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# Morphology, Biometry, Ecology and Biogeography of Five Species of *Difflugia* Leclerc, 1815 (Arcellinida: Difflugiidae), from Tiete River, Brazil

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**Summary.** *Difflugia corona* Wallich, 1864; *D. gramen* Penard, 1902; *D. lanceolata* Penard, 1890; *D. claviformis* Penard, 1899 and *D. gigantea* Chardez, 1967 collected at the Tiete River Ecological Park (Brazil) are redescribed and compared to previous descriptions regarding morphology, biometry, ecology and geographical distributions. The biometrical measures and analysis based on a large number of individuals shows that natural populations of testate amoebae may be highly variable. Distinguishing traits for each species are discussed. The five *Difflugia* species inhabit quite a large range of micro habitats. The geographic distribution for each species is reviewed.

**Key words:** biometry, ecology, *Difflugia*, geographic distribution, morphology

**Abbreviations used:** ad - aperture diameter, al - number of apertural lobes, desc - description of the taxon, dim - dimensions of the test, eco - ecological associations, geo - geographic distribution, LM - light microscopy, ns - number of spines, SEM - scanning electron microscope, sl - spine length, syn - synonymic list, td - test diameter, tl - test length.

## INTRODUCTION

The genus *Difflugia* Leclerc, 1815 is well defined as a genus of testate amoebae (Protista), but there is considerable uncertainty as to what distinguishes the species. There are descriptions of about 300 species and 200 subspecific and infrasubspecific taxa (Ogden 1983, Meisterfeld 2000). These descriptions were made mostly by light microscopy which does not allow detailed comparisons of test structure, form and composition (Ogden and Živković 1983). The identity of species is unclear partly because few species have types. Gauthier-Lièvre and Thomas (1958), in the most complete survey of the genus (Ogden 1983), described 129 species based on African specimens. Forty-five taxa were new to science, but none had a designated type. Few lists of synonyms have been compiled.

There have however been a number of improvements. The application of electron microscopy to protistology from the early 1960's, has improved the descriptions of protists (Patterson 1994, 1999; Patterson and

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Brugerolle 1988). Scanning electron microscopy allows more detailed examination of many characters of testate amoebae and more consistent definitions at the species level (Ogden 1983, Ogden and Živković 1983, Meisterfeld 2000). *Difflugia* has benefited from this approach (Ogden 1979, 1980a, b, 1983, 1990; Ogden and Fairman 1979; Ogden and Hedley 1980; Ogden and Živković 1983; Ogden and Meisterfeld 1989; Dekhtyar 1993). Finally, biometric analysis has been used increasingly to characterize species (Schönborn *et al.* 1983, Bobrov *et al.* 1999, Bobrov and Mazei 2004).

Good taxonomic work, redescribing and establishing the synonymies of poorly described species (Bobrov and Mazei 2004), is important to understand other aspects of the biology of these organisms such as their distribution, dispersal, ecology, and will help solve issues such as species concepts (Schlegel and Meisterfeld 2003, Bobrov and Mazei 2004).

Studies of testate amoebae to date emphasize species from Europe and Africa. Yang and colleagues have extended the observations to China (e.g.: Yang *et al.* 2004, Yang and Shen 2005) applying ultrastructural characters, morphometry and ecology. There are few studies on testate amoebae from South America and most have been conducted using LM only (e.g.: Boltovskoy 1956; Boltovskoy and Lena 1966, 1971; Dioni 1970; Vucetich 1970, 1972, 1973, 1978). There have been a few studies on the genus *Difflugia* from Brazil (Velho and Lansac-Tôha 1996, Lansac-Tôha *et al.* 2001).

The present paper adds data on five species of the genus *Difflugia* from the much polluted waters of the Tiete River. These observations extend species circumscriptions, and bring new light to bear on the morphology, biometry, ecology and distribution of *Difflugia*.

## MATERIALS AND METHODS

Samples were taken from two localities in the Ecological Park of the Tiete River, Sao Paulo - Brazil in February (summer) and August (winter) 2004. The first locality was in the River itself (23°29'374"S 46°31'500"WO), a lotic environment, and the second was at a marginal lake (23°29'055"S 46°30'939"WO), a lentic environment. In both localities, two sampling methods were used: for organisms associated to the bottom sediment, 2 liters of the top 5 cm of the bottom sediment and water were collected and fixed using 4% neutralized formaldehyde. For organisms associated to the roots of aquatic plants, the method of Dioni (1967, 1970) was adapted in which 2 liters of water containing *Azolla* sp. and *Salvinia* sp. were collected, roots removed and fixed with 4% neutralized formaldehyde.

Collected materials were filtered through a series of sieves and the 40 µm to 500 µm size fraction was preserved in alcohol 70% for analysis. Sub-samples were placed in Petri dishes and stained with Bengal's Rose. Testate amoebae were separated from sediment under a stereomicroscope. Detailed analysis and measures were obtained using dissecting and compound microscopes. All procedures were done by the same person, following the recommendations of Patterson and Lee (2000) and Finlay *et al.* (2001).

The scanning electron microscopy procedures follow Ogden and Hedley (1980) and Yang *et al.* (2004). A diversity of individuals were selected and cleaned by successive transfers through distilled water and placed on a glass slide to dry. The individuals were transferred, using the static electricity of a single hair brush, to an aluminum-stub covered with adhesive from commercial adhesive tape dissolved in chloroform. The cells were oriented as required. Specimens were covered by a thin gold layer. The materials were analyzed and photographed using a Zeiss DSM 940 scanning electron microscope operating at 10 kV, at the Electronic Microscopy Laboratory, Institute of Biosciences, University of Sao Paulo (IBUSP). Materials are deposited at Laboratorio de Malacologia (IBUSP).

The terminology in testate amoebae is poorly established (Foissner and Korganova 2000). The present paper uses the following standard for descriptions: form of the test, composition of the test, pattern of the cementing organic matrix, color of the test, texture of test's surface, apertural complex (structures that delimit and surrounds the aperture). Polarity: apertural region and abapertural region (Fig. 1) is suggested terminology to substitute the use of oral and aboral. The present paper also uses anterior to refer to the apertural end of the cell, and posterior, regarding abapertural.

The review of the literature includes data previously reported. Some of this has been reported under different names. The abbreviations in parenthesis indicate the nature of the information given. Comprehensive tables regarding morphometry, localities and ecology have been compiled by the authors and are available upon request.

For all five species, three morphometric characters, as indicated in Fig. 1, were measured: test length, test diameter and aperture diameter. For *Difflugia corona*, two additional characters were measured: number of spines (ns) and spine length. Statistics were performed using the program STATISTICA 6.0.

## RESULTS

### *Difflugia corona* Wallich, 1864

Synonyms: *Difflugia proteiformis* Ehrenberg subspecies *globularis* Dujardin variety *corona* Wallich 1864, *Difflugia corona ecornis* Gauthier-Lièvre et Thomas, 1958 (published as *Difflugia corona* var. *ecornis*) *syn. nov.*

Data previously reported: *Difflugia proteiformis* Ehrenberg *globularis* Dujardin var. *corona* Wallich 1864:240-241 pl. 15, figs. 4b, c (desc); *Difflugia corona* Daday 1905:13-14 (desc, dim, geo); Deflandre 1926:523-525 (desc, dim, geo, syn); Štepánek 1952:16-17 (desc,

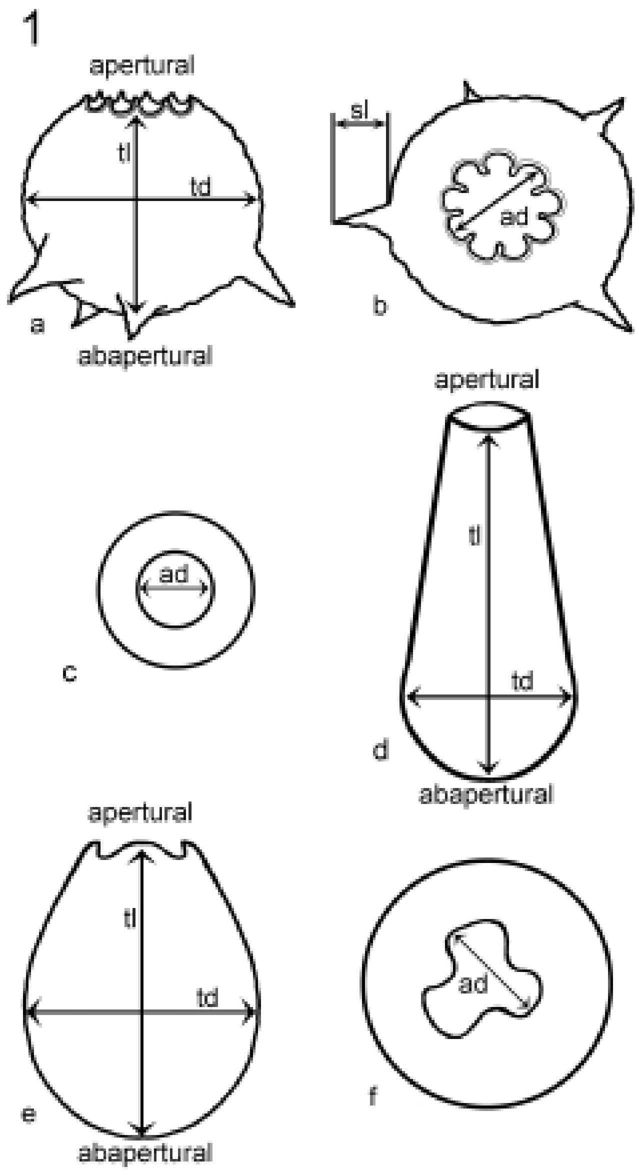



**Fig. 1.** Schematic outline of tests, showing position of measured axis and polarity. Not drawn in scale. *Difflugia corona* a - side view, b - apertural view; generalized pyriform *Difflugia*, c - apertural view, d - side view; *Difflugia gramen* e - side view, f - apertural view. tl - test length, td - test diameter, ad - aperture diameter, sl - spine length.

dim); Boltovskoy 1956:307-308 fig. 5 (desc, geo, syn); Gauthier-Lièvre and Thomas 1958:254-256, fig. 2a (desc, dim, eco, geo); Closs and Madeira 1962:8-9 (desc, dim, geo, syn); Closs and Madeira 1967:14 (desc, dim, geo); Vucetich 1972:277-278 (desc, dim, eco, geo, syn); Vucetich 1973:301 (desc, dim, eco, geo, syn); Ogden and Hedley 1980:128-129, figs. A-D (desc, dim, geo, SEM); Ogden and Živković 1983:346-348, fig. 4a-d (desc, dim, eco, geo, SEM); Medioli and Scott 1985:27 fig. 2a, b (desc, syn); Dekhtyar 1993:11-13, figs 4a-f (not *Protocucurbitella coroniformis* Gauthier-Lièvre and Thomas 1960, desc, dim, eco, geo, syn, SEM); Torres and Jebram 1994:72 (desc, dim, eco); Velho and Lansac-Tôha 1996:179-181 figs. 1, 1a (desc, dim, geo); Blanco 2001 (desc, dim); Zapata *et al.* 2002:24 (desc, dim, geo); Bobrov and Mazei 2004:136 (dim); *Difflugia corona ecornis* - Gauthier-Lièvre and Thomas 1958:256-258, fig. 3 (published as *Difflugia corona* var. *ecornis*)

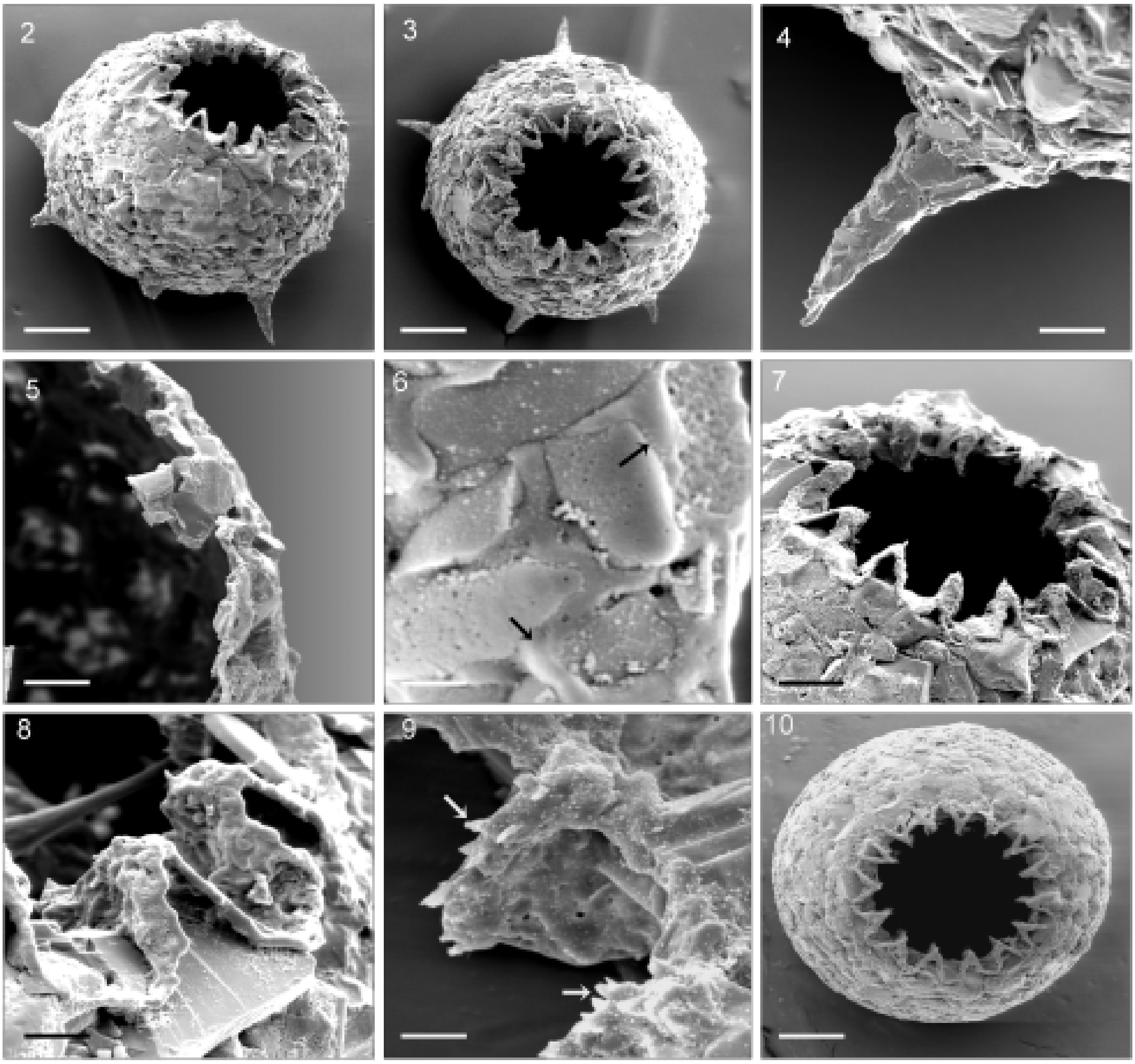
Examined material: 683 specimens by LM, 26 by SEM; 42 micrographs taken by Colin G. Ogden deposited at the Natural History Museum, London (NHM)

**Morphology.** Test sub-spherical to spherical (Figs 1a, 2). Circular in apertural view (Figs 1b, 3). Variable number (0-10) of conical hollow spines (Fig. 4) inserted from the mid-line to the abapertural end (Figs 2, 3). Surface of test smooth (Fig. 5), component aggregated particles flat, rarely breaking the circular contour. Test walls even in thickness (Fig. 5), except for a thicker aperture. Whole test composed of fine sand granules, rounded particles of quartz and cylindrical structures of exogenous nature (Figs 5, 6) all agglutinated by smooth organic cement (Fig. 6). Inside of test covered by organic matrix.

Aperture terminal, circular, with 9 to 15 denticulate lobes (Fig. 7). Apertural complex delimited by a particulate outline of the test and a rim of organic cement. Particles delimit a somewhat circular border, and an organic matrix covers this free edge to form lobes (Fig. 8). Apertural cover thick, each lobe is a hollow structure at the outer most part. Each lobe extends to the immediate inner surface of the test as a solid column, observed by LM as rod-like structures perpendicular to the outline of the aperture. Using SEM, it is possible to see that each lobe is provided, on the edge of each column, with one or two rows of spiny protuberances. These vary in number, shape and length (around 3-4 µm) from lobe to lobe (Fig. 9). The protuberances can be randomly distributed, not necessarily organized in rows.

**Biometry.** All measurements (Table 1) are highly variable (CV = 25.2-67.0). The number of conical spines presents the lowest standard error of the mean (0.11).

Size frequency distribution analyses indicate that *D. corona* has a main-size class and a large size-range for all characters. The smaller tests are less than a third of the size of bigger ones. The test height varies from 80 to 330 µm, but 58% are within the limits of 160 to 240 µm. The frequency analysis of test diameter gives




**Figs 2-10.** SEM micrographs of *Difflugia corona*. **2** - oblique apertural view of *D. corona*, showing test and spine form, and apertural outline; **3** - apertural view of *D. corona*, showing circular outline of the aperture; **4** - detail of one of the conical spines of *D. corona*; **5** - detail of test wall, this specimen was intentionally broken to show that walls are even in thickness; **6** - detail of agglutination in one of the spines, exactly as in all other areas of the test. Arrows indicate areas where the cement organic matrix is visible; **7** - detail of the aperture, completely outlined by organic material; **8** - detail of a denticulate lobe. Notice that the aperture is roughly defined by particle deposition, and the fine outline is made only by organic matrix; **9** - edge of denticulate lobe as seen from the inside of the test. Arrows indicate the spiny protuberances that are inserted at the outer-most edge of the lobe column; **10** -apertural view of *Protocucurbitella coroniformis ecornis*, showing test form, aperture outline and lack of spines. Scale bars: 60 µm (2, 3); 15 µm (4); 10 µm (5,8); 2 µm (6); 30 µm (7); 5 µm (9); 45 µm (10).

similar results. In regard to aperture diameter, the main-size class range is even smaller, where 67% of measurements are within the limits of 70-120 µm. The size of the conical spines varies from 20 to 40 µm in 70% of the analyzed tests. The number of spines varies from 0 to 10, but in 86.4 % of the tests is restricted to 3-5.

All measurements of *D. corona* are positively correlated to each other (Table 2), except for number of

**Table 1.** Biometric characterization of the investigated *Difflugia* species from Tiete River. Characters are as designated in Fig 1. Measurements in µm. $\bar{X}$ - arithmetic mean, M - median, SD - standard deviation, SE - standard error of the mean, CV - coefficient of variation in %, Min - minimum, Max - maximum, n - number of investigated specimens.

| Characters | $\bar{X}$ | Median | SD | SE | CV | Min | Max | n |
|---|---|---|---|---|---|---|---|---|
| *Difflugia corona* | | | | | | | | |
| tl | 192.1 | 200.0 | 48.36 | 1.85 | 25.2 | 80.0 | 380.0 | 683 |
| td | 189.8 | 200.0 | 48.76 | 1.87 | 25.7 | 80.0 | 360.0 | 683 |
| ad | 91.6 | 100.0 | 26.22 | 1.00 | 28.6 | 30.0 | 200.0 | 683 |
| sl | 37.5 | 40.0 | 12.07 | 0.49 | 32.2 | 10.0 | 100.0 | 607 |
| ns | 3.0 | 4.0 | 2.03 | 0.11 | 67.0 | 0.0 | 10.0 | 337 |
| *Difflugia gramen* | | | | | | | | |
| tl | 108.0 | 100.0 | 20.21 | 1.46 | 18.7 | 80.0 | 160.0 | 192 |
| td | 92.3 | 80.0 | 29.07 | 2.10 | 31.5 | 50.0 | 160.0 | 192 |
| ad | 38.0 | 40.0 | 10.94 | 0.79 | 28.7 | 20.0 | 80.0 | 192 |
| *Difflugia lanceolata* | | | | | | | | |
| tl | 174.8 | 180.0 | 26.37 | 2.16 | 15.1 | 80.0 | 240.0 | 149 |
| td | 75.1 | 80.0 | 13.83 | 1.13 | 18.4 | 40.0 | 120.0 | 149 |
| ad | 39.6 | 40.0 | 6.84 | 0.56 | 17.3 | 20.0 | 80.0 | 149 |
| *Difflugia claviformis* | | | | | | | | |
| tl | 226.5 | 220.0 | 65.05 | 6.12 | 28.7 | 110.0 | 380.0 | 113 |
| td | 88.0 | 80.0 | 24.27 | 2.28 | 27.6 | 60.0 | 200.0 | 113 |
| ad | 49.3 | 40.0 | 11.83 | 1.11 | 24.0 | 40.0 | 90.0 | 113 |
| *Difflugia gigantea* | | | | | | | | |
| tl | 380.0 | 420.0 | 78.10 | 26.03 | 20.6 | 280.0 | 460.0 | 9 |
| td | 204.4 | 200.0 | 32.83 | 10.94 | 16.1 | 180.0 | 280.0 | 9 |
| ad | 86.7 | 80.0 | 14.14 | 4.71 | 16.3 | 80.0 | 120.0 | 9 |

**Table 2.** Correlation coefficient between morphometric characteristics in the studied *Difflugia* species from Tiete River. Characters are as designated in Fig 1. n - number of studied specimens, NA - non applicable.

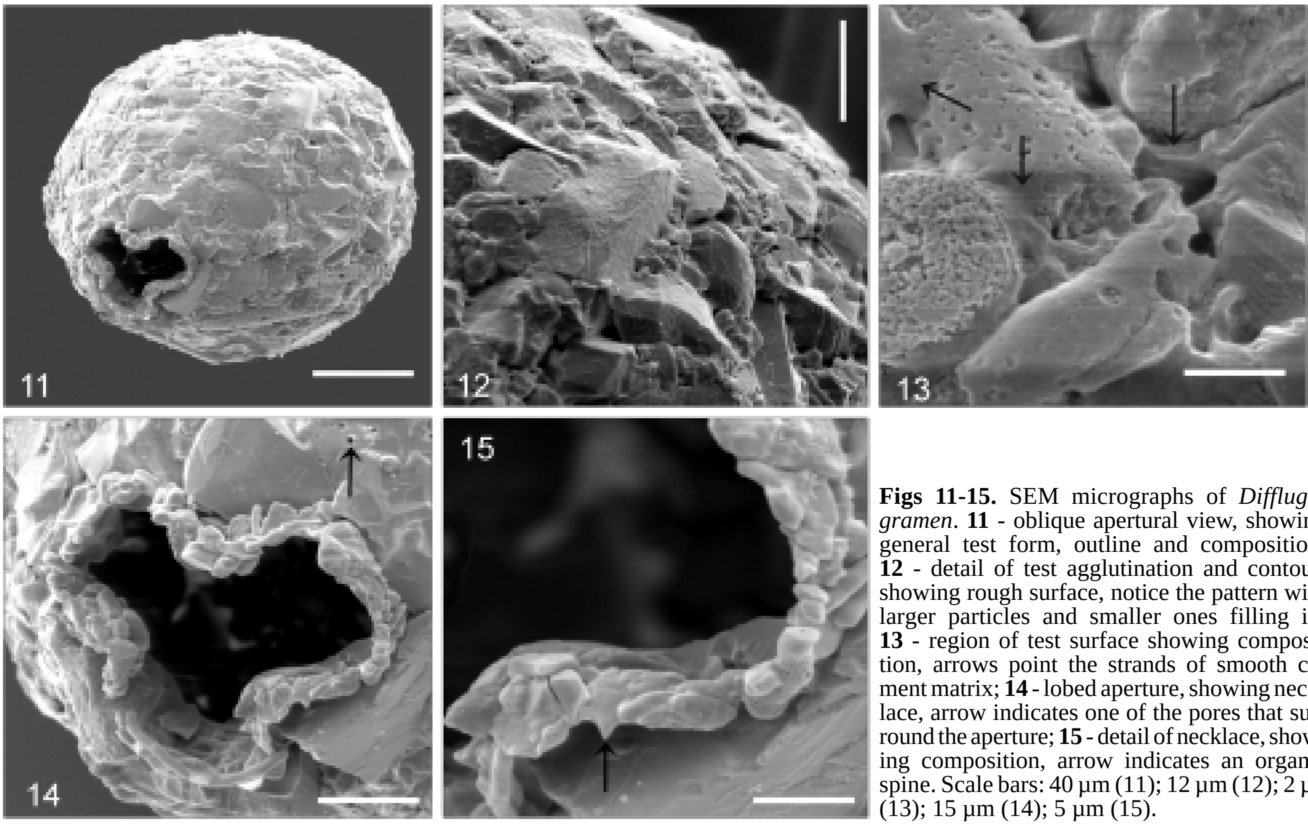
| Species | tl-td | tl-ad | td-ad | sl-tl | sl-td | sl-ad | ns-tl | ns-td | ns-ad | ns-sl |
|---|---|---|---|---|---|---|---|---|---|---|
| *Difflugia corona* (n=♣) | 0.93* | 0.84* | 0.85* | 0.41* | 0.44* | 0.42* | 0.16** | 0.12$^{NS}$ | 0.17** | 0.18$^{NS}$ |
| *Difflugia gramen* (n=192) | 0.72* | 0.50* | 0.58* | NA | NA | NA | NA | NA | NA | NA |
| *Difflugia lanceolata* (n=149) | 0.66* | 0.45* | 0.75* | NA | NA | NA | NA | NA | NA | NA |
| *Difflugia claviformis* (n=113) | 0.70* | 0.72* | 0.58* | NA | NA | NA | NA | NA | NA | NA |

♣ - for characters tl-td, tl-ad and td-ad n=683; for sl-tl, sl-td and sl-ad n=608; for ns-tl, ns-td, ns-ad and ns-sl n=261. * $p < 0.001$, ** $p < 0.01$. NS - not significant

spines, which is not significantly correlated to test diameter and spine length. Table 3 shows morphometric ratios.

**Remarks.** These observations agree with recent studies (Ogden and Hedley 1980, Ogden and Živković 1983, Dekhtyar 1993) regarding test form and composition of this species. Although previous workers had not reported the apertural complex architecture described here, the authors of the present paper had access to Colin G. Ogden's micrographs of *D. corona* deposited in the Natural History Museum (NHM) Protist Collection, and the specimens registered are in agreement with the description given above. Some of the specimens registered by Ogden showed an apertural rim constructed mostly with agglutinated particles, while the ones encountered in the Tiete River always made it exclusively with organic cement material.

Compared with previous studies *D. corona* world-wide has a wide size range. The present work encountered a larger variation within the same population, and we attribute this to the size of sample (n=683).



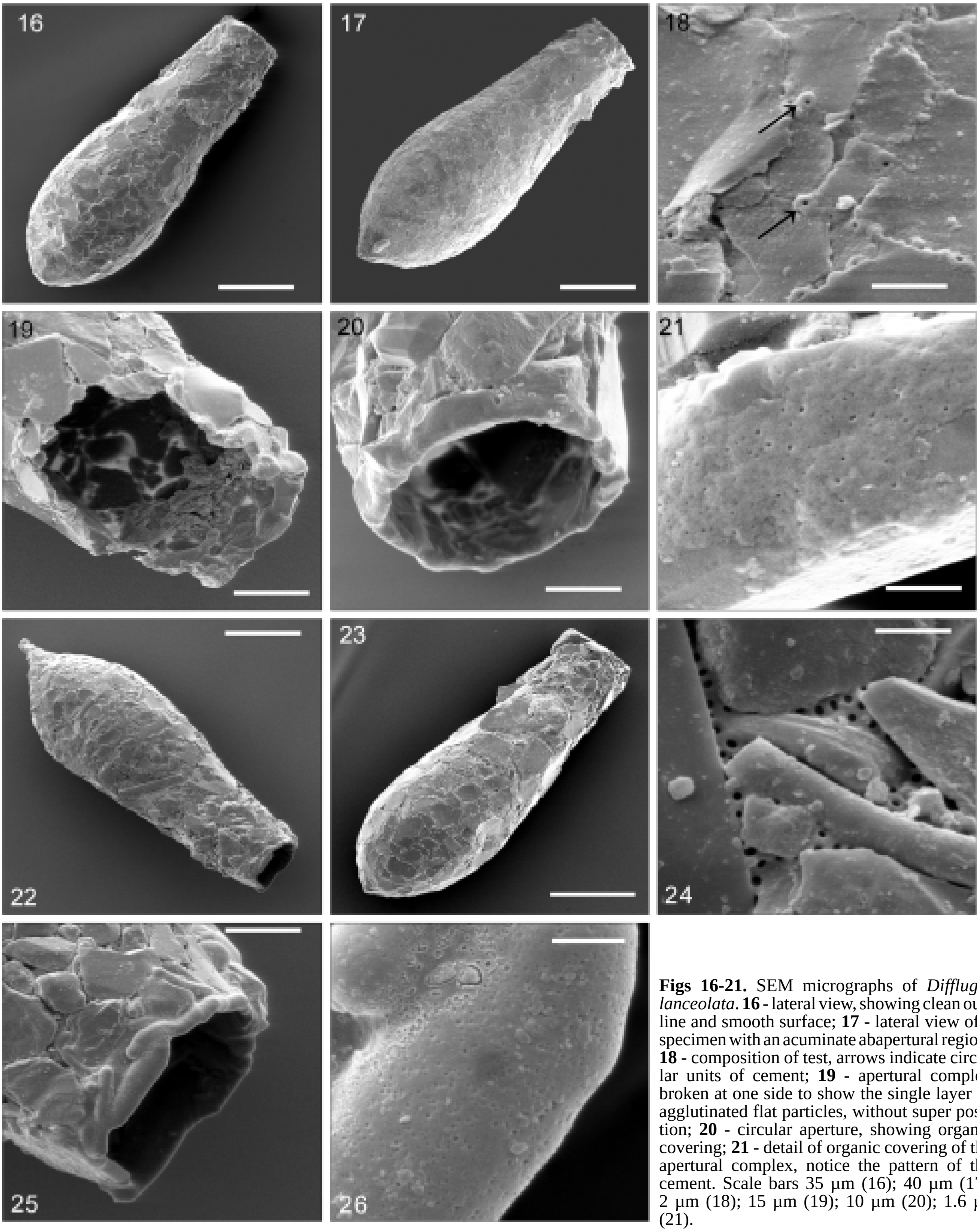

**Figs 11-15.** SEM micrographs of *Difflugia gramen*. **11** - oblique apertural view, showing general test form, outline and composition; **12** - detail of test agglutination and contour, showing rough surface, notice the pattern with larger particles and smaller ones filling in; **13** - region of test surface showing composition, arrows point the strands of smooth cement matrix; **14** - lobed aperture, showing necklace, arrow indicates one of the pores that surround the aperture; **15** - detail of necklace, showing composition, arrow indicates an organic spine. Scale bars: 40 µm (11); 12 µm (12); 2 µm (13); 15 µm (14); 5 µm (15).

**Table 3.** Size ratios between morphometric characters in the studied populations of *Difflugia* from Tiete River. Characters are as designated in Fig 1. SD - standard deviation, n - number of studied specimens.

| Species | td/tl (SD) | ad/tl (SD) | ad/td (SD) |
|---|---|---|---|
| *Difflugia corona* (n=683) | 0.99 (0.11) | 0.48 (0.08) | 0.49 (0.08) |
| *Difflugia gramen* (n=192) | 0.85 (0.20) | 0.35 (0.09) | 0.43 (0.11) |
| *Difflugia lanceolata* (n=149) | 0.43 (0.07) | 0.23 (0.04) | 0.53 (0.08) |
| *Difflugia claviformis* (n=113) | 0.40 (0.10) | 0.23 (0.06) | 0.58 (0.13) |
| *Difflugia gigantea* (n=9) | 0.55 (0.11) | 0.24 (0.05) | 0.43 (0.08) |

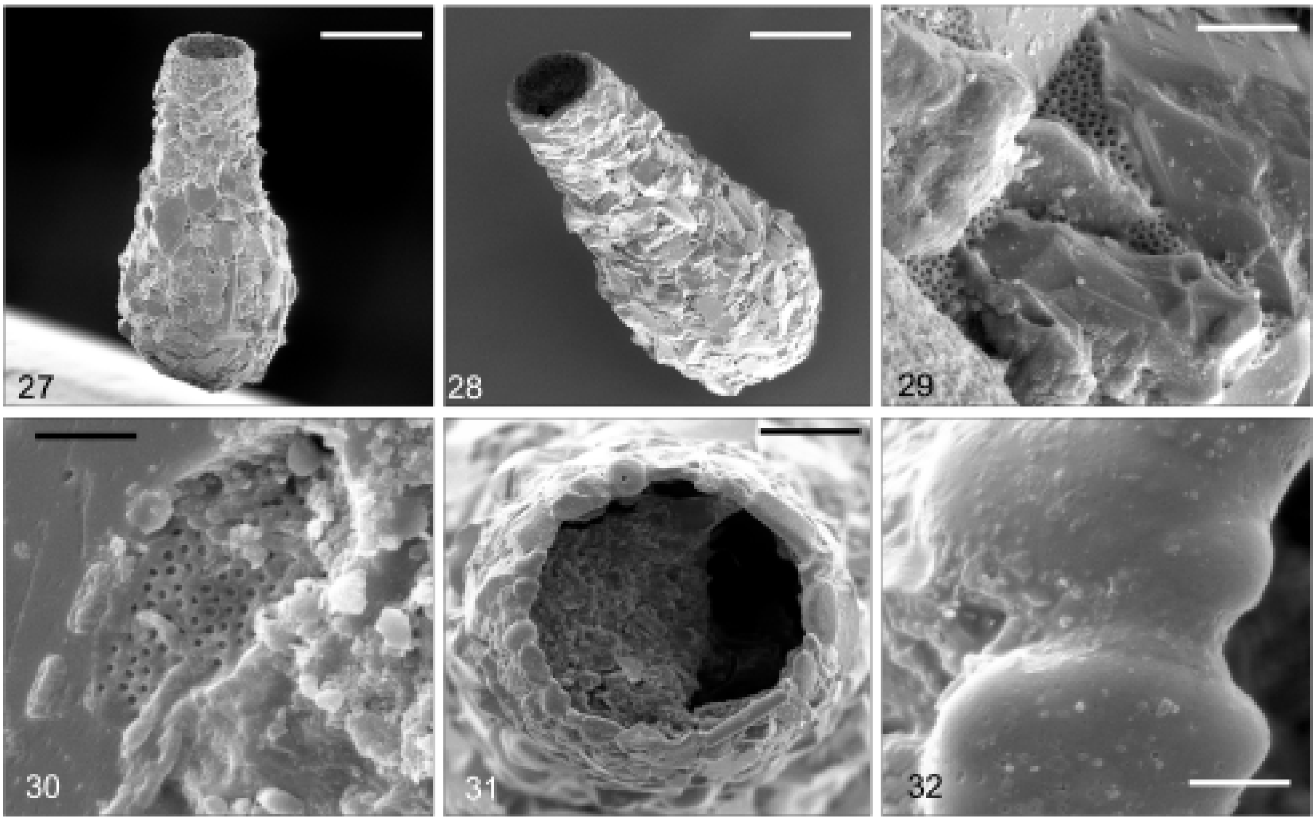
**Table 4.** Habitat exploration of *Difflugia* from Tiete River. Numbers in %. LS - lake sediment, LR - lake roots, RS river sediment, RR - river roots.

| Species | LS | LR | RS | RR |
|---|---|---|---|---|
| *Difflugia corona* | 26.06 | 46.76 | 9.15 | 18.03 |
| *Difflugia gramen* | 40.51 | 22.05 | 3.08 | 34.36 |
| *Difflugia lanceolata* | 0.00 | 3.57 | 9.29 | 87.14 |
| *Difflugia claviformis* | 5.60 | 6.40 | 64.00 | 24.00 |
| *Difflugia gigantea* | 33.33 | 33.33 | 33.33 | 0.00 |




**Figs 16-21.** SEM micrographs of *Difflugia lanceolata*. **16** - lateral view, showing clean outline and smooth surface; **17** - lateral view of a specimen with an acuminate abapertural region; **18** - composition of test, arrows indicate circular units of cement; **19** - apertural complex broken at one side to show the single layer of agglutinated flat particles, without super position; **20** - circular aperture, showing organic covering; **21** - detail of organic covering of the apertural complex, notice the pattern of the cement. Scale bars 35 µm (16); 40 µm (17); 2 µm (18); 15 µm (19); 10 µm (20); 1.6 µm (21).

**Figs 22-26.** SEM micrographs of *Difflugia claviformis*. **22** - lateral view showing general test form, outline and composition; **23** - another individual, to show variation on spine morphology; **24** - detail of test composition, showing particles embedded in a network like cement; **25** - circular aperture, showing organic covering; **26** - detail of the organic covering of the apertural complex, notice the pattern of the cement. Scale bars: 35 µm (22, 23); 2 µm (24); 15 µm (25); 2 µm (26).




**Figs 27-32.** SEM micrographs of *Difflugia gigantea*. **27** - lateral view, showing general test form, outline and composition; **28** - oblique apertural view shows the lack of distinction between the fundus and the neck; **29** - composition of test; **30** - detail of cement organization, notice the smooth layer underlying the perforations; **31** - circular aperture, showing organic covering; **32** - detail of the organic covering of the apertural complex, notice the pattern of the cement. Scale bars: 100 µm (26); 90 µm (27); 4 µm (28); 2 µm (29); 25 µm (30); 2 µm (31).

**Ecology and geographic distribution.** *Difflugia corona* has been reported from Africa, Asia, Europe, North and South America (Ogden and Hedley 1980, Ogden and Živković 1983). Records were made from plankton, sediment and periphyton samples (Table 4). In this study, the majority of organisms (46.76%) were in periphyton environments, such as the roots of aquatic plants in the River, a lotic habitat, confirming previous observations.

**Taxonomic remarks about *Difflugia corona* var. *ecornis* Gauthier-Lièvre *et* Thomas, 1958.** According to the ICZN (Ride *et al.* 1999), *Difflugia corona* var. *ecornis* must be regarded as *Difflugia corona* subspecies *ecornis*, because it was described before 1961 as a variety and is deemed to be treated as subspecies (article 45.6.4).

We are unable to distinguish *Difflugia corona ecornis* from the individuals observed here (and elsewhere) as *Difflugia corona*. The present survey found 76 specimens without spines, 15 of them were examined by SEM. All morphological features (Fig. 10) and biometrical values (Table 1) are otherwise the same as in *D. corona*. Our view is that the spineless specimens are part of a continuum within *Difflugia corona* and the names *Difflugia corona* var. *ecornis*, *Difflugia corona ecornis* and *Difflugia ecornis* are synonymized with *D. corona corona* (created through the principle of co-ordination, article 43.1).

We suspect this is indicative of a more general trend. Many early descriptions are inadequate to distinguish taxa to todays standards (Meisterfeld 2000). Small sample sizes will underestimate the biometric range in

**Table 5.** Morphometric comparisons among different surveys that used large sample sizes of the investigated *Difflugia* species. Characters are as designated in Fig 1. Measurements in µm.CA - character absent, ? - not informed.

| References | td | Tl | ad | al | sl | ns | N |
|---|---|---|---|---|---|---|---|
| *Difflugia corona* | | | | | | | |
| Blanco (2001) | 50-230 | 50-220 | 20-90 | ? | ? | 0-7 | 200 |
| Bobrov and Mazei (2004) | 132.5-184.5 | 141-210 | 8.5-86.1 | ? | ? | ? | 65 |
| present work | 80-330 | 80-380 | 30-200 | 9-15 | 10-100 | 0-10 | 683 |
| *Difflugia gramen* | | | | | | | |
| Stepanek and Jiri (1958) | 53.4-64.7 | 70.8-92.4 | 17.5-28 | 3 | CA | CA | 100 |
| Ogden (1983) | 42-75 | 61-97 | 18-33 | 3-4 | CA | CA | 44 |
| Bobrov and Mazei (2004) | 39.5-77.5 | 57.0-85.5 | 15.9-36.0 | 3 | CA | CA | 40 |
| present work | 50-160 | 80-160 | 20-80 | 3-4 | CA | CA | 192 |
| *Difflugia lanceolata* | | | | | | | |
| Ogden (1983) | 56-92 | 108-155 | 22-32 | CA | CA | CA | 38 |
| Bobrov and Mazei (2004) | 40.5-84.5 | 87.5-189.5 | 21-54 | CA | CA | CA | 82 |
| present work | 40-120 | 80-240 | 20-80 | CA | CA | CA | 149 |
| *Difflugia claviformis* | | | | | | | |
| Ogden and Hedley (1980) | 97-196 | 247-393 | 33-62 | CA | CA | CA | 12 |
| present work | 60-200 | 110-380 | 40-90 | CA | CA | CA | 113 |
| *Difflugia gigantea* | | | | | | | |
| Ogden and Fairman (1979) | 168-231 | 341-480 | 55-84 | CA | CA | CA | 7 |
| present work | 180-280 | 280-460 | 80-120 | CA | CA | CA | 9 |

populations. As larger numbers of cells are included, the size ranges and ranges for other attributes within a species will increase. The concept of the species will expand, and taxa previously deemed to be distinct as varieties or subspecies will lose their identity.

### *Difflugia gramen* Penard, 1902

Data previously reported: *Difflugia gramen* - Penard 1902:281; Deflandre 1926:518 (eco, syn); Štepánek 1952:17 (desc, dim, eco); Gauthier-Lièvre and Thomas 1958:257 Pl I figs a-e (desc, dim, eco, geo, syn); Štepánek and Jiří 1958:139, fig. 1 (desc, dim, eco, syn); Green 1975:550 (eco, geo); Vucetich 1970:45 (syn), 1972:275, fig. 4 (desc, dim, eco, geo, syn), 1973:302, fig. 25 (desc, dim, eco, geo, syn), 1978:81 (eco); Velho and Lansac-Tôha 1996:182 Pl I figs 6, 6a (desc, dim, eco, syn); Ogden 1980b:125 figs 1-6 (desc, eco, dim, SEM), 1983:50 (desc, eco, dim); Ogden and Meisterfeld 1989:121 figs 5, 6, 23, 24 (desc, dim, eco, geo, syn, SEM)

Examined material: 192 specimens by LM, 16 by SEM.

**Morphology.** Test spherical to ovoid (Figs 1e, 11), slightly tapered at apertural end. Circular in apertural view. Contour regular, sometimes broken by a larger agglutinated particle (Fig. 12). Whole test composed of some large and many medium sized mineral grains, mostly particles of quartz and between these are smaller ones, usually spherical, cemented by an organic matrix (Fig. 13). Surface of test slightly rough. Smooth cement matrix between the particles (Fig. 13) sometimes with perforations.

Aperture terminal, with 3 to 4 petal-shaped lobes (Fig. 14), delimited by a slightly raised irregular ridge (necklace), made of small agglutinated particles - small sand grains or cylindrical structures - and smooth organic matrix, evidenced by spiny protuberances (Fig. 15). A ring of pores in the organic matrix, outside the raised ridge, surrounds the aperture (Fig. 15).

**Biometry.** All characters exhibit much variability (Table 1, CV between 18.7 and 31.5). A low standard error of the mean is observed only in aperture diameter (0.79). Size frequency analysis shows that all measurements have a large size range, however, all present a main size class: for test length, 54.6% of observed measures are within the limits of 90 to 110 µm; for test diameter, 52.58% are within 60-100 µm; for aperture diameter 77.04% are within 20-40 µm. Table 2 shows that all characters are positively correlated to each other.

**Remarks**. *Difflugia gramen* was redescribed by Ogden (1980b). His observations agree with those of the present paper and other works (Penard 1902; Gauthier-Lièvre and Thomas 1958; Štepánek and Jiří 1958; Vucetich 1972, 1973; Ogden and Hedley 1980; Ogden 1983; Ogden and Meisterfeld 1989; Velho and Lansac-Tôha 1996). Nevertheless, there is some morphological variance in the Tiete River population.

Ogden (1980b) and Ogden and Meisterfeld (1989) observed that texture and composition of the shell varies from a smooth outline composed of fine sand granules to a rough outline, composed of medium sized granules, sometimes breaking the contour of the test, whilst the present study observed an intermediate situation where the test is composed of both small and medium granules, and when the test does present a rough texture, the contour is rarely broken.

The apertural ridges observed here are more clearly delineated than previously described (Ogden 1980b, 1983; Ogden and Hedley 1980; Ogden and Meisterfeld 1989), and composed of both small grains and organic cementing material. The pores surrounding the aperture are smaller and sparser than those observed by Ogden (1980b).

The greatest variation observed regards cementing material. Ogden (1980b) and Ogden and Meisterfeld (1989) show cement matrix arranged in a mesh network, while the present work revealed a very smooth texture for the observed individuals.

The current study includes more individuals than previous studies. Most previous observations provide measurements that fall within the size range observed for all characters, except for Gauthier-Lièvre and Thomas (1958) and Ogden (1983), who observed smaller individuals. The present study extends the maximum size for this species. Ogden (1983) observed that a common feature for two British populations in Norfolk (Ogden 1980b, 1983) was the ratio aperture diameter/test length ($0.32 \pm 0.04$ and $0.34 \pm 0.04$), the present study concurs with that ratio: $0.35 \pm 0.09$ (Table 3).

**Ecology and geographic distribution.** *Difflugia gramen* is recorded for all continents (Ogden and Hedley 1980). Species can be found in plankton, periphyton and in the sediment. In South America, records have been made by Deflandre (1926), Decloitre (1955), Vucetich (1972, 1973, 1978). In Brazil, records are for Rio de Janeiro (Wailes 1913, Cunha 1913), Mato Grosso (Green 1975), Minas Gerais (Dabés 1995), and Mato Grosso do Sul (Velho and Lansac-Tôha 1996) where the species was associated with the roots of aquatic plants.

Most organisms (Table 4) were in lentic habitats (62.58% of total), preferably associated to the sediment (40.53%). Previous workers have not observed this. An association with periphyton was indicated by Vucetich (1973), Ogden (1980b) and Ogden and Meisterfeld (1989).

### *Difflugia lanceolata* Penard, 1890

Data previously reported: *Difflugia lanceolata*: Penard 1890:145-146, pl. IV, figs 59,60; Decloitre 1954:107-108, fig. 25 (dim); Vucetich 1973: 311, pl. V, fig. 43 (desc, dim, geo); Ogden and Hedley 1980:140, pl 59, figs A-D (desc, dim, geo, SEM); Ogden 1983:11, figs 6a-6f (desc, dim, geo, syn, SEM)

Examined material: 149 specimens by LM, 10 by SEM.

**Morphology.** Test pyriform, elongate (Fig. 16). Circular in apertural view. Abapertural region varies from regular semi-circumferential to acuminate (Figs 16, 17), tapering evenly from widest diameter at the posterior third towards the aperture. Tapering angle varies, in some cases slender, conical tear-shaped, in others the diameter is maintained along the test and individuals are more cylindrical. Test contour completely regular. Test made of flat particles of quartz (Fig. 18) and flat diatom frustules, arranged in a puzzle way, so that no superposition occurs (Fig. 19), hence, the test is thin and surface smooth, with polished appearance. Particles are agglutinated by circular units of organic cement (Fig. 18), seen between test particles.

Aperture terminal, circular. Complex constructed with flat or rounded quartz particles agglutinated and covered by several layers of organic cement (Fig. 20). Pattern of organic matrix is conserved indicating that this cover is made with the same cementing material (Fig. 21).

**Biometry.** Details are given in Table 1. All characters are variable (CV between 15.1 and 18.4), high standard error of the mean (SE = 0.56-2.16). Size frequency analyses show that all characters have a wide size range, but a main size classes can be defined as follows: test diameter, 63.75% of measures are within the limits of 80 to 90 µm; test length, 70.46% are within 180-200 µm; and aperture diameter, 73.82% are within 30-40 µm. Table 2 shows that all measured characters are high and positively correlated to each other.

**Remarks.** The characteristics of the test are in complete agreement with the diagnostic features of *D. lanceolata* described in Ogden (1983) and Ogden and Hedley (1980). Variations have been observed in the Tiete River population in regard to the general test form and abapertural region. Even though these variations have been reported in the literature, they have not previously been reported within the same population.

The smallest and also the largest known individuals for this species were found in this study, extending the known size-range.

**Ecology and geographic distribution.** *D. lanceolata* has been reported from Africa, Asia, Europe, North and South America. In South America, there are reports from Argentina (Vucetich 1973), Venezuela (Grospietsch 1975) and Brazil for Mato Grosso (Hardoim and Heckman 1996) and Goiás (Lansac-Tôha *et al.* 2000). In the present study 87.2% of individuals associated to the roots of aquatic plants in the River (Table 4).

### *Difflugia claviformis* Penard, 1899

Synonyms: *Difflugia pyriformis* var. *claviformis* Penard, 1899; *Difflugia oblonga* var. *claviformis* Cash, 1909; *Difflugia (oblonga) claviformis* Štepánek, 1952.

Data previously reported: *Difflugia pyriformis* var. *claviformis* - Penard, 1899 figs. 12-14; 1902: 218 fig. 3, 4; *Difflugia oblonga* var. *claviformis* - Cash *et* Hopkinson, 1909; Chardez 1967:594 Pl II fig 18 (desc, dim, syn); *Difflugia* (*oblonga*) *claviformis* - Štepánek 1952: 25 (desc, syn); 1967: 39 fig. 8. 6,8 (syn); *Difflugia claviformis* - Ogden 1979:145, 146, 148 fig. 4 (desc, SEM); Ogden and Hedley 1980:126 Pl. 52 figs A-D (desc, dim, geo, SEM)

Examined material: 113 specimens examined by LM, 12 by SEM.

**Morphology.** Test pyriform, elongate. Circular in apertural view. The abapertural region ends in a conical spine that varies in size and shape (Figs 22, 23), tapering evenly. Test contour regular, outline rarely broken by a larger particle. Apertural end composed of rounded particles of quartz and cylindrical structures, so the surface is slightly rough, while the abapertural end is composed of flatter particles of quartz, so the surface is smoother (Figs 22, 23). In both cases, particles are embedded in a network-like organic cement matrix (Fig. 24).

Aperture terminal, circular. Apertural complex made of cylindrical and spherical particles smaller than those in other regions of the test, agglutinated and covered by organic cement matrix (Fig. 25). Several layers of organic matrix are deposited on the free edge, this covering looks smooth, but still some of the meshed pattern can be seen (Fig. 26).

**Biometry.** All characters are very variable (Table 1, CV = 24.0-28.7), with a high standard error of the mean (1.11-6.12). Size frequency analysis shows that all measurements have a large size range, however, all have main size classes: 67.24% of all measured test diameter are within the range of 80 to 100 µm; 56.59% of all test lengths are within 180 to 240 µm; 91.13% of all aperture diameters are within 40 to 60 µm. Table 2 shows that all characters are positively correlated to each other.

**Remarks**. The morphology of tests observed here agree with *D. claviformis* as described by Ogden and Hedley (1980) and (Ogden 1979), except for additional observation of an apertural organic cover, and larger variation on conical spine size and shape. Changes of circumscription in this species are mainly due to variation in spine form. Cash and Hopkinson (1909) restricted *D. claviformis* to those provided with a nipple-like abapertural spine. However, Ogden (1979) and Ogden and Hedley (1980) lump different spine morphologies under this taxon - sometimes the spine is reduced or even absent - based on ultrastructural identity of the cement, which agrees to specimens observed here. Specimens from Tiete River, opposing to European populations, are mostly provided with a tubular conical spine, but other forms are also present. Differential diagnosis from similar species, namely *D. acuminata* Ehrenberg, 1838, *D. elegans* Penard, 1890 and *D. curvicaulis* Penard, 1899, is given elsewhere (Ogden 1979). The population of Tiete River shows more variation in size than all previous studies.

**Ecology and geographic distribution.** *Difflugia claviformis* has been reported from Europe and North America (Ogden and Hedley 1980), from Venezuela (Grospietsch 1975), and from bottom samples in coastal lagoons of Rio Grande do Sul state, Brazil (Madeira-Falcetta 1974). In The present study 88% of all individuals were found in the River, 64% associated with the roots of aquatic plants and 24% associated with the sediment (Table 4).

### *Difflugia gigantea* Chardez, 1967

**Synonym: *Difflugia oblonga* v. *gigantea* Chardez, 1967**

Data previously reported: *Difflugia oblonga* v. *gigantea* - Chardez 1967:593 fig. 2 (dim, syn);

*Difflugia gigantea* - Ogden and Fairman 1979:375 figs 12-15 (desc, dim, SEM).

Examined material: 9 specimens examined by LM, 5 by SEM.

**Morphology.** Test elongate, pyriform (Figs 27, 28). Circular in apertural view. Abapertural region spherical. Sides taper evenly towards the aperture from middle of total test length (Fig. 28). Test contour smooth, outline sometimes broken by larger particles. Test composed of agglutinated quartz particles and diatom frustules, so the test has an irregular outline (Fig. 29). Particles embedded in network-like organic cement with a meshed pattern (Fig. 30). The depressions that make up the mesh do penetrate through the cement, as there is an underlying continuous layer where smaller perforations can be seen.

Aperture terminal, circular. Apertural complex made of cylindrical and spherical particles agglutinated together and covered by organic cement matrix (Fig. 31). Several layers of organic matrix are deposited on the free edge so it is smooth but the meshed pattern can be seen (Fig. 32).

**Biometry.** All characters are variable (Table 1, CV between 16.1 and 20.6) and with a high standard error of the mean (4.71-26.03).

**Remarks.** *Difflugia gigantea* was originally proposed by Chardez (1967) as *Difflugia oblonga* v. *gigantea*, and elevated to species status by Ogden and Fairman (1979). In *D. oblonga* the test outline is more irregular due to the large particles agglutinated, the test surface, therefore, is rough. There is a well defined neck, characteristic of the species, and the organic matrix is only seen as a strand binding the particles together (Ogden and Fairman 1979, Ogden and Hedley 1980), both characteristics differ in *D. gigantea*. The specimens studied are generally more slender, especially in the anterior region, but for other characteristics are in complete agreement to those studied by Ogden and Fairman (1979). Population of Tiete River shows greater variation in size than reports in the literature.

**Ecology and geographic distribution.** Reported from England and North America, *Difflugia gigantea* is rare. The present work surveyed many samples where other pyriform species were abundant (*D. claviformis* and *D. lanceolata*) and found only nine specimens. Ogden and Fairman (1979) also found small numbers associated with *Sphagnum* mosses. Table 4 shows that the specimens were associated with the bottom sediment of the marginal lake (lentic environment), with the roots of aquatic plants in the same lake and with the sediment of the Tiete River.

## DISCUSSION

As most prior studies of *Difflugia* lack type material and involve small sample sizes (see introduction), the identities of species is not clear. The most proximate genus is *Netzelia* Ogden, 1979, species of which build their test with autogenous material (idiosomes), or with siliceous structures found in nature (Ogden 1979, Anderson 1987, Meisterfeld 2000). *Netzelia* tests have regular contours, and a smooth surface. The particles that make up the test of *Difflugia* are picked up from the environment.

In this study we distinguished among spherical species by their form, size, and apertural complex architecture. *D. corona* can be distinguished by the presence of spines. *D. gramen* may be confused with some other testacea, such as *Difflugia lobostoma*, but can be distinguished by nuclear organization (*D. gramen* has a vesicular nucleus while *D. lobostoma* has an ovular nucleus with many nucleoli).

Distinctions between pyriform *Difflugia* are not easy to make (Chardez and Decloitre 1973; Ogden 1979, 1980a, b, 1983; Ogden and Fairman 1979). *D. claviformis* and *D. lanceolata* have similar LM appearances, with almost the same dimensions (Table 1) and are indistinguishable by size ratios (Table 3). The most important difference is the patterning of the organic matrix. It is treated as a reliable distinguishing character for species (Ogden 1983, 1990; Ogden and Živković 1983; Meisterfeld 2000). In *D. lanceolata* the cement is composed of circular, ring-like units, which connect the flattish agglutinated particles at some points. In *D. claviformis*, the cement is a network-like matrix where the particles are embedded. *Difflugia gigantea* can be distinguished by its larger size, but once again, the pattern of the organic cement matrix is also unique. At the outer side of the test, there is a perforated layer, and subjacent to it there is another one with smaller pores. Any uncertainties about this character would undermine the distinctions among these pyriform species.

The morphometric variation found in the Tiete River is the most extensive ever reported. Table 5 shows surveys with largest sample sizes to date, as well as the biometrical data obtained. High variations have also been observed for some species in another biometrical survey (Bobrov and Mazei 2004). They observed that the most variable character is the aperture diameter. The present study differed, with a lower variance coefficient for this character when compared to other characters. Bobrov and Mazei (2004) used a dataset from numerous localities and the variation they observed may reflect selection pressures of many ecological factors. A single population, such as the one found in Tiete River, may therefore show less variation in highly selected features.

The high variation observed is in part a consequence of the large number of individuals investigated. It can also be attributed to ecological factors such as micro-habitats and great environmental ranges, since the Tiete River is highly polluted. Consequently more niche adaptation would follow. Attributes of each species show normal distributions. A main-size class is always present, and studies that rely on fewer cells are likely to report individuals with a limited size range. This will give good estimates of the mean, but underestimate the range.

The increase in range for attributes leads to increased overlap between nominal species described earlier, and should lead us to question the validity of some of those taxa.

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