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Autoecological Approaches to Resolve Subjective Taxonomic Divisions within Arcellacea

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Arcellacea (testate lobose amoebae) are important lacustrine environmental indicators that have been used in paleoclimatic reconstructions, assessing the effectiveness of mine tailings pond reclamation projects and for studying the effects of land use change in rural, industrial and urban settings. Recognition of ecophenotypically significant infra-specific 'strains' within arcellacean assemblages has the potential to enhance the utility of the group in characterizing contemporary and paleoenvironments. We present a novel approach which employs statistical tools to investigate the environmental and taxonomic significance of proposed strains. We test this approach on two identified strains: *Difflugia protaeiformis* Lamarck strain 'acuminata' (DPA), characterized by fine grained agglutination, and *Difflugia protaeiformis* Lamarck strain 'claviformis' (DPC), characterized by coarse grained agglutination. Redundancy analysis indicated that both organisms are associated with similar environmental variables. No relationship was observed between substrate particle size and abundance of DPC, indicating that DPC has a size preference for xenosomes during test construction. Thus DPC should not be designated as a distinct strain but rather form a species complex with DPA. This study elucidates the need to justify the designation of strains based on their autecology in addition to morphological stability.

Key words: Arcellacea; ecophenotype; multivariate analysis; strain; testate amoebae; thecamoebian.

Introduction

Arcellacea Ehrenberg, 1832 are rhizopods that construct a unilocular test. Also informally known as thecamoebians (Patterson and Kumar 2002) or testate lobose amoebae (Mitchell et al. 2008) they are characterized by lobose pseudopods, which

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protrude from the test aperture, and perform the dual functions of facilitating motility and feeding. They form small tests (5-500 μm) comprised primarily of agglutinated material (e.g. mineral grains and diatom frustules with autogenous cement). These tests preserve very well, and in great abundance (500-3000 specimens per ml; Patterson and Kumar 2002). Arcellaceans are important paleolimnological indicators, particularly in the lower pH conditions that typically characterize organic rich sediments (Medioli and Scott 1988; Patterson and Kumar 2002; Wall et al. 2009). Arcellacean assemblage data have been used to develop paleoclimatic reconstructions (Boudreau et al. 2005; Collins et al. 1990; Dallimore et al. 2000; McCarthy et al. 1995), to investigate mine-impacted lake sediments (Kumar and Patterson 2000; Patterson et al. 1996, 2013; Reinhardt et al. 1998), to assess the effectiveness of mine tailings pond reclamation (Neville et al. 2010a, b, 2011) and to study the effects of urban development on lakes (Patterson et al. 2002, 2012; Roe et al. 2010).

Traditionally a 'morphospecies' approach (i.e. species are distinguished based solely on morphologic traits) has been adopted to delineate arcellacean taxa since sexual reproduction is thought to be rare or completely absent and the protoplasm decays rapidly precluding preservation (Charman 1999; Medioli and Scott 1983). Of late the "chastity" of testate lobose amoebae has come into question and it appears sexual reproduction is more common than previously thought (Lahr et al. 2011). The few molecular studies on living arcellaceans confirm that general test shape is a reliable taxonomic indicator (Gomaa et al. 2012). Matters are complicated though as several arcellacean taxa are characterized by considerable morphologic variability. This has in the past resulted in taxonomic confusion (Medioli and Scott 1983; Medioli et al. 1987) revolving principally around the inability to properly quantify the degree of acceptable infra-specific variation (Charman 1999; Mazei and Warren 2012). Infra-specific variation refers to variation in morphology that is not significant enough to elevate a phenotype to the species level (Charman 1999; Wanner 1999), and is assumed to reflect ecophenotypic variation related to variable environmental conditions (Asioli et al. 1996; Patterson et al. 2002; Reinhardt et al. 1998; Wanner 1999). Smith et al. (2007) proposed that the within-species 'lumping' may be more genetically valid than 'splitting' as arcellaceans are characterised by phenotypic plasticity. Mazei and Warren (2012) recommended that taxa which are morphologically difficult to distinguish be lumped together into species "complexes", but cautioned that information pertaining to environmental variability could be lost. In order to circumvent these issues phenotypes showing stable morphologies at the infra-specific level are designated as 'strains' (Asioli et al. 1996; Reinhardt et al. 1998). Strains are not valid taxonomic divisions under the International Code of Zoological Nomenclature (ICZN), but their use increases the utility of using Arcellacea as environmental indicators (Asioli et al. 1996; Neville et al. 2010b; Patterson and Kumar 2002; Reinhardt et al. 1998). Laboratory experiments involving multigenerational morphological analysis of arcellacean clones under differing environmental conditions has confirmed that ecophenotypic variants exist (Medioli et al. 1987), and that in many cases these changes are controlled by environmental variables (Wanner 1999), as first proposed by Wallich (1864). For example, Asioli et al. (1996) demonstrated that arcellaceans living within contaminated lacustrine sediments in northern Italy responded to this environmental stress by displaying infra-specific variability.

The criteria for the designation of strains are based on a phenetic framework, where designations are based on the overall similarity of all observable traits (Medioli and Scott 1983). Although Asioli et al. (1996) employed several observable traits in their classification of strains including size, shape, processes and collar, the associated environmental conditions, which are assumed to solicit strain response, were not included. Advances in the ability to record environmental conditions in lakes and the development of new statistical approaches (e.g. canonical ordination), have led to significant improvements in the understanding of the association of various arcellacean taxa with particular sets of environmental conditions (cf. Legendre and Birks 2012a). Thus it is possible for strain designations to not only include a morphological description but also a description of the associated environmental conditions controlling expression of the observed infra-specific variation. This would enhance their ability to provide useful ecological information.

This study utilizes a distributional analysis of two long-recognized (Reinhardt et al. 1998) strains of *Difflugia protaeiformis* Lamarck 1816 strain 'acuminata' (DPA) and 'claviformis' (DPC), to demonstrate the applicability of statistical approaches to assess morphologic variants.

Both DPA and DPC produce xenogenous tests (Medioli and Scott 1988), but the distinguishing feature between them is the size of grains used to construct their tests (Kumar and Dalby 1998;

Reinhardt et al. 1998). DPC uses coarse silt size grains, giving the test a rough outline (Fig. 5) and making it opaque under transmitted light. In contrast, DPA uses medium and fine silt size grains, giving the test a smooth outline (Fig. 5) and making it translucent under transmitted light (Patterson and Kumar 2002; Reinhardt et al. 1998). These two morphological variants have previously been designated as strains on the assumption that selection of grain size in test construction, although distinct, was an infra-specific trait (Reinhardt et al. 1998). Possible correlation between available grain size in the environment and the distribution of DPC was never tested. The purpose of this research is to determine whether the observed variation between DPA and DPC is an ecophenotypic variation, an expression of substrate bias, or whether the variation reflects a preference, on the part of DPC, for coarser grains in test construction. The research was carried out using the distribution of arcellaceans as previously documented in a set of lakes that spanned a variety of urban agricultural and forested settings in southern Ontario (Patterson et al. 2012; Roe et al. 2010).

Results

Interpretation of the Q-mode cluster analysis (Fig. 2) resulted in recognition of four distinct arcellacean assemblages: 'Nutrient Loaded' (NLA); 'Nutrient Poor' (NPA); 'High Conductivity' (HCA), and; 'Low Conductivity' (LCA). R-mode cluster analysis (Fig. 2) permitted recognition of seven arcellacean species and strains that had a significant influence on assemblage composition: *Centropyxis aculeata* (Ehrenberg 1832) strain "aculeata"; *Centropyxis aculeata* (Ehrenberg 1832) strain "discoides"; *Cucurbitella tricuspis* Carter 1864; *Difflugia corona* Wallich 1864; *Difflugia oblonga* Ehrenberg 1832 strain "oblonga"; *Difflugia oblonga* Ehrenberg 1832 strain "tenuis"; and DPA. DPA and DPC occur within separate branches of the R-mode cluster analysis, branching at the highest level (Fig. 2) with DPA found within the cluster that contains other significant arcellacean species and strains. DPC characterized a distinct cluster along with two other strains of *Difflugia protaeiformis*: strain 'protaeiformis' and strain 'scalpellum', as well as *Difflugia oblonga* strain 'lanceolata' and *Centropyxis constricta* (Ehrenberg, 1843) strain 'spinosa'. Cluster analysis using multiscale bootstrap resampling (Suzuki and Shimodaira 2006) found these clusters to be statistically significant ($p < 0.05$).

The RDA results indicated that a compilation of the first (Eigenvalue = 0.253) and second (Eigenvalue = 0.139) axes explained 39.2% of the variance in the species data and 56.1% of the species-environment relationship, while the measured environmental variables together explained 38.1%. Monte-Carlo permutation tests indicated that the first and all canonical axes were significant at $P < 0.002$ [see Roe et al. (2010) and Patterson et al. (2012) for an in depth discussion of the arcellacean assemblages used in this research]. The centroids of the DPA and DPC correlations both displayed an intermediate fit within the plot. They are associated with dissolved oxygen, pH, calcium, oxygen reducing potential, and conductivity. Calcium, conductivity and pH showed the strongest correlation with DPA and DPC in this area of the RDA. DPA and DPC plotted orthogonally with sedimentary phosphorus in this study. This indicates that neither variant is sensitive to phosphorus loading, in contrast to other arcellacean species (Patterson et al. 2012; Roe et al. 2010).

A survey of scanning electron microscope images taken of DPC revealed that a significant proportion of grains on the order of 20 to 40 microns are incorporated into their growth plan (Fig. 5). The coarse axes of the ternary diagrams represent the abundance of this class of particles (Fig. 4). The spread of abundances along this axis are thus especially important. The cloud of samples fell within the 10-75% coarse particle size, 35-45% medium particle size and the 15-35% fine particle size (Fig. 4). Inspection of the ternary diagrams revealed that "Above Average" samples for DPC were found toward the lower portion of the sample cloud, associated with substrates with lower abundances of coarse particles (Fig. 4). "Average" and "Barren" samples are found spread equally throughout the sample cloud. Scatterplots (Fig. 4b-d) of the abundance of DPC versus each of the axes show there to be no clear relationship. DPA is present in the majority (35 out of 42) of samples, while DPC was found in fewer (22 out of 42) (Fig. 4b-d). Most samples that contained DPC also contained DPA except for a few exceptions (3 out of 42) and in most cases DPA is in greater abundance except in a minority of samples where DPC is most abundant (3 out of 42).

Discussion

Environmental Response

Arcellacean strains have generally been defined based on morphologic traits that are stable within

a given population (Asioli et al. 1996; Patterson and Kumar 2002; Reinhardt et al. 1998). These are assumed to represent infra-specific variation resulting from distinct environmental pressures (Asioli et al. 1996; Patterson and Kumar 2002; Reinhardt et al. 1998), and thus environmental parameters associated with a given strain could also be used in defining it. Reinhardt et al. (1998) employed R-mode cluster analysis to gauge the validity of strain classifications in arcellacean populations from two small lakes impacted by mine tailings near Cobalt, Ontario. They concluded that in order for infra-specific phenotypes to be recognized as distinct strains, they needed to cluster apart, as different clusters were assumed to represent different ecological conditions. Reinhardt et al. (1998) found DPA and DPC to cluster distinctively and thus concluded that they were distinct ecophenotypic strains. The cluster analysis results in this study are in agreement with those of Reinhardt (1998) and show DPA and DPC clustering distinctively (Fig. 2). In contrast, Patterson and Kumar (2000), in an analysis of arcellacean populations along a pH gradient in James Lake, northern Ontario, found that DPA and DPC clustered very close to each other, suggesting that the strains, at least in that lake, shared a nearly identical autecology.

Although R-mode cluster analysis can be used to distinguish between different arcellacean assemblages, which may reflect distinct environmental conditions, it does so indirectly. Caution must therefore be taken in interpreting the results of a R-mode cluster analysis since the analysis itself may produce partitions irrelevant to any real discontinuities in the data (Legendre and Birks 2012a). We employed PVCLUST (Suzuki and Shimodaira 2006) a multiscale bootstrap resampling cluster analysis technique that assigns significance values to branching points. The results from the PVCLUST analysis confirmed that the clusters were statistically significant suggested that these two morphotypes should be classified as distinct strains.

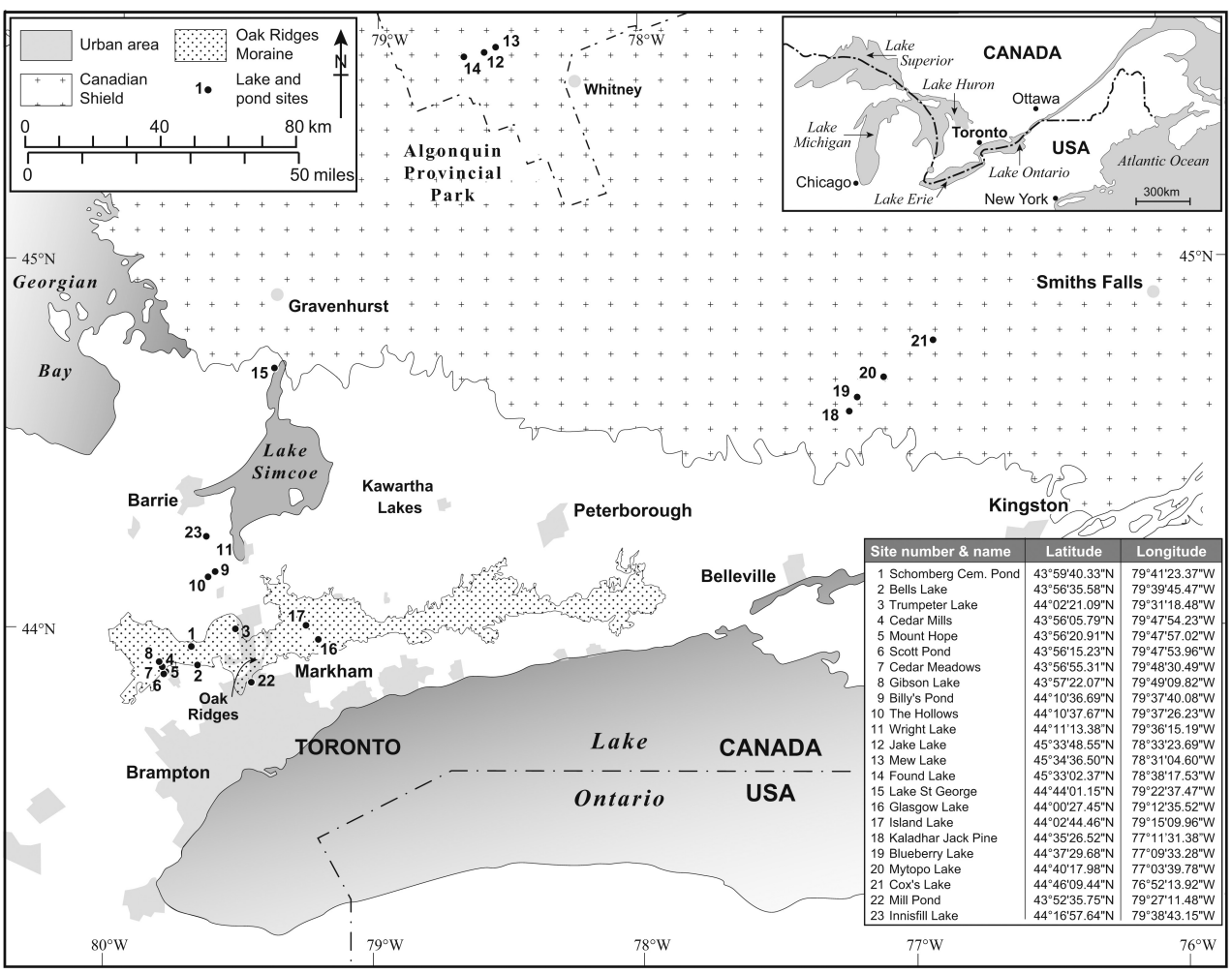
Legendre and Birks (2012b) suggested that clustering techniques to not be solely applied to assemblage data, but rather to use in conjunction with ordination techniques such as RDA. Ordination techniques, which measure environmental variability directly, are much more robust in assessing whether two or more phenotypes share similar autecologies (Legendre and Birks 2012a). The RDA results do not support an ecological distinction between DPA and DPC (Fig. 3). Both plot near to one another, and thus are associated with similar environmental parameters. These results correlate well with other ordination analyses results, where DPC and DPA were observed to plot close together (Kihlman and Kauppila 2012; Neville et al. 2010a; Patterson et al. 2012). It must be noted that the environmental parameters incorporated are only those that could be measured, thus important environmental variables that might influence the autecology of these organisms could be missing. Also subtle differences in their ecology could exist, and the RDA plot might not be sensitive enough to fully capture these subtleties.

Correlation with Substrate

As discussed above DPC was defined as a separate strain based on its incorporation of coarse silt in its growth plan (Reinhardt et al. 1998). Based on an analysis of the mode of test construction observed in *C. tricuspis*, Medioli et al. (1987) had reservations about this approach as they experimentally determined that *C. tricuspis* was opportunistic in selecting materials to build their tests. Their results predict that DPC would be preferentially found in environments with higher percentages of coarse silt. Our results show no such trend, as all abundance categories (i.e. Above Average, Average, Below Average, and Barren) are spread across the sedimentary gradient (Figs 2, 4). The same observation can be made for the distribution of DPA. This result suggests that DPC selectively chooses coarse grains to incorporate into its growth plan, regardless of the actual abundance of coarse silt in the substrate. Although grain size selectivity has not been widely studied in arcellaceans, it has been commonly seen in agglutinated foramifera (Bowser and Bernhard 1993; and references therein). Bowser and Bernhard (1993) observed that *Astrammina rara* Rhumbler selected large grains (>300 μm) for test construction from surrounding sediment characterized by a higher percent weight of smaller grain sizes (<300 μm). This preferential grain selection was attributed to an evolutionary pressure to build stronger tests to reduce the amount of cement needed to fasten the grains. As the cement is produced in the cytoplasm (Bender and Hemleben 1988) and therefore autogenous in nature – any mechanism that will reduce the energy requirement (e.g. using larger grains) to produce cement may present an evolutionary advantage. The recognition that Arcellacea will not undergo nuclear division in the absence of appropriate agglutinating material suggests that similar genetic linkages exist in this group as well (Stump 1936). Thus it can be said that DPC displays a selective behaviour when choosing grains to incorporate into its test.

Molecular data is needed in order to justify the elevation of DPC from a subspecies classification to the species level. Gomaa et al. (2012) published the first molecular data concerning *Difflugia* and found that general shell shape was a reliable indicator. The same could not be said about the structure of the organic cement used in test agglutination. Whether the variation in the amount of cement used in test construction represents specific variation still needs to be elucidated. It is the opinion of the authors that DPC be no longer considered a strain. The inclusion of coarser grains during test construction is not an ecophenotypical response, but could represent genetic underpinnings. In lieu of molecular data to support the elevation to a species level, DPC and DPA should be complexed together, after Mazei and Warren (2012), as they share similar morphologies and similar autoecologies.

Including a description of environmental parameters that are associated with the morphospecies along with a morphological description is a pragmatic method that will increase our understanding of the ecophenotypic variability within arcellaceans. Cluster analysis, both R-mode and multi-scale bootstrap analysis, separates DPA and DPC into separate clusters. RDA analysis showed that the separation of these strains was not due to measured environmental variables. Scatterplots and ternary diagrams revealed there was no relationship between substrate particle size and abundance of DPC. The results indicate that DPC are most likely selecting certain grain sizes, regardless of substrate abundance or environmental conditions. Thus further work is needed to see if this behaviour reflects a genetic basis. In lieu of this information it is the opinion of the authors that



Site number & name	Latitude	Longitude
1 Schomberg Cem. Pond	43°59'40.33"N	79°41'23.37"W
2 Bells Lake	43°56'35.58"N	79°39'45.47"W
3 Trumpeter Lake	44°02'21.09"N	79°31'18.48"W
4 Cedar Mills	43°56'05.79"N	79°47'54.23"W
5 Mount Hope	43°56'20.91"N	79°47'57.02"W
6 Scott Pond	43°56'15.23"N	79°47'53.96"W
7 Cedar Meadows	43°56'55.31"N	79°48'30.49"W
8 Gibson Lake	43°57'22.07"N	79°49'09.82"W
9 Billy's Pond	44°10'36.69"N	79°37'40.08"W
10 The Hollows	44°10'37.67"N	79°37'26.23"W
11 Wright Lake	44°11'13.38"N	79°36'15.19"W
12 Jake Lake	45°33'48.55"N	78°33'23.69"W
13 Mew Lake	45°34'36.50"N	78°31'04.60"W
14 Found Lake	45°33'02.37"N	78°38'17.53"W
15 Lake St George	44°44'01.15"N	79°22'37.47"W
16 Glasgow Lake	44°00'27.45"N	79°12'35.52"W
17 Island Lake	44°02'44.46"N	79°15'09.96"W
18 Kaladhar Jack Pine	44°35'26.52"N	77°11'31.38"W
19 Blueberry Lake	44°37'29.68"N	77°09'33.28"W
20 Mytopo Lake	44°40'17.98"N	77°03'39.78"W
21 Cox's Lake	44°46'09.44"N	76°52'13.92"W
22 Mill Pond	43°52'35.75"N	79°27'11.48"W
23 Innisfill Lake	44°16'57.64"N	79°38'43.15"W

Figure 1. Map of Sampling Sites. Location of sampling sites.

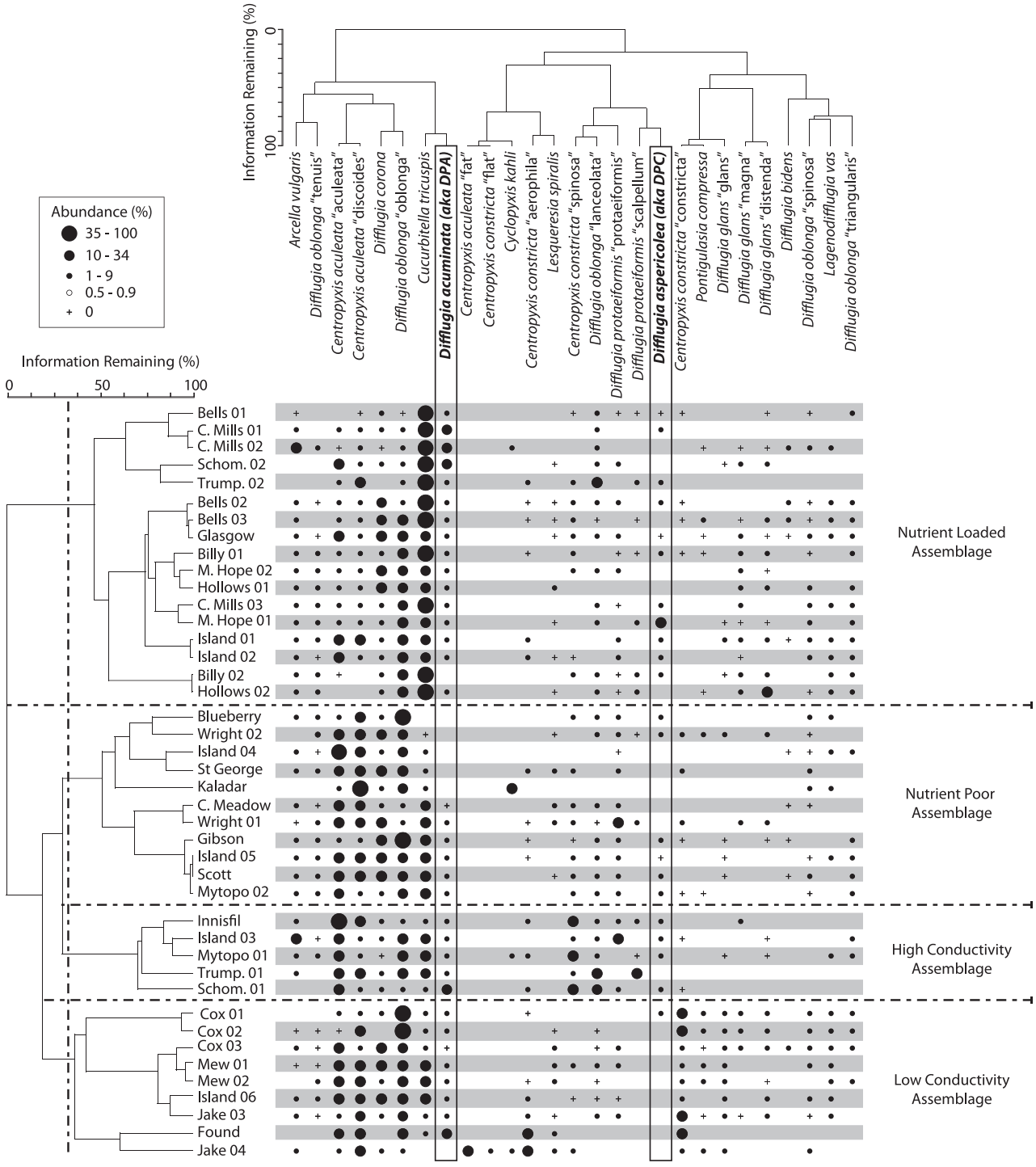
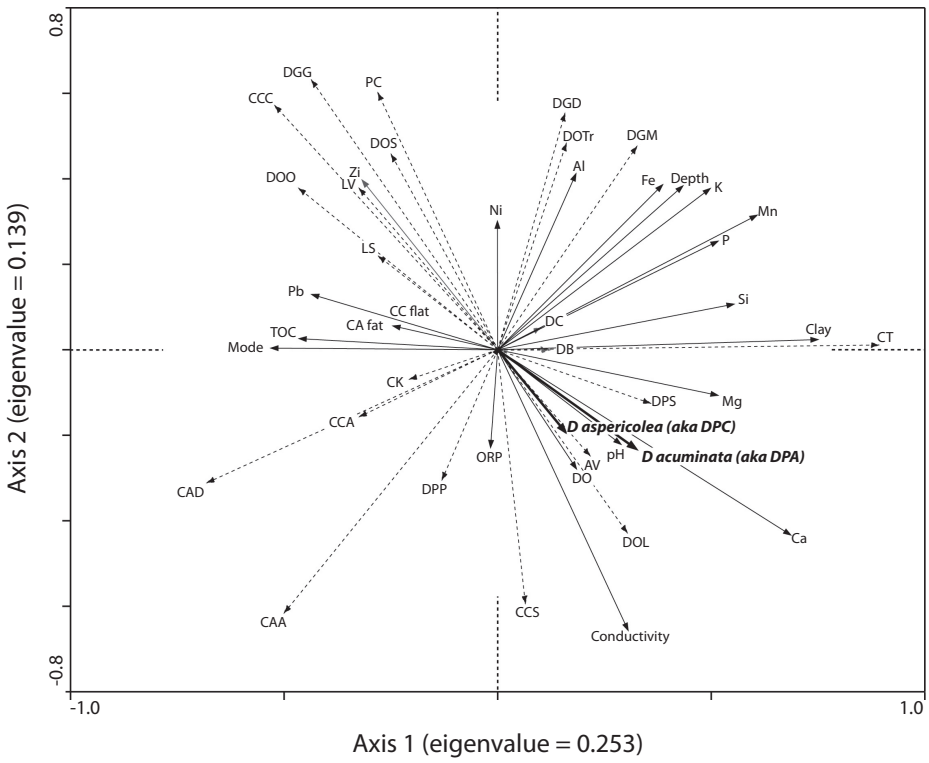


Figure 2. Cluster Analysis Results. R-mode vs. Q-mode cluster diagram for the 42 samples with statistically significant arcellacean counts. Four faunal assemblages are indicated. The *dashed lines* demarcate clusters of samples with correlation coefficients greater than the selected level of significance. C. Mills = Cedar Mills; Schom. = Schomberg Cemetery; Trump. = Trumpeter; M. Hope = Mount Hope; C. Meadows = Cedar Meadows.



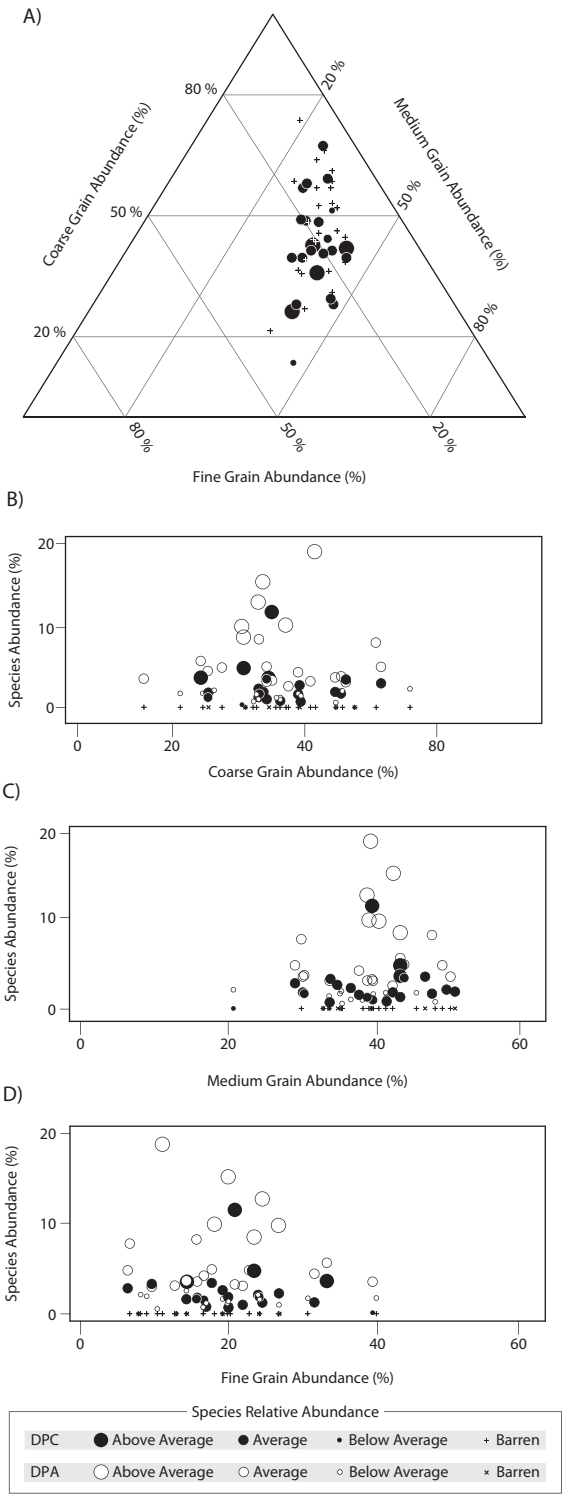
VARIABLE	%	P<
Al	4.72	0.006
Depth	4.15	0.032
Ni	4.15	0.032
K	3.86	0.026
DO	3.86	0.046
P	3	0.136
ORP	2.86	0.164
Ca	2.86	0.176
Mn	2.72	0.202
Cond.	2.72	0.204
pH	2.43	0.266
Zn	2.29	0.314
Mode	2.15	0.36
Mg	2.15	0.36
Fe	2.15	0.364
Pb	2.15	0.378
Clay	1.86	0.496
Si	1.86	0.604
TOC	1.43	0.706
Intercor.	45.5	n / a

Figure 3. Redundancy Analysis Results. Redundancy analysis (RDA) species–environment biplot for the 42 samples that yielded statistically significant arcellacean populations and were analyzed by ICP-MS. Percentage variance explained by each environmental variable and its corresponding p value as calculated by variance partitioning (p=RDA) are shown in the table. DO – dissolved oxygen (%), ORP – oxidation reduction potential, TOC – total organic carbon. *AV – Arcella vulgaris*, *CAA – Centropyxis aculeata* "aculeata", *CAD – Centropyxis aculeata* "discoides", *Centropyxis aculeata* "fat", *CCA – Centropyxis constricta* "aerophila", *CCC – Centropyxis constricta* "constricta", *Centropyxis constricta* "flat", *CCS – Centropyxis constricta* "spinosa", *CK – Cyclopyxis kahli*, *DB – Difflugia bidens, DC – Difflugia corona, DPB – Difflugia protaeiformis* "scalpellum", *DGD – Difflugia glans* "distenda", *DGG – Difflugia glans* "glans", *DGM – Difflugia glans* "magna", *DOO – Difflugia oblonga* "oblonga", *DOS – Difflugia oblonga* "spinosa", *DOTr – Difflugia oblonga* "triangularis", *DPP – Difflugia protaeiformis* "protaeiformis", *LV – Lagenodifflugia vas, LS - Lesquereusia spiralis, Pontigulasia compressa*.

DPC should be complexed with DPA. This study further elucidates the need to justify arcellacean strains based on a distinct environmental response in addition to morphological stability.

Methods

The data set comprises 42 sediment-water interface samples collected from lakes and ponds in the Greater Toronto Area (GTA), Algonquin Park to the north and adjacent areas in eastern Ontario, Canada (Fig. 1). The arcellacean species and strains, associated environmental properties of each station and methodologies employed were previously documented in Roe et al. (2010) and Patterson et al. (2012). Samples for this study were carefully selected to represent diverse limnological environments and contain statistically significant populations of DPA and/or DPC (after Patterson and Fishbein 1989; Boudreau et al. 2005). These samples in total contain 27 arcellacean species and strains present in statistically significant populations. The arcellacean faunal diversity within each sample was assessed using the Shannon Diversity Index (SDI), (Shannon 1948), which has been found to provide an indication of the relative health of lakes and ponds (Patterson and Kumar 2000; Roe and Patterson 2006). Q- and R-mode cluster analysis was carried out to identify arcellacean assemblages (after Fishbein and Patterson 1993). Results were organized into a two-way cluster diagram (Fig. 2) using the PC-ORD (Version 6 for Windows) software package. PVCLUST (Suzuki and Shimodaira 2006) was used to calculate probability values for each cluster using multiscale bootstrap resampling techniques. Ordination analysis was carried out using CANOCO version 4.5 and CANODRAW (ter Braak 2002; ter Braak and Smilauer 2002). Detrended Correspondence Analysis (DCA) was used to explore the inter-site characteristics of arcellacean communities, and defined the gradient lengths characterizing the dataset. Datasets having gradient lengths < 2 SD indicated a linear response, while those with gradient lengths > 2 SD denoted a unimodal response (Birks 1995). DCA results indicated that our dataset had a unimodal response (2.73 SD). Subsequently Pearson product-moment correlation coefficient analysis was carried out to determine the intercorrelations between environmental variables and to provide an assessment of the degree of



redundancy in the dataset (Birks 1995). Redundancy Analysis (RDA) was carried out to determine the relationships between arcellacean taxa, sample sites and environmental variables (ter Braak 2002; ter Braak and Smilauer 2002). In these canonical ordination-type analyses, the data are constrained to correspond to a portion of the variation reported in a table of response variables (e.g. arcellacean percent abundance) that is maximally related to a set of explanatory variables (e.g. measured environmental parameters) (Legendre and Birks 2012a). As a successful RDA analysis requires datasets to have a linear response our unimodal dataset was transformed using the Hellinger transformation (Legendre and Gallagher 2001). As per the work of Medioli et al. (1987) it is assumed that the abundance of DPC should parallel the abundance of coarser grains in the substrate. This assumption was tested by plotting the normalized percent abundance data was plotted on a ternary diagram representing the grain size composition of the sites (Fig. 4) (Tri-plot Version 1.4; Graham and Midgley 2000). Percent abundance data was normalized to facilitate the groupings on the ternary plot (Fig. 4). The size ranges plotted for each axis were based upon a survey of scanning electron microscope images taken of DPC and DPA. The survey revealed that a significant proportion of grains on the order of 20 to 40 microns are incorporated into the growth plan of DPC (Fig. 5). Plotted data points were organized into four series: "Barren Samples" (0% abundance); "Below Average" (less than -0.5 standard deviations); "Above Average" (greater than 1 standard deviation); and "Average" (between 1 and -0.5 standard deviations).

SYSTEMATICS

Subkingdom PROTOZOA
Phylum AMOEBOZOA Lühe 1913
Class LOBOSIA (Carpenter 1861)
Order ARCELLINIDA (Kent 1880)
Superfamily ARCELLACEA (Ehrenberg 1830)
Family DIFFLUGIDAE (Stein 1859)
Genus *Difflugia* Leclerc 1815

Difflugia protaeiformis Lamarck 1816 strain 'acuminata'

(Figure 5, A-D)

Difflugia acuminata EHRENBERG, 1830, p. 95.
Difflugia acuminata Ehrenberg EHRENBERG, 1838, pl. 9, fig. 3.

Figure 4. Substrate Bias Results. **A**) Ternary plot of the 42 samples. Axes of the plot represent the abundance of certain grain sizes in the samples. Coarse Grain = 63 to 16 microns; Medium Grain = 16 to 4 microns; Fine Grain = 4 microns and smaller. Sample points are coded to represent relative abundance of *Difflugia protaeiformis* Lamarck strain 'claviformis'. Above Average = greater than 1 standard deviation (SD); Average = 1 to -0.5 SD; Below Average = less than -0.5 SD; Barren = not present in the sample. (**B-D**) scatterplots of ternary plot axes versus percent abundance of both DPA (open circle) and DPC (closed circle). DPC is equally distributed throughout the sedimentary gradient and thus displays no substrate bias.

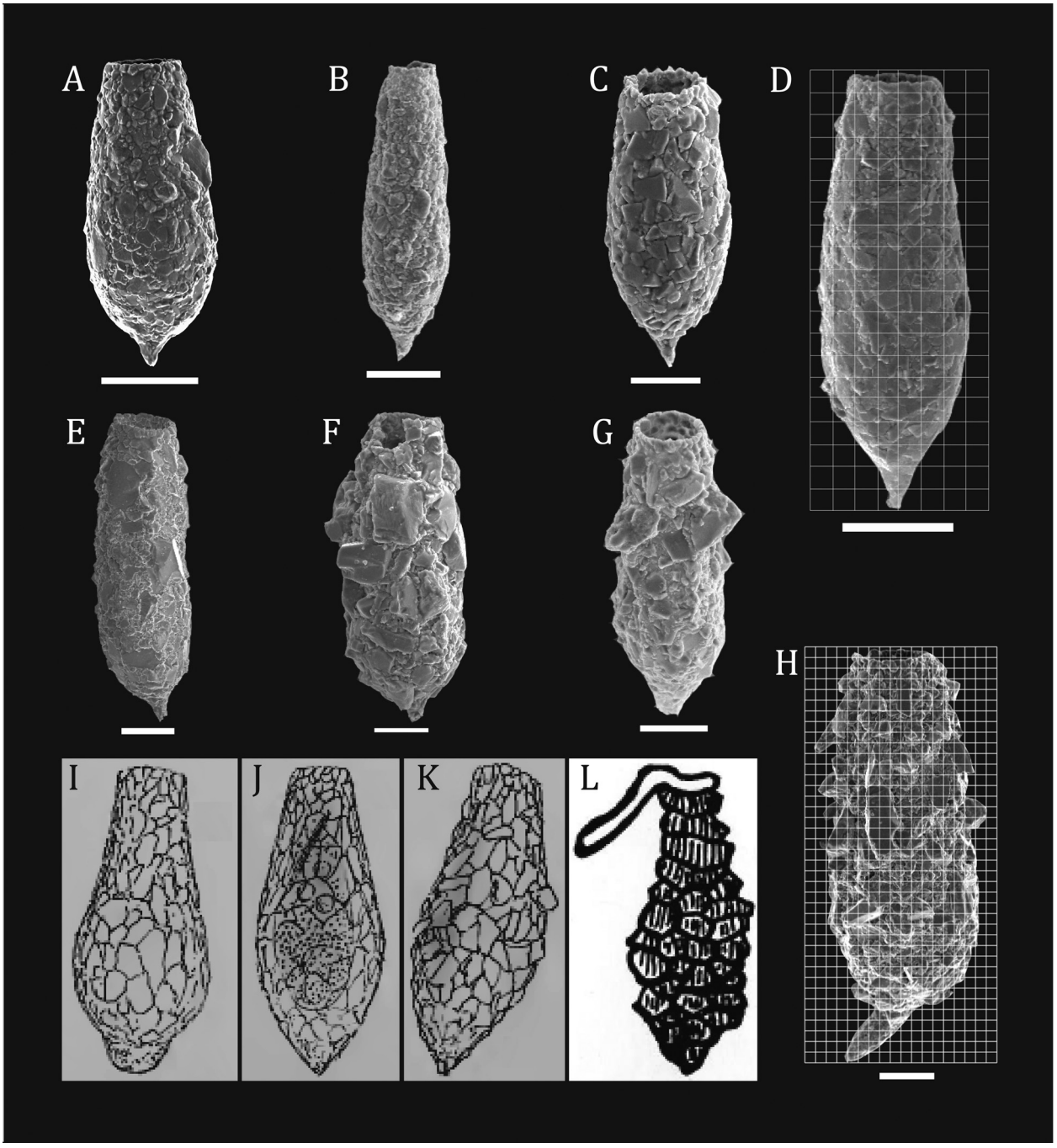


Figure 5. Photoplate. Scanning electron micrographs of tests from species treated in this study: **(A - E)** *Difflugia protaeiformis* Lamarck strain 'acuminata', **(F – H)** *Difflugia protaeiformis* Lamarck strain 'claviformis'. Hand drawn pictures of tests from species treated in this study: **(I – K)** *Difflugia pyriformis* var. claviformis Penard 1899, **(L)** *Difflugia protaeiformis* Lamarck. All scale bars represent 50 μm. The lines placed over **(D)** and **(I)** are spaced at 10 μm.

Difflugia acuminata Ehrenberg var. inflata PENARD, 1899, pl. 3, fig.1.
Difflugia curvicaulis PENARD, 1899, pl. 3, figs. 2-5.
Difflugia acuminata Ehrenberg CASH AND HOPKINSON, 1909, p. 22, fig. 43, not fig. 42.
Difflugia acuminata Ehrenberg SCOTT AND MEDIOLI,1983, p. 818, fig. 9d.
Difflugia protaeiformis Lamarck MEDIOLI AND SCOTT, 1983, pl. 1, figs. 19-20 (not pl. 1, figs 15-18).
Difflugia protaeiformis Lamarck strain 'acuminata' REINHARDT ET AL., 1998, pl. 2, fig. 5.
Difflugia protaeiformis Lamarck strain 'acuminata' ROE ET AL., 2010, p. 960, Fig. 2p.
Difflugia protaeiformis Lamarck strain 'acuminata' PATTERSON ET AL., 2012, p. 39, Fig. 4.25.
Difflugia acuminata Ehrenberg MAZEI AND WARREN, 2012, not fig. 7 or 8, fig. 9 all except f and g, fig. 10 all except a or f or g.

Description. Test free, elongate, almost cylindroconical, fundus acuminate, tapering to form blunt basal process; anterior portion truncated at a right angle to form apertural opening, neck absent; aperture encompasses entire to almost the entire width of the test, aperture circular, narrow without lip; wall agglutinated, test smooth almost hyaline (i.e. transparent).

Remarks. Distinguished from *Difflugia protaeiformis* Lamarck strain 'claviformis' (DPC) by having a thinner wall, which appears transparent under a light microscope and by having a smooth outline. DPC is also much more coarsely agglutinated. *Difflugia curvicaulis* Penard, 1899 is identical to *Difflugia protaeiformis* Lamarck strain 'acuminata' (DPA) with the exception of a curved basal process. We do not consider this minor variation a specific trait and thus consider *D. curvicaulis* to be a junior synonym of DPA.

***Difflugia protaeiformis* Lamarck strain 'claviformis'**

(Figure 5, E-I)

Difflugia protaeiformis Lamarck morph 'protaeiformis' ASIOLI ET AL., 1996, p. 255, pl. 2, figs.1a,1b.
Difflugia protaeiformis Lamarck strain 'claviformis' REINHARDT ET AL., 1998, pl. 2, fig. 3.
Difflugia protaeiformis Lamarck strain 'protaeiformis' DALLIMORE ET AL., 2000, fig. 11.1,2.
Difflugia protaeiformis Lamarck HOLCOVÁ, 2008, pl. 2, fig. 26.
Difflugia protaeiformis Lamarck LORENCOVÁ, 2009, Fig. 13 (M,N).
Difflugia protaeiformis Lamarck strain 'claviformis' KIHLMAN AND KAUPPILA, 2012, Fig. 3.26.
Difflugia protaeiformis Lamarck strain 'claviformis' PATTERSON ET AL., 2012, p. 39, Fig. 4.27.
Difflugia acuminata Ehrenberg MAZEI AND WARREN, 2012, fig. 7, fig. 8, fig. 9 f and g, fig. 10 a, f, g.

Description. Test free, elongate, almost cylindroconical, fundus acuminate, tapering to form blunt spine, anterior portion truncated at a right angle to form apertural opening, neck absent; aperture encompasses entire to almost entire width of the test, aperture circular, narrow without lip; wall agglutinated, test rough in outline, formed of irregular and angular clasts of medium to coarse grained silt, opaque under a light microscope and small.

Remarks. *Difflugia protaeiformis* Lamarck strain 'claviformis' (DPC) is distinguished from *D.protaeiformis* Lamarck strain 'acuminata' (DPA) in having a coarser test made up of medium to coarse-grained silt particles, and is opaque under a light microscope. The use of strain names to refer to morphologic variants within the Arcellacea first appeared in the literature in the 1990s (e.g. Asioli et al. 1996; Reinhardt et al., 1998) and was used to delineate ecophenotypic populations where there was uncertainty as to whether distinct species existed (Patterson and Kumar 2002). DPC is distinct from *D. protaeiformis*, as specimens of the latter species are typically characterized by an elongate test with a globular posterior region, which tapers over a very short interval to form a long shaft. This shaft, comprising 2/3^{rds} of the test, does not taper and is truncated perpendicularly, having an aperture that spans the width the test. Reinhardt et al. (1998) based identification of their DPC on the *D. claviformis* (Penard) of Ogden and Hedley (1980, p. 118, pl. 52, figs A-D). The specimens illustrated by Ogden and Hedley (1980) are actually the same as specimens illustrated by Reinhart et al. (1998) as *Difflugia oblonga* strain 'spinosa' (Pl. 2, figs. 11ab). Ogden and Hedley (1980) in turn attributed their *D. claviformis* (Penard) to *Difflugia pyriformis* var. claviformis Penard 1899 (p. 25, pl. 2, figs 12-14). Penard illustrated two different species in these figures. Although Pl. 2, fig 12 is attributable to the current concept of *D. claviformis*, the specimens illustrated on pl. 2, figs. 13, 14 are more likely attributable to *Difflugia scalpellum* Penard, 1899, described in the same volume.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2014.03.004.

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