrichous protozoa, sessile ciliates, Sequencing Batch Reactor (SBR), tezontle, protozoan staining techniques

Introduction

The biological systems of wastewater treatment involve microorganisms that include mainly bacteria, protozoa, rotifers, and in some cases, fungi. Bacteria play a fundamental role in the degradation of organic matter, while protozoa perform the function of purifying effluents by consuming particulate (suspended) material that remains after bacterial degradation. That is, protozoa are efficient in purifying of wastewater due to their ability to act as predators that feed on dispersed bacteria (Rakshit et al. 2014). Also, it has been demonstrated that protozoa indirectly influence the clarification of effluents by forming flocs, increasing bacterial activity, contributing directly to the secretion of exopolymer substances, and participating in the development of the structure and biological activity of the flocs (Papadimitriou et al. 2010). It has also been documented that the appearance, abundance, and diversity of protozoa are related to the chemical conditions of the systems, like the presence of toxic substances, oxygen load, etc. Physical conditions also influence the characteristics of the protozoan community, such as the type of packaging (if used) for support purposes. Support materials may be synthetic or natural (Yáñez-Ocampo et al. 2011; Dzionek et al. 2016) and may serve as elements for the retention of suspended particles (mechanic effect) and to underpin microbial activity (biochemical and microbiological effects) (Liu et al. 2015). Some authors have stated that, in contrast

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to synthetic materials like plastics, ceramic, and diatomaceous earth, the tezontle is a low-cost material that may be less costly and easier to manipulate than the former ones (Yáñez-Ocampo et al. 2011). Tezontle is a natural, economic, and abundant (in some parts of the world) volcanic rock commonly used in Mexico as a construction material, which presents a characteristic reddish color due to the presence of Fe ions (Dzionek et al. 2017). Specifically in Mexico, this rock is abundantly produced in the proximities of the volcanic belt of Central Mexico, composed of SiO_2 (60%), Al_2O_3 (12%), MgO (8%), Fe_2O_3 (5%), CaO (4%), Na_2O (3%), and other oxides at low concentrations (Acevedo-Davila et al. 2007). Protozoa are related to the efficiencies of wastewater treatment facilities. For example, the presence of the protozoan *Euglena* spp. in treatment systems is related to Cr^{6+} (20 μg/ml) and Pb^{2+} (30 μg/ml) tolerance (Rehnam 2011). Peritrichous protozoa like *Vorticella* spp., *Epistylis* spp., and *Opercularia* spp. show resistance to hydrocarbons present in residual waters (Kachieng'a and Momba 2018). Protozoan number and diversity differ between continuous and Sequencing Batch Reactors (SBR), or between aerobic and anaerobic conditions (Ogleni et al. 2010; Papadimitriou et al. 2010; Pérez-Uz et al. 2010). An SBR is a system of activated sludge for wastewater treatment, and it has been successfully used to treat both municipal and industrial wastewater. In this system, the wastewater is used to fill a single batch reactor, and the water is treated to remove the non-desirable components, and both are finally discharged. The homogenization, aeration, and clarification can be achieved using a single batch reactor. Two or more discontinuous reactors in a specific operational order can be used to optimize the system's yield (EPA 1999). The efficiency of the SBR is evaluated with determinations such as organic material, total solids, suspended solids, and the presence of pathogens and parasites, among others.

The most representative protozoa in SBR are *Arcella* spp. and *Difflugia* spp. from the amoeba group, *Didinium* spp. (ciliated free-living protozoa), *Aspidisca* spp. (walking ciliate), *Peranema* spp. (flagellate species), and sessile ciliates, such as *Carchesium* spp., *Epistylis* spp., and *Vorticella* spp., are considered indicators of the excellent quality of activated sludges (Ogleni et al. 2010). Sessile protozoa are those adhered or attached by a peduncle, stalk, or lorica to sediment, other organisms, or even the interior of a lorica. Mucus secretion plays an important role in adhesion capability (Arndt et al. 2003). For protozoan identification, it is important to evaluate their size, the shapes of the zoids and nucleus, and their movements, among other characteristics.

Due to the importance of protozoa for wastewater treatment systems, many studies have focused on their identification to a species level. Different staining methods are used to fulfill this task, such as the pyridinated silver carbonate or protargol methods or even molecular techniques, which can be more time-consuming and expensive. Nevertheless, sometimes protozoa can be only identified at a genus level with these staining methologies (Azovsky and Mazei 2018) due mainly to the low nuclei definition achieved, causing easy confusion among species (Robertson et al. 2019). Besides, some protozoan species can resist the stains used in conventional techniques. These facts indicate that establishing novel, quick, and accurate techniques may be helpful. The present study assessed the relationship between the density and diversity of peritrichous protozoa with water quality in the SBR with tezontle as a packaging material.

Additionally, two new and effective nuclei staining techniques for identifying solitary and peritrichous protozoa were proposed. The first used Morrison's fixative, Freitas mordant, and Delafield's hematoxylin (MFD), and the second one employed only Freitas mordant and Delafield's hematoxylin (FD). It could complement the traditional techniques used for this purpose. A molecular approach for protozoan identification was also performed to validate the staining techniques established.

Experimental

Materials and Methods

Sequencing Batch Reactor. Tezontle stones with a mean particle size of 2–2.5 cm were used as fillers in an automatic SBR. The inoculum (bacteria and protozoa) and the wastewater used to feed the reactor were obtained from the municipal activated sludge treatment plant (Tecamac, State of Mexico). Protozoan samples, once taken, were preserved under aerated conditions with over 2 mg/l of oxygen, and oxygen concentration was kept higher than 2.3 mg/l during experimentation. The environmental temperature oscillated from 22–26°C, and the pH of the residual water was adjusted to 7.6 with 0.1N HCl solution. The system functioned with typical cycles: feeding, aeration-reaction, sedimentation, and discharge, with corresponding times of 1 min, 23 h, 20 min, and 1 min. The hydraulic retention time was 23 h, and the volumes of wastewater and tezontle were 1.8 and 2 l, respectively.

Physicochemical analyses. Chemical oxygen demand (COD) and total solids (TS) were determined following the standard methods of water analyses (Baird et al. 2017). These two parameters were assessed to determine the efficiency of the reactor and their relationship with the density and diversity of protozoa. pH and temperature were determined in situ; pH was measured using a potentiometer (HANNA Instruments, model pH 211; USA), while the temperature was

measured with a Branan mercury thermometer. These parameters were determined in both the influent and the effluent during the 60 days.

Analysis of the packaging material (Tezontle). The density of tezontle was calculated by Archimedes' principle. First, dried tezontle stones were weighed, then again weighed when submerged in distilled water at 22°C. Tezontle density was calculated using the following equation:

$$\rho_{tez} = \frac{M_d}{M_d - M_w} \rho_{pwz} \quad (1)$$

where: ρ_{tez} is the tezontle density, M_d is the mass of the dried tezontle, M_w is the mass of the tezontle submerged in water, and ρ_{wz} is the density of distilled water at 22°C.

The scanning electron microscopy (SEM) images of tezontle stones were recorded using a JCM-6000 Plus NeoScope operating under a low accelerating voltage (5 kV). The dried tezontle, without further preparation, was placed on the sample holder of the microscope.

Staining techniques. Two novel staining techniques were developed to observe peritrichous protozoan nuclei, both colonial and solitary, and complement the traditional techniques of protozoan identification. The first used Morrison's fixative, Freitas mordant, and Delafield's hematoxylin (MFD), and the second one employed only Freitas mordant and Delafield's hematoxylin (FD). This latter dye was prepared as indicated by Beltran et al. (2014) with a modification: the addition of 80 ml of methyl alcohol and 80 ml of glycerol. Metazoans' identification was achieved with the help of Baird et al. (2017) based on morphological observations.

For MFD, Morrison's Fixative was added to a 25-µl sample (previously dried for 2 hours) and let stand for 6 min. After this time, the sample was rinsed with a moderate water flow, and it was drained and entirely dried by slightly pressing the sample with absorbent paper. Once totally dried, the sample was covered with Freitas' Mordant and let stand for 5 min. Afterward, the sample was rinsed and dried in the same way. The sample was then covered with Delafield's hematoxylin for 5 min and subsequently rinsed. A drop of water was added and spread over the sample to prevent the zoids from becoming dehydrated. After staining, the sample was observed under the optic microscope at 20× or 40×, as specified for each case.

In the FD technique, 0.5% hydrochloric acid was added to the sample (pre-dried for 2 hours), covering it for 6 min. After this time, it was rinsed with moderate water flow. Afterward, the sample was drained and dried entirely by slightly pressing the sample with absorbent paper. Once completely dried, the sample was covered with Freitas' Mordant and let stand for 5 min, subsequently rinsed and dried in the same way. The sample was covered with Delafield's hematoxylin for 6 min; then, it was rinsed, drained, and a drop of water was added and spread over the sample to prevent it from drying. Then, protozoa were observed through the microscope under the same conditions described above. Both FMD and FD staining techniques were repeated at least six times for each specimen to ensure reproducibility.

Assessment of protozoan density. To determine the density and diversity of protozoa in the SBR, tezontle samples were taken at the beginning and the end of the experimentation period (after 60 days); the assessment was independently performed twice, taking two replicates in each repetition.

Each sample consisted of the biomass scrapped from two tezontle stones using for this purpose a sterilized brush. Samples were homogenized, adding 50 ml of wastewater and shaking them for 2 min. 25-µl samples were taken with an automatic micropipette (Socorex, Switzerland) to quantify the density of both protozoa and metazoans. Afterward, the samples were observed in-fresh for cell counting using a Neubauer chamber (in triplicate) and observed for a period no longer than 3 h (Dubber and Gray 2009), using a clear-field microscope Ni-U NIKON® (USA) with 40× and 100× objectives.

Samples were observed in vivo using MFD and FD techniques (permanent preparations) to determine protozoan diversity in the SBR. To this end, the different organelles and structures (cilia, flagella, zoids, peduncles, and nuclei) were highlighted by right-field microscopy and Nomarski (DIC, Differential Interference Contrast) techniques using a Ni-U NIKON® (USA) microscope. Species were identified according to Isac et al. (2008), Lynn (2010), and Küppers et al. (2020). As for rotifers, genera were identified according to their movement and morphology, following Baird et al. (2017).

Molecular approach for protozoan identification. The *Vorticella campanula*'s identity was confirmed by using a conserved region of the ITS 18S rRNA, for which total RNA was extracted by the TRIZOL reagent technique (Sigma Aldrich). The RNA obtained was reconstituted with RNAase-free water, aliquoted in Eppendorf tubes at a concentration of 50 µg/µl, and stored at –70°C. For amplification, an RT-PCR was developed using a Sensicript™ Reverse Transcription Kit (QIAGEN) and a Taq PCR Core Kit (QIAGEN), using the set of primers designed to identify the conserved region within the ITS 18S of the microorganism, which is VCS302 3'-ACGGCTACCACATCTACGG-5' and the VCA103 5'-GTGCAAAACCGTCAATTCCT-3'. Amplification was effected using the following protocol: reverse transcription at 37°C for 60 min; PCR at 94°C for 4 min, followed by 30 cycles of 60 s at 94°C, 30 s at 55°C and 60 s at 74°C, and a final extension at 74°C for 10 min. The RT-PCR product was visualized by electrophoresis on a 1.2% agarose gel and quantified by spectrophotometry.

The amplicons were extracted from the agarose gel, purified directly using a QIAquick Gel Extraction Kit (QIAGEN, Germany), and sequenced automatically using the dideoxynucleotide chain-termination method.

The phylogenetic analysis was performed using data from the entire ITS 18S regions obtained from GenBank. Sequences were aligned using CLUSTALX and adjusted with the BIOEDIT v.7.0.9 (Sun et al. 2013). The aligned matrix was analyzed by using three methods: maximum likelihood (ML), neighbor-joining (NJ), and maximum parsimony (MP). The analysis was performed with MEGA X (Kumar et al. 2016) and Modeltest 3.7 (Posada and Crandall 1998), using the GTR + I and GTR + G model, and Kimura of 2-parameters with a 1,000 bootstrap (Liao et al. 2018).

Statistical analysis. Each experiment was replicated to obtain protozoan density, and the results were analyzed using R software. To determine the difference between the total densities of the sessile ciliated protozoan species present in the different replicates, a Student's *t*-test was performed ($p = 0.01$). An ANOVA was performed to evaluate the difference between the densities of each species ($p = 0.01$). To corroborate these significant differences between the densities, data was analyzed by a Tukey test ($p = 0.01$). Also, a multivariate analysis was conducted (Principal Components Analysis, PCA) to statistically analyze the results, using the Statgraphic Plus v.5.1 software (Statgraphics Technologies Inc., USA); this analysis allowed the detection of the relationship between the density of each protozoan species with the operating time of the reactor.

Results

Establishment of protozoan staining techniques. Two different protocols were assessed to establish novel, quick, and accurate techniques that may be useful for identifying solitary and colonial peritrichous protozoa. To this end, protozoan samples were taken from the facilities of a municipal activated sludge treatment plant in Tecamac, State of Mexico. First, Fig. 1 shows a colony of peritrichous protozoa in fresh (Fig. 1a) and stained with the Harris Hematoxylin traditional technique (Fig. 1b). It was observed that nuclei could not be distinguished, and only dark spots could be seen. Therefore, it was corroborated that using conventional staining protocols, it is difficult to determine protozoan species. In contrast, there are examples of protozoan samples in fresh (Fig. 1c and 1e) and stained with MFD (Fig. 1f and 1h) and FD (Fig. 1d and 1g) protocols. In the first technique, MFD, the cell body was stained intensively. In contrast, while the nucleus was stained in lighter shades than the rest of the zoid, so the size, shape, and position of the macronucleus can be distinguished (Fig. 1f and 1), which allowed the identification of the species *Epistylis plicatilis*. On the other hand, the FD technique (Fig. 1d) allowed the dyed of the macronucleus in a stronger color. At the same time, the rest of the zoid was dyed in lighter shades, also allowing the identification of *E. plicatilis*.

Identification of protozoan species in the SBR. Identifying colonial peritrichous protozoa is not easy, as training is necessary regarding microscopical and specific staining techniques; furthermore, long-lasting fixing and preserving methods for free-living protozoa are still required, as was indicated by Küppers et al. (2020). Using the MFD and FD protocols described above, the colonial and solitary peritrichous protozoan species present in the SBR were detected: *Zoothamnium paraentzii*, *E. plicatilis*, *V. campanula*, *Epistylis*

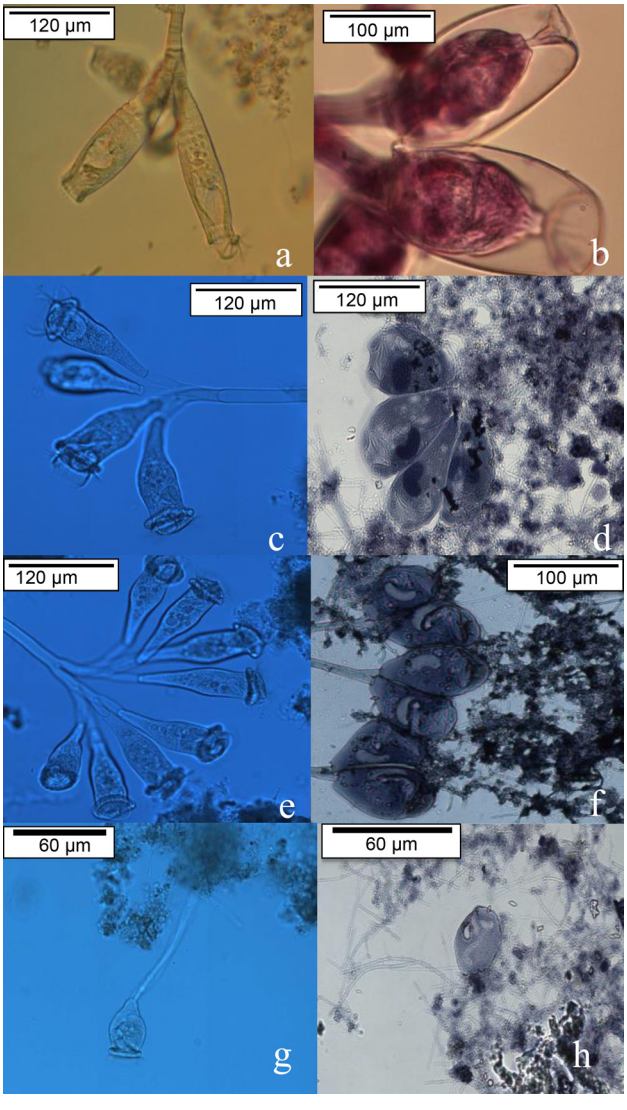

Fig. 1. Micrographs of colonial peritrichous protozoa.
a) Fresh sample and b) stained with Harris hematoxylin technique at 40x; c) 4-zoid fresh colony using Nomarski technique and d) FD staining at 40x; e) 8-zoid fresh colony using Nomarski technique and f) the MFD staining at 40x; and g) fresh samples of *Vorticella campanula* and h) stained with the MFD technique ("J" shaped nucleus) at 20x magnification.

Table I
The density of peritrichous protozoa (peritrichous protozoa $\times 10^3$/ml).

Time (days)	*Zoothamnium paraentzii*	*Epistylis plicatilis*	*Vorticella campanula*	*Epistylis rotans*	*Charchesium polypinum*	*Vorticella convallaria*	*Vorticella aquadulcis*	Total density ($\times 10^3$ protozoa/ml)
1	11	8	10	7	33	1.5	1	72
10	142	34	57	47	17	2	2	299
20	267	34	57	47	17	2	2	425
30	220	92	96	76	11	5	2	500
40	244	63	76	61	14	3	2	462
60	232	77	86	68	12	4	2	481

rotans, *Carchesium polypinum*, *Vorticella convallaria*, and *Vorticella aquadulcis*. Other genera of protozoa, different from ciliated peritrichous, were identified by their morphology and movement as *Cyclidium* sp., *Colpidium* sp., *Litonotus* sp., *Paramecium* sp., *Chilodonela* sp., *Aspidisca* sp., *Euplotes* sp., *Euglypha* sp., and *Centropyxis* sp. Metazoans such as *Philodina* sp., *Rotaria rotatoria*, and *Chaetonotus* sp. were identified based on their morphology.

Micrographs included as supplementary material (Fig. S1–S6) present some protozoan species identified in the SBR by MFD and FD techniques. In *V. campanula*, stained using the MFD technique (Fig. 1g and 1 h), it presents very wide inverted bell-shaped zoids, with measures of 50–157 µm for the zoid, 35–100 µm for the peristomal width and peduncles of 250–350 µm long.

V. campanula is abundant in the SBR system and tolerant to some heavy metals. Therefore, this species is essential for degrading some toxic compounds (Vilas-Boas et al. 2020). It is also subsequently used to complement, by a molecular approach, the precision of staining techniques for identifying the protozoan species. To this end, the ITS 18S region of this protozoan sample was amplified and aligned (Table SI). A phylogenetic analysis was performed (Fig. 2) using the procedures described in Materials and Methods. The identity percentage of the aligned region (98.5%), and the phylogenetic analysis indicated on *V. campanula*, and the strain was codified as *V. campanula* 01mex. Since protozoa are commonly found in consortia, it is important to mention that it may be challenging to isolate strains and subsequently perform molecular biology techniques for their identification. Even though, the identification of the *V. campanula* 01mex specimen by a molecular approach was successfully achieved in the current study as a complement to the staining methods proposed.

Protozoan density and SBR efficiency. Table I shows the density and diversity of protozoa found during the 60 days of SBR functioning. The highest densities of sessile ciliates were found for the species *Z. paraentzii*, *E. plicatilis*, *V. campanula*, *E. rotans*, and *Carchesium polypinum* with maximum values of 244×10^3 protozoa/ml, 92×10^3/ml, 96×10^3/ml, 76×10^3/ml, and 33×10^3/ml respectively. *V. convallaria* and *Vorticella aquadulcis* were detected at low density: 5×10^3 and 2×10^3 protozoa/ml, respectively. The densities of other relevant microorganisms related to water quality were also assessed and are as follows: *Euglypha* sp. and *Centropyxis* sp. from the amoebae group were present at the constant density (a maximum of 4.5×10^4 amoebae/ml). The behavior of the free-swimming ciliated protozoa, like *Cyclidium* sp., *Colpidium* sp., *Litonotus* sp., *Paramecium* sp., and *Chilodonella* sp. was different, as their density remained of up to 4.1×10^4 of free-swimming ciliates/ml. Metazoans, considered good indicators of treatment quality in activated sludge systems, were also identified and it was *Philodina* sp., *R. rotatoria*, and *Chaetonotus* sp. Regarding the density of total protozoa (ciliated sessile, free-swimming, and amoebae), two consecutive stages were observed; an adaptation stage followed by a stabilization one (Fig. 3), as the total density of the sessile protozoa increased steadily from day 1 to day 20. After day 20 and up to day 60, the protozoan density remained constant. The *t*-test analysis showed no significant differences when comparing the total densities of peritrichous protozoa between the two independent experiments. Additionally, ANOVA and Tukey analyses demonstrated the significant differences between the densities of the diverse protozoan species (Table II). The density

Table II
The mean abundance of peritrichous protozoan species.

Species	Average density (protozoa $\times 10^3$/ml)
Zoothamnium paraentzii	185.79[a]
Epistylis plicatilis	51.18[b]
Vorticella campanula	63.56[b]
Epistylis rotans	50.79[b]
Charchesium polypinun	17.31[c]
Vorticella convallaria	2.85[d]
Vorticella acuadulcis	1.64[d]

[a–d] – lower-case letters represent groups of data that were significantly different by the Tukey's test ($\alpha = 0.01$)

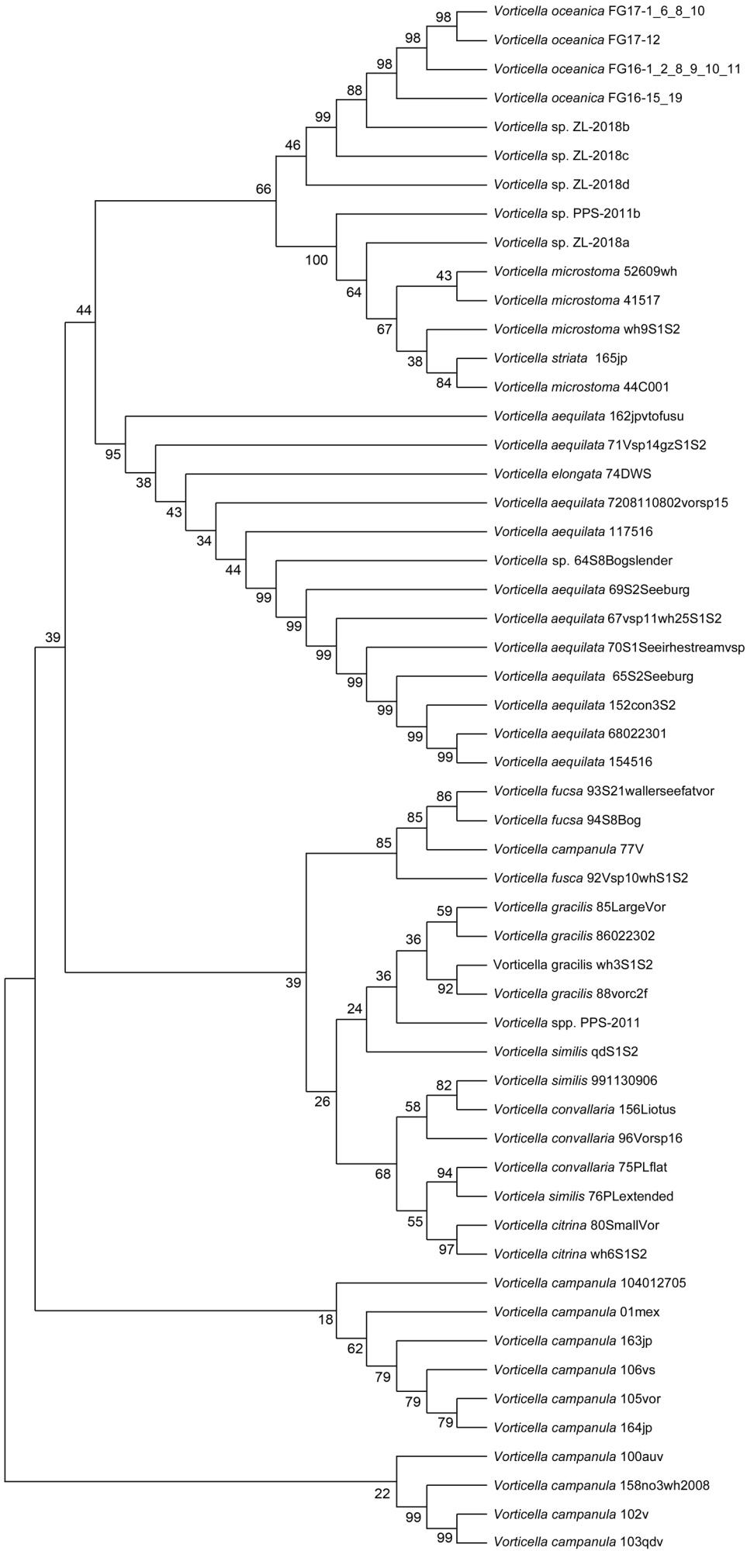

Fig. 2. Identification of *Vorticella campanula* 01mex by a phylogenetic analysis based on its ITS 18S rRNA sequence.

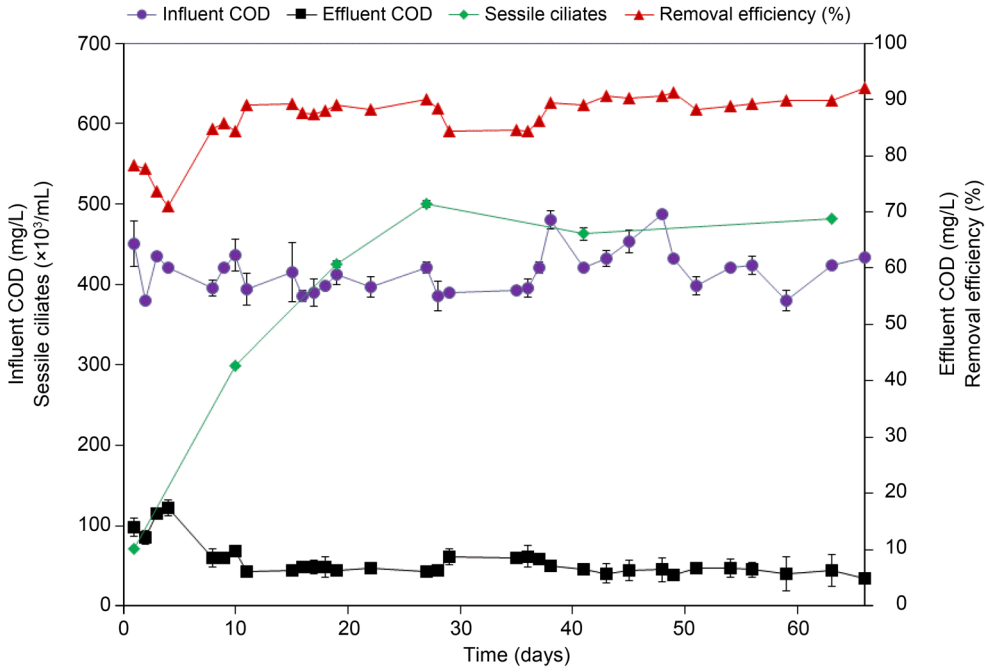

Fig. 3. Relationship between protozoan density and COD in the SBR.
Protozoa density; Influent COD; Effluent COD; Removal efficiency (%).

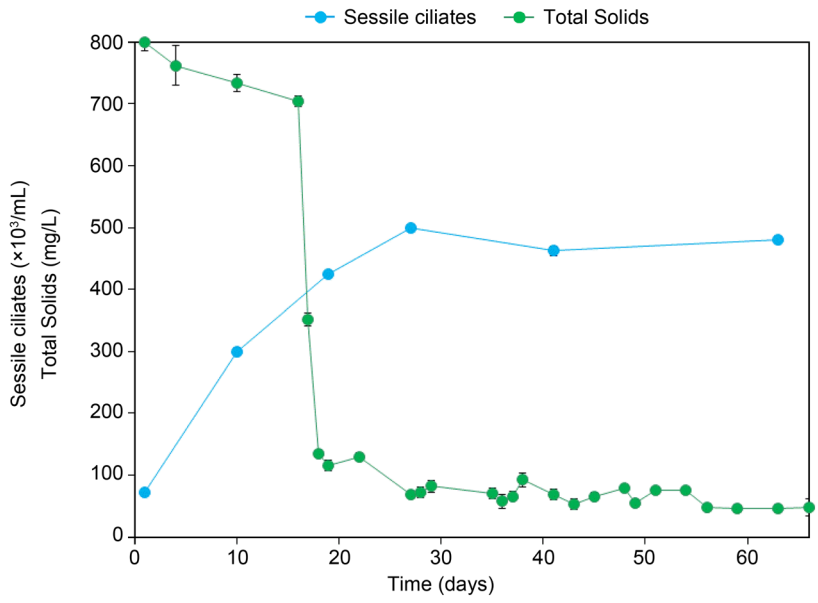

Fig. 4. Behavior of sessile ciliates with respect to the concentration of total solids.

of *Z. paraentzii* was the highest, while the densities of *E. plicatilis*, *V. campanula*, *V. convallaria*, and *Vorticella aquadulcis* were similar. Since the COD is indicative of the effluent quality and the efficiency of the removal of organic matter, the COD concentrations were measured in the influent and effluent (Fig. 3). They were in the ranges of 377–480 mg/l, and 38–122 mg/l, for days 10 to 60, respectively. During the first stage (days 1–20), protozoa/ml densities of 7.2×10^3 to 4.25×10^5 were obtained, with COD average removal efficiencies of 83.5%. In the second stage (days 21–60), the maximum density obtained was $5{,}0 \times 10^5$ protozoa/ml, with a mean removal efficiency of 88.7%. In the stabilization stage, efficiency percentages increased from 84.27 to 91.93%, while the protozoan density remained nearly constant ($4.63–5.0 \times 10^3$/ml).

The density of the sessile ciliates and total suspended solids (TSS) in the effluent were determined (Fig. 4), since suspended solids indirectly quantify the organic matter and the number of microorganisms

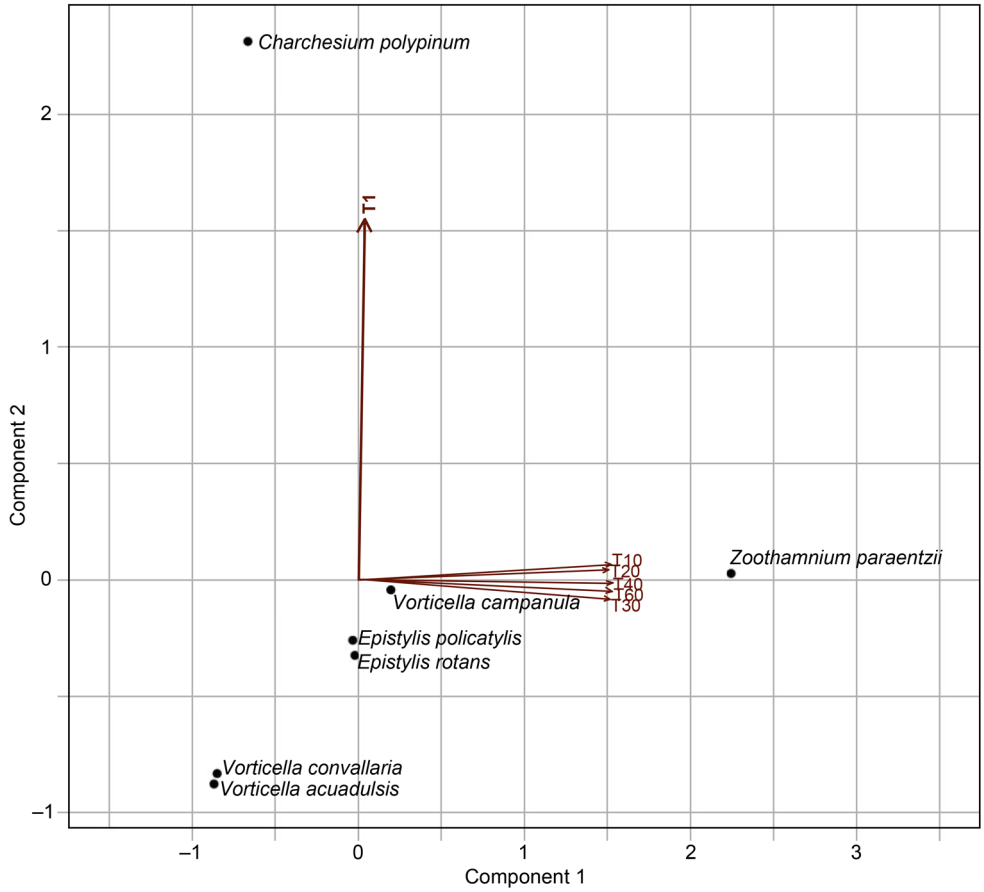

Fig. 5. Principal Component Analysis (PCA): relationship between the abundance of protozoa and other microorganisms, time, and removal percentage. Axes 1 and 2 account for 54.9% and 22.1% of the total variation presented, respectively.

(bacteria, protozoa, rotifers, and algae in some cases) in the effluent. A decrease in the TSS concentration suggests a decrease in the number of microorganisms and enhances water quality by diminishing the floating solids. At the beginning of the process (day 1), the TSS concentration in the effluent was higher than 700 mg/l. On the other hand, concentrations decreased to the range of 46–96 mg/l during the stabilization stage.

Principal Component Analysis (PCA). A PCA analysis was performed to determine the relationship between the functioning time of the reactor and the densities of diverse species of colonial and solitary peritrichous protozoa (Fig. 5). The first component of the PCA explains 80% of the data obtained, while the second one explains the 17%; thus, 97% of all the data obtained could be explained with these first two components. The analysis showed that *Z. paraentzii* had a strong correlation with the functioning time of the reactor. At the same time, species of colonial peritrichous protozoa like *V. campanula*, *E. plicatilis*, and *E. rotans* presented a moderate correlation with the functioning time of the reactor.

Tezontle in the SBR. The weight and porosity of tezontle were determined to assess the potential use of tezontle as supporting material (as a filler) in the SBR. The tezontle weight was calculated using Archimedes' principle, which provided a value of 2.537 ± 0.060 g/ml, with a porosity of 43.605 ± 2.046. Afterward, an SEM analysis of the tezontle was performed to observe its internal structure (Fig. 6). Tezontle showed a broad, porous diameter distribution (from tens to hundreds of micrometers) in the tezontle stone. Besides their size, the porous varied in shape and profundity among them.

Discussion

Both staining techniques proposed in the present study seemed economical, efficient, and quick options for staining protozoan nuclei of biological wastewater treatment systems, which could represent a relevant tool for identifying these microorganisms. Besides, all the microorganisms found in the SBR system using these novel staining methods have been earlier found in the systems of good removal efficiencies (> 90%), as full-scale operations activated sludge treatment plants, rotating biological contactors, and wetlands (Ginoris et al. 2007; Papadimitriou et al. 2010). Metazoans are highly sensitive to physical, chemical, and operational conditions (Ginoris et al. 2007); the metazoan

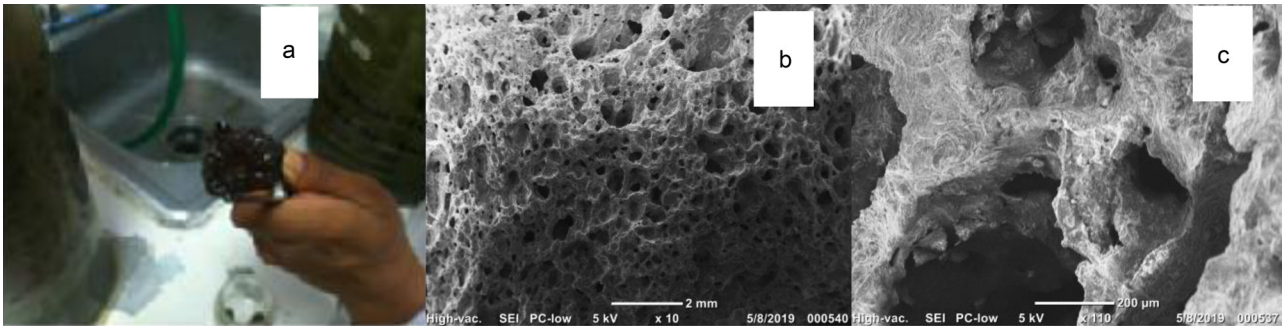

Fig. 6. Properties of tezontle as supporting material of the SBR. a) A piece of tezontle containing adhered peritrichous colonies; b) Scanning Electron Microscopy (SEM) of tezontle stones; c) closeup of tezontle pores.

identified *R. rotatoria* has been previously found in wetlands (Priya et al. 2007) and activated sludge systems (Ginoris et al. 2007).

As can be observed, *Z. paraentzii* (MFD technique) (Fig. S1) developed colonies with various zoids; in some cases, small colonies were shown, while other samples presented huge colonies with hundreds of zoids. A common dichotomous stalk connected the colonies, and the contractile spasmonema run uninterrupted throughout the colony, allowing contraction as a single unit. The body was highly variable in shape but usually elongated, measuring about 50–80 × 25–45 µm. The peristomial lip was single-layered, contractile vacuole apically located, and macronucleus generally C-shaped and horizontally oriented (Sun et al. 2005). It is important to note that the MFD staining technique proposed within this study is economical, quick, and allows the correct observation of the nucleus of *Z. paraentzii* for its identification. It represents a significant contribution, as in previous studies, only macronuclei drawings could be observed; other staining methods do not allow clear nuclei observations, so the identification of this microorganism could only be made at the genus level (Isac et al. 2008; Lynn 2010). This staining technique allows for obtaining permanent samples.

The MFD technique also allowed identifying *E. plicatilis* (Fig. 1d and Fig. S2); colonial ciliated protozoa with branching peduncles and without contractile capability (without myonema), whose zooids had a peristomial lip. They had dichotomous ramification colonies of up to 2–3 mm, with malleus-shaped zoids ranging from 70–90 mm, with nonhollow peduncle and horseshoe-shaped transversal macronucleus. It has been reported that the ciliated *Epistylis* spp. was related to the stabilization of activated sludge systems that work well with optimal loading (Isac et al. 2008).

On the other hand, the FD staining technique allowed *Carchesium polypinum* staining (Fig. S3) and, in agreement to Isac et al. (2008), its colony presented bell-shaped zoids, with independent and spiral contraction, peduncle without septa and with visible discontinuous spasmonema, dichotomous branches, and marked peristomal lip. The zoids' width was 46 µm, with a length of 72 µm. The colony showed a horseshoe-shaped macronucleus located at the top of the zoid. This kind of protozoa indicates that the sludge system of the plant is in optimal aeration conditions and has a high biological quality (Isac et al. 2008). Besides, nuclei of telotroch larvae were also efficiently stained with the proposed techniques: MFD and FD (Fig. S4). It is known that a telotroch is a mobile form that disperses when peritrichous protozoa divide. After the telotroch chooses a site, it begins to secrete a stalk from the scapula at the aboral pole of the body (Bradbury 1994).

The FD technique allowed the nuclei staining of *V. aquadulcis* (Fig. S5), which had an inverted cup-shaped zoid; this was a sessile organism with a contractile vacuole located in the upper part of the body, and its very elongated macronucleus was "C" shaped. This organism measured 55 µm long and 35 µm wide, and the diameter of the peristomatic lip was 30 µm (Isac et al. 2008; Lynn 2010). The FD staining technique also allowed the identification of *V. convallaria* (Fig. S6), which had a flared shape and a peristomial lip, with a width equal to or greater than that of the zoid, a fine and elongated peduncle (100–500 µm), a macronucleus in the form of "J", and a contractile vacuole in the anterior third of the cell (Isac et al. 2008). This microorganism is also related to transitory conditions (unstable or colonization), indicating nitrification absence in biological reactors (Martín-Cereceda et al. 2001). *V. campanula* is commonly associated with low organic loads (Isac et al. 2008, Küppers et al. 2020). It has been reported that the genus *Vorticella* has substantial environmental importance, like in the degradation of petroleum hydrocarbons in wastewater (Kachieng'a and Momba 2018), and the tolerance to heavy metals (Vilas-Boas et al. 2020).

The genus *Z. paraentzii* was also found to be one of the most abundant species in some phases of the process. Similar results were found in rotating biological contactors and activated sludge systems (Martín-Cereceda et al. 2002). This behavior is very interesting because tezontle conditions may have allowed the

growth of protozoa that are difficult to grow in the laboratory or have relevant biotechnological potential. Thus, this system could serve to grow colonial and solitary peritrichous ciliated protozoa, such as *Vorticella* sp. and *Epistylis* sp. that currently degrade hydrocarbons (Kachieng'a and Momba 2018), or some ciliated protozoa that may be used for the bioremediation of waters contaminated with heavy metals (Kumar et al. 2017; Vilas-Boas et al. 2020). Madoni (2010) mentioned that in a plant of activated sludge with an appropriate operation, the protozoan community is dominated by peritrichous specimens (*Vorticella* spp., *Carchesium* spp., *Zoothamnium* spp., *Epistylis* spp.) and hypotricks (*Aspidisca* spp., *Euplotes* spp.). In the case of amoeba, the genus *Euglypha* sp. was the most abundant, being these amoebae associated with good effluent quality (Ginoris et al. 2007). Additionally, the presence of the metazoan *Philodina* sp. is interesting because it has been reported that it is a rotifer that secretes polymeric extracellular substances that act as promoters of bacterial growth (Kachieng'a and Momba 2018).

The results regarding protozoan densities indicate an adaptation stage of the sessile protozoa. They could reflect one of the relevant advantages that tezontle may have: its porous structure, which seemed to allow the establishment of a microbial consortium. These results agree with those obtained from real-scale systems, such as wetlands, SBR, and activated sludge processes with advanced nitrogen-reducing systems (Papadimitriou et al. 2010). Besides, it has been reported that some protozoa are present in wastewater treatment systems, especially in activated sludge operations, rotating biological contactors, percolating filters, wetlands, and coastal areas (Ogleni et al. 2010; Papadimitriou et al. 2010; Charpentier 2014).

Overall, the results observed in the SBR system support previous reports that the most common protozoa in wastewater treatment systems are flagellates, free- swimming ciliates, crawling and sessiles, including the genera *Paramecium* sp., *Colpidium* sp., *Peranema* sp., *Tetrahymena* sp., *Euplotes* sp., *Aspidisca* sp., *Trachelophyllum* sp., *Vorticella* sp., *Epistylis* sp., *Difflugia* sp., *Arcella* sp., *Zoothamnium* sp., and *Carchesium* sp., among others (Ginoris et al. 2007; Canals et al. 2017). The observations also suggest a relationship between protozoan density and COD removal efficiencies in the SBR. During the growth stage, the removal efficiencies increased progressively from 71.02% to 89.08%, and the protozoan density increased from 7.2×10^4 protozoa/ml to 4.81×10^5 protozoa/ml. These results agree with those obtained by Priya et al. (2007) in continuously stirred anaerobic reactors that the density of ciliated protozoa was strongly related to the COD removal. The pH of the influent remained constant at 7.5 (data not shown), which is the same value reported by other researchers, such as Martín-Cereceda et al. (2001), who worked with a system of rotating biological contactors. They found species like *Zoothamnium* sp., *Vorticella* sp., *Epistylis* sp., *Euplotes* sp., and *Opercularia* sp. In the present study, the SBR worked in a range of 22–26°C throughout the experimentation, allowing bacteria and protozoa to grow.

Notably, the pH values and temperature were adequate with the metabolism of protozoa, thus promoting the correct functioning of the reactor. This observed behavior is logical since the sessile ciliated protozoa are bacterial and organic matter predators, so it can be inferred that when protozoan density increases, they feed on bacteria, and therefore TSS will diminish. This behavior is consistent with the fact that most protozoa were sessile attached to the support material (tezontle). Li et al. (2013) mentioned that *Vorticella* spp. and some rotifers are linked to the ingestion of fine particles, which positively affects the reduction of suspended solids. Also, the observed correlation in the PCA could be related to sessile protozoan stalks, which may have led to a better adhesion to the tezontle; these microorganisms may have had better adaptation opportunities and hence, presented an increase in their abundance. Besides, the PCA confirmed that free-living protozoa, amoebas, and metazoans (rotifers) always occurred in low abundance, indicating that they had no chance of survival compared to sessile protozoa (with stalk). Papadimitriou et al. (2010) stated that protozoan abundance could be used as a bioindicator of the treatment efficiency in constructed wetlands. In this regard, the present study elucidated a similar conclusion.

In addition, it is suggested that a greater abundance and diversity of protozoa may correlate with the effluent's good quality. It has also been mentioned that a correlation between the phosphorus and the rates of removal of total coliforms was observed in the presence of increased protozoan taxa, while the removal of the organic load and the inorganic nitrogen increased in the case of high protozoan diversity in the soil/water interface. Some authors have pointed out that each group of protozoa is associated with different factors influencing the process. For example, the ciliate group is related to good organic matter removal, while the flagellates are closely related to nitrogen elimination (Papadimitriou et al. 2010). The present study considers the high densities of the sessile protozoa (*E. rotans*, *E. plicatilis*, *Z. paraentzii*, *C. polypinum*, and *O. coarctata*) as high-quality effluent indicators (Li et al. 2017).

Additionally, the quick identification of the species present in the system (the proposed MFD and FD staining techniques) led determining the relationship between the presence of specific protozoan species and some system conditions, such as the quality of the effluent, removal efficiencies, and amount of organic matter. Previously, Li et al. (2017) also obtained a moderate

correlation between all protozoan communities and environmental parameters, such as the concentrations of ammonia nitrogen (NH^{4+}-N), total nitrogen (TN), total phosphorus (TP), and COD. Additionally, Xu et al. (2014) mentioned that the biofilms formed by the spatial patterns of the ciliated communities were significantly correlated with environmental variables, especially COD and nutrients, in coastal waters.

Concerning the support material used in this study, the "tezontle" word is derived from the Nahuatl "teztzontli", where "tezt" means stone and "zontli" means hair. Tezontle is a volcanic stone native to the State of Morelos, Mexico, which has a water retention capacity of 12.91–43.3%. In addition, its high porosity provides a large contact surface area, so it can be used as a substrate for many applications; the viability for the establishment of micro-bacterial colonies in tezontle stone due to its micropores has been previously reported (Liu et al. 2015). Besides, tezontle has good absorption properties and high mechanical resistance (Yáñez-Ocampo et al. 2011), which may be relevant for the region in which the study took place because Mexico has large tezontle deposits. The material could be used as a natural, environmental-friendly, and economic support for different wastewater treatment systems. Based on its characteristics, it may allow the adherence of protozoa, which may serve as indicators of different conditions in water treatment systems, besides promoting good removal efficiencies of organic and particulate matter (bacteria). The results obtained about the density and porosity of tezontle match very well with the density and porosity ranges reported before for this material (2.93 g/ml) (Li et al. 2017) and 55.5% (Rodríguez-Díaz et al. 2013), respectively. These characteristics are very important in absorption applications, as they represent the surface area and confirm that the tezontle's porosity might have been strongly related to the adhesion of the peritrichous ciliated protozoa. Also, it has been previously reported that tezontle mainly comprises of iron, aluminum, and silicon oxides, representing more than 70 wt % of its composition. It also contains magnesium, calcium, and sodium oxides (around 30 wt %). Kachieng'a and Momba (2018) obtained more than 90% of COD removal after 20 days, This percentage was obtained due to their sessile and free nature and the interaction among the protozoan isolates (consortium).

On the other hand, Nacheva et al. (2008) observed more than 95% biodegradation (COD removal) when activated carbon and tezontle were used as biofilm supports in anaerobic biofilters. Specifically, more than 95% biodegradation was obtained with both support materials at organic loads lower than 1.7 $kg/m^3 \times d$ in tezontle, and with loads of up to 13.3 $kg/m^3 \times d$ in granulated activated carbon. In the present research, the density of the colonial and solitary peritrichous ciliates was much higher than that of free-living ciliates during the process. It suggests that the silicon oxide contained in the tezontle promotes good conditions for the growth of sessile ciliates. Some organisms, from protists to sponges, employ silicon sources to build internal or external skeletons and/or scale structures (Perry et al. 2003; Foissner et al. 2009). Moreover, according to Foissner et al. (2009), silicon granulates regulate light perception and are a protective mechanism against mechanical stress and protozoan predation.

Some studies show the suitability of using packaging materials (known as carriers) to immobilize microorganisms to obtain high removal efficiencies of water contaminants; such materials include tezontle, bagasse, sawdust, coconut fiber, and cotton fiber, among others. However, these packaging materials have been mainly used with bacteria, algae, and fungi but not with protozoa (Dzionek et al. 2016). In contrast to publications on the association of bacteria and algae with substrates, knowledge about the behavior of protozoa associated with substrates is scarce, although protozoa occur in high numbers in biofilms (Arndt et al. 2003). It has been mentioned that packaging materials for wastewater treatment purposes should possess specific characteristics present in tezontle, like being insoluble, non-toxic (for the system and the environment), accessible, economical, stable, and appropriate for regeneration. The matrices used for adsorption or attachment should be of high porosity to ensure the high contact area, as has been determined for tezontle. Besides, for wastewater treatment processes, packaging materials must have high mechanical resistance, as they can be exposed to diverse types of physical stress factors (Dzionek et al. 2016).

Thus, the activity of protozoa in the present study seems to be influenced by the presence of the tezontle. On one side, this substrate has the appropriate characteristics (theoretically and experimentally established) to be used as a suitable packaging material. On the other side, protozoa, as the sessile pedunculated ones, survive on various substrates like solid-air (soil grains, rocks), water-air, or solid-water (stones, macrophytes, animals, leaf litter, etc.) (Arndt et al. 2003). The protozoa's capability to adhere, colonize substrates, or temporally separate from biofilms provides them with clear advantages because food concentration (bacteria, algae, and other protists) may be significantly higher than in the surrounding water, and biofilms can serve as a refuge against predation (Arndt et al. 2003).

The proposed nuclei staining techniques for colonial and solitary peritrichous ciliated protozoa were simple, fast, and economical. Both techniques seem reproducible and reliable, allowing the observation of well-defined nuclei in all the cases evaluated and identifying the most abundant species that colonized tezontle in

the system. On the other hand, tezontle is an economical, natural, and abundant material in Mexico; it has a large number of pores and, therefore, a large surface area that allowed good adhesion of bacteria and peritrichous protozoa, consequently obtaining good removal efficiencies of organic matter (91.93%). Therefore, the present work confirmed that tezontle is an economical material with favorable composition and porosity for the abundant growth of solitary and colonial peritrichous ciliated protozoa, indicators of the excellent quality of treated wastewater.

The protozoan species that grew in the system could later be used to degrade toxic compounds, such as hydrocarbons or metals. Ciliated protozoa with peduncles, like *Z. paraentzii*, *E. plicatilis*, *V. campanula*, and *E. rotans*, showed a moderate to strong relationship with the functioning time of the reactor, which allowed the obtention of high removal efficiencies of both organic matter and suspended bacteria. The novel FMD and FD staining techniques highlighted the nuclei in all solitary and colonial peritrichous protozoa. It helped to identify them at the species level; therefore, they could be used for protozoan identification or as complementary techniques to those already existing for staining nuclei of species resistant to other dyes.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

Acevedo-Davila J, Torres-Trevino LM, Lauren Y, Gómez Z. Tezontle aggregate substitute optimization in building blocks mixture. Paper presented at: Electronics, Robotics and Automotive Mechanics Conference (CERMA 2007); 2007 September 25–28; Cuernavaca, Mexico. p. 307–311. https://doi.org/10.1109/cerma.2007.4367704

Arndt H, Schmidt-Denter K, Auer B, Weitere M. Protozoans and biofilms. In: Krumbein WE, Paterson DM, Zavarzin GA, editors. Fossil and recent biofilms. Dordrecht (Netherlands): Springer; 2003. p. 173–189. https://doi.org/10.1007/978-94-017-0193-8_10

Baird RB, Eaton AD, Rice EW. Standard methods for the examination of water and wastewater. Washington DC (United States): American Public Health Association; 2017. p. 2–68–10–17.

Azovsky AI, Mazei YA. Diversity and distribution of free-living ciliates from high-arctic Kara Sea sediments. Protist 2018 Apr; 169(2):141–157. https://doi.org/10.1016/j.protis.2018.01.001

Beltran FME, Otarola MJ, Tarqui KT. [Laboratory procedures for feces diagnosis] (in Spanish). In: Zavaleta Martinez-Vargas A, editor. [Manual of laboratory procedures for the diagnosis of intestinal parasites in humans] (in Spanish). Lima (Peru): National Institute of Health; 2014. p. 9–35.

Bradbury PC. Ciliates of fish. In: Kreier JP, editor. Parasitic protozoa. Massachusetts (USA): Elsevier; 1994. p. 81115.

Canals O, Massana R, Riera JL, Balagué V, Salvadó H. Microeukaryote community in a partial nitritation reactor prior to anammox and an insight into the potential of ciliates as performance bioindicators. N Biotechnol. 2018 Jul 25;43:3–12. https://doi.org/10.1016/j.nbt.2017.05.003

Charpentier J. [Treatment of residual waters with activated sludges] (in Spanish). Buenos Aires (Argentina): National Institute of Industrial Technology; 2014. p. 1–43.

Dubber D, Gray NF. Enumeration of protozoan ciliates in activated sludge: Determination of replicate number using probability. Water Res. 2009 Aug;43(14):3443–3452. https://doi.org/10.1016/j.watres.2009.05.006

Dzionek A, Wojcieszynka D, Guzik U. Natural carriers in bioremediation: A review. Electron J Biotechnol. 2016 Sep;23:28–36. https://doi.org/10.1016/j.ejbt.2016.07.003

EPA. Wastewater, technology fact sheet: sequencing batch reactor. Office of Water, Washington DC (USA): United States Environmental Protection Agency; 1999 [cited 2022 Apr 29]. Available from https://www3.epa.gov/npdes/pubs/sbr_new.pdf

Foissner W, Weissenbacher B, Krautgartner WD, Lütz-Meindl U. A cover of glass: first report of biomineralized silicon in a ciliate, *Maryna umbrellata* (Ciliophora: Colpodea). J Eukaryot Microbiol. 2009 Nov–Dec;56(6):519–530. https://doi.org/10.1111/j.1550-7408.2009.00431.x

Ginoris YP, Amaral AL, Nicolau A, Coelho MA, Ferreira EC. Development of an image analysis procedure for identifying protozoa and metazoa typical of activated sludge system. Water Res. 2007 Jun;41(12):2581–2589. https://doi.org/10.1016/j.watres.2007.02.006

Isac L, Rodriguez E, Salas L, Fernández N. [Atlas of protists and metazoans present in activated sludge] (in Spanish). Paper presented at: Jornada Internacional de Tratamiento y Reutilización en Aguas Residuales; 2008 June 5; Sevilla, Spain. p. 1–76.

Kachieng'a L, Momba MNB. The synergistic effect of a consortium of protozoan isolates (*Paramecium* sp., *Vorticella* sp., *Epistylis* sp. and *Opercularia* sp.) on the biodegradation of petroleum hydrocarbons in wastewater. J Environ Chem Eng. 2018 Aug;6(4):4820–4827. https://doi.org/10.1016/j.jece.2018.07.005

Kumar N, Saini R, Chaudhary K, Bansal M, Kanika, Pandey N. Correlation between toxic stress by heavy metal toxicants and periphytic protozoan colonization rates. Int J Adv Res. 2017 May;5(5): 529–534. https://doi.org/10.21474/IJAR01/4140

Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016 Jul;33(7):1870-1874. https://doi.org/10.1093/molbev/msw054

Küppers GC, Kosakyan A, Siemensma F, Claps MC, da Silva Paiva T, Fernández LD, Burdman L, Krashevska V, Lara E, Damborenea C.

Protozoa. In: Thorp JH and Rogers CD, editors. Amsterdam (Netherlands): Elsevier; 2020. p. 9–77.
https://doi.org/10.1016/B978-0-12-804225-0.00002-2
Li J, Ma L, Wei S, Horn H. Aerobic granules dwelling vorticella and rotifers in an SBR fed with domestic wastewater. Sep Purif Technol. 2013 June;110:127–131. https://doi.org/10.1016/j.seppur.2013.03.022
Li YD, Chen Y, Wang L, Yao L, Pan XM, Lee DJ. Pollution tolerant protozoa in polluted wetland. Bioresour Technol. 2017 Sep;240: 115–122. https://doi.org/10.1016/j.biortech.2017.02.051
Liao CC, Shin JW, Chen LR, Huang LLH, Lin WC. First molecular identification of *Vorticella* sp. from freshwater shrimps in Tainan, Taiwan. Int J Parasitol Parasites Wildl. 2018 Oct 11;7(3):415–422. https://doi.org/10.1016/j.ijppaw.2018.10.002
Liu J, Chen S, Ding J, Xiao Y, Han H, Zhong G. Sugarcane bagasse as support for immobilization of *Bacillus pumilus* HZ-2 and its use in bioremediation of mesotrione-contaminated soils. Appl Microbiol Biotechnol. 2015 Dec;99(24):10839–10851.
https://doi.org/10.1007/s00253-015-6935-0
Lynn DH. Phylum CILIOPHORA – conjugating, ciliated protists with nuclear dualism. In: Lynn DH, editor. The ciliated protozoa. Dordrecht (Netherlands): Springer; 2010. p. 89–120.
https://doi.org/10.1007/978-1-4020-8239-9_4
Madoni P. Protozoa in wastewater treatment processes: A minireview. Ital J Zool. 2010 Mar;78(1):3–11.
https://doi.org/10.1080/11250000903373797
Martín-Cereceda M, Pérez-Uz B, Serrano S, Guinea A. Dynamics of protozoan and metazoan communities in a full scale wastewater treatment plant by rotating biological contactors. Microbiol Res. 2001;156(3):225–238. https://doi.org/10.1078/0944-5013-00105
Martín-Cereceda M, Zamora J, Pérez-Uz B, Guinea A. Ciliate communities of rotating biological contactor biofilms: A multivariate approach. Syst Appl Microbiol. 2002 Aug;25(2):301–313.
https://doi.org/10.1078/0723-2020-00090
Nacheva PM, Moeller Chávez G, Bustos C, Garzón Zúñiga MA, Hornelas Orozco Y. Comparison of bioreactors with different kinds of submerged packed beds for domestic wastewater treatment. Water Sci Technol. 2008;58(1):29–36.
https://doi.org/10.2166/wst.2008.321
Ogleni N, Topaloglu D, Dere T, Ileri R. Microbiological investigation of activated sludge in sequencing batch bioreactor for urban wastewater treatment. J Sci Ind Res. 2010 Aug;69(8):643–646.
Papadimitriou CA, Papatheodoulou A, Takavakoglou V, Zdragas A, Samaras P, Sakellaropoulos GP, Lazaridou M, Zalidis G. Investigation of protozoa as indicators of wastewater treatment efficiency in constructed wetlands. Desalination 2010 Jan;250(1):378–382.
https://doi.org/10.1016/j.desal.2009.09.060
Pérez-Uz B, Arregui L, Calvo P, Salvadó H, Fernández N, Rodríguez E, Zornoza A, Serrano S. Assessment of plausible bioindicators for plant performance in advanced wastewater treatment systems. Water Res. 2010 Sep;44(17):5059–5069.
https://doi.org/10.1016/j.watres.2010.07.024
Perry CC, Belton D, Shafran K. Studies of biosilicas; structural aspects, chemical principles, model studies and the future. In: Müller WEG, editor. Silicon Biomineralization. Progress in Molecular and Subcellular Biology. Berlin, Heidelberg (Germany); Springer: 2003(33). p. 269–299. https://doi.org/10.1007/978-3-642-55486-5_11
Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. Bioinformatics. 1998;14(9):817–818.
https://doi.org/10.1093/bioinformatics/14.9.817
Priya M, Haridas A, Manilal VB. Involvement of protozoa in anaerobic wastewater treatment process. Water Res. 2007 Dec;41(20): 4639–4645. https://doi.org/10.1016/j.watres.2007.06.047
Rakshit D, Biswas SN, Sarkar SK, Bhattacharya BD, Godhantaraman N, Satpathy KK. easonal variations in species composition, abundance, biomass and production rate of tintinnids (Ciliata: Protozoa) along the Hooghly (Ganges) River Estuary, India: A multivariate approach. Environ Monit Assess. 2014 May;186(5):3063–3078.
https://doi.org/10.1007/s10661-013-3601-9
Rehnam A. Heavy metals uptake by *Euglena proxima* isolated from tannery effluents and its potential use in wastewater treatment. Russ J Ecol. 2011 Jan;42:44–49.
https://doi.org/10.1134/S1067413611010085
Robertson LJ, Clark CG, Debenham JJ, Dubey JP, Kváč M, Li J, Ponce-Gordo F, Ryan U, Schares G, Su C, et al. Are molecular tools clarifying or confusing our understanding of the public health threat from zoonotic enteric protozoa in wildlife? Int J Parasitol Parasites Wildl. 2019 Feb 13;9:323–341.
https://doi.org/10.1016/j.ijppaw.2019.01.010
Rodríguez-Díaz E, Salcedo-Pérez E, Rodríguez-Macias R, González-Eguiarte DR, Mena-Munguía S. [Tezontle reuse: effect on physical properties and tomato *(Lycopersicon esculentum* Mill) production] (in Spanish). Terra Latinoam. 2013 Oct;31(4):275–284.
http://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S0187-57792013000500275&lng=es&nrm=iso
Sun P, Ji D, Song W. Notes on a new marine peritrichous ciliate (Ciliophora: Peritrichida), *Zoothamnium xuianum* n. sp., with redescription of *Z. paraentzii* Song, 1991 from northern China. Zootaxa 2005 Nov;1075(1):41–53. https://doi.org/10.11646/zootaxa.1075.1.2
Sun P, Clamp JC, Xu D, Huang B, Shin MK, Turner F. An ITS-based phylogenetic framework for the genus *Vorticella*: finding the molecular and morphological gaps in a taxonomically difficult group. Proc Biol Sci. 2013 Oct 2;280(1771):20131177.
https://doi.org/10.1098/rspb.2013.1177
Vilas-Boas JA, Cardoso SJ, Senra MVX, Rico A, Dias RJP. Ciliates as model organisms for the ecotoxicological risk assessment of heavy metals: A meta-analysis. Ecotoxicol Environ Saf. 2020 Aug;199: 110669. https://doi.org/10.1016/j.ecoenv.2020.110669
Yáñez-Ocampo G, Sánchez-Salinas E, Ortiz-Hernández ML. Removal of methyl parathion and tetrachlorvinphos by a bacterial consortium immobilized on tezontle-packed up-flow reactor. Biodegradation. 2011 Nov;22(6):1203–1213.
https://doi.org/10.1007/s10532-011-9475-z
Xu H, Zhang W, Jiang Y. Do early colonization patterns of periphytic ciliate fauna reveal environmental quality status in coastal waters? Environ Sci Pollut Res Int. 2014;21(11):7097–7112.
https://doi.org/10.1007/s11356-014-2615-3

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