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First application of PDMPO to examine silicification in polycystine Radiolaria

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Abstract: 2-(4-pyridyl)-5-[(4-(2-dimethylaminoethylaminocarbamoyl)methoxy)-phenyl] oxazole (PDMPO) is a fluorescent compound that accumulates in acidic cell compartments. PDMPO is accumulated with silica under acidic conditions, and the newly developed silica skeletons show green fluorescent light. This study is the first to use PDMPO in polycystine radiolarians, which are unicellular planktonic protists. We tested *Acanthodesmia* sp., *Rhizosphaera trigonacantha*, and *Spirocyrtis scalaris* for emission of green fluorescence. Entire skeletons of *Acanthodesmia* sp. and *Sr. scalaris* emitted green fluorescent light, whereas only the outermost shell and radial spines of *Rz. trigonacantha* showed fluorescence. Two additional species, *Spongaster tetras tetras* and *Rhopalastrum elegans* did not show fluorescence. Green fluorescence of the entire skeleton is more like the "skeletal thickening growth" defined by silica deposition throughout the surface of the existing skeleton. The brightness of the fluorescence varied with each cell. This difference in fluorescence may reflect the rate of growth in these cells. Green fluorescence in PDMPO-treated polycystines suggests the presence of similar metabolic systems with controlled pH. The results of this study shed light not only on the feasibility of using PDMPO in diatoms and siliceous sponges, but also in polycystines. Further application of PDMPO could refine polycystine skeletogenic hypotheses and offer new insight on the physiological functions of silica assimilation.

Key words: PDMPO, polycystine, radiolaria, silicification, skeletogenesis

Introduction

Polycystine radiolaria (unicellular planktonic Protista) have a very geometric, siliceous skeleton, with spherical, discoidal, spiral, and multi-segmented conical forms. As shown in Fig. 1A, some spherical polycystines have concentric, spherical shells (e.g., Order Spumellaria, Superfamily Actinommoidea), as if each of their shells grows from the inside, whereas multi-segmented conical polycystines (Fig. 1B) (e.g. Order Nassellaria, Suborder Cyrtellaria) appear as though they make their segments one-by-one (Hollande & Enjumet 1960, Petrushevskaya 1962).

The skeletogenesis of polycystines is little known. The form of skeletogenesis is generally hypothesized from observations of skeletal forming signs recorded in plankton and fossil specimens (e.g. Anderson & Swanberg 1981, Thurow & Anderson 1986, Nishimura 1990, Itaki & Bjørklund 2007). However, these studies examined the skeletogenesis only of fully grown polycystines, and the growing process from young to fully grown cells has not yet been observed. One of the most effective methods for studying polycystine skeletogenesis is to observe the growing process, but it is difficult because long-term culture experiments of polycystines have not yet been successful (e.g., Anderson 1983, Anderson et al. 1989, Matsuoka & Anderson 1992, Sugiyama & Anderson 1997).

One effective method for studying polycystine skeletogenesis is the 2-(4-pyridyl)-5-[(4-(2-dimethylaminoethylaminocarbamoyl)methoxy)-phenyl] oxazole (PDMPO; Molecular Probe, Lysosensor™ DND-160 Yellow/Blue) technique (Shimizu et al. 2001), which has been applied for investigations of the biological silicification of demosponges (Schröder et al. 2004) and diatoms (Hazellar et al. 2005). PDMPO is a fluorescent compound and an aci-

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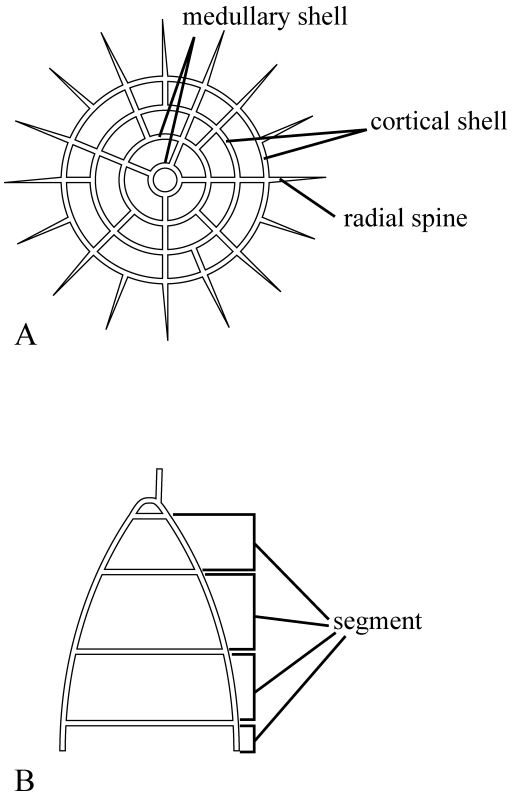

Fig. 1. Radiolarian test terminology. A: Order Spumellaria, Superfamily Actinommoidea; B: Order Nassellaria, Suborder Cyrtellaria.

dotropic pH indicator that accumulates in acidic cell compartments. In diatoms, the silica deposition vesicle (SDV) is an acidic cell compartment (Vrieling et al. 1999). PDMPO accumulates with silica under acidic conditions in the SDV, and is then deposited in the skeleton. Due to PDMPO, the newly developed silica skeletons emit green fluorescence light.

Although the biochemical conditions and growth rate during silicification and skeletogenesis cannot be directly observed, the application of PDMPO-silica fluorescence is worth implementing. Because SDVs are likely to exist in the polycystines, based on transmission electron microscope (TEM) images of probable silica aggregate granules (0.2–0.3 μm in diameter) between the siliceous test and the inner membrane of the cytokalymma in *Collosphaera globuralis* Haeckel (Family Collsphaeridae) (Anderson 1981, 1994), we believe that PDMPO is applicable for staining the newly developed skeleton of polycystines. Our results clearly show the existence of an acidic cell compartment in radiolarian cells, and this compartment is likely related to silica deposition.

Materials and Methods

The material used in this study was collected from surface waters (ca. 4 m depth) on 25 November 2008 by a 3-min tow using the No. 3 plankton net of Matsuoka (2002) (a 2-m long net with a 44-μm mesh and a 50-cm square opening) at a locality (Station 081125; 26°35′41″N, 127°50′30″E) off Sesoko Island, Okinawa, southwestern Japan. This area is commonly affected by the warm Kuroshio Current. The temperature and salinity of the seawater were 24.6°C and 33, respectively. The collected samples were processed at the Sesoko Tropical Biosphere Research Center, University of the Ryukyus (STBRC). Terminology used to describe radiolarians follows Suzuki (1998) and De Wever et al. (2001).

The collected materials were stained with PDMPO and observed by the following method. As this is the first report to apply PDMPO to polycystine radiolarians, the detailed procedures are explained as follows (Fig. 2):

1) The collected material was filtered through a sieve with a 1-mm opening to remove >1 mm plankton, predominantly Copepoda, for keeping polycystines in good condition. Polycystines weaken and die in the presence of >1 mm plankton (Fig. 2A).
2) The filtered material was settled for a few hours in a bottle full of seawater, because polycystine radiolarians tend to mostly settle to the bottom.
3) A small portion of the settled radiolarians was removed from the bottles with a Pasteur pipette and transferred into a small glass tube with 16 mL of seawater filtered with a 0.45-μm membrane filter. (Fig. 2B).
4) The undiluted PDMPO (1 mM) was dissolved in 20 mL of percolated seawater. Dissolved PDMPO (2.5 μM, 4 mL) was added to the radiolarian tube (final concentration: 0.5 μM PDMPO) (Fig. 2D).
5) The tubes were incubated under an artificial day-night cycle (12 h day/12 h night) with red and blue LED lights for 24 h at 26°C (Fig. 2E).
6) The samples were fixed in the incubation tubes with 37.5% formalin solution (Wako Pure Chemical Industrials) (final concentration: 2% formaldehyde).
7) The incubated samples were moved to petri dishes (Fig. 2F), and the polycystines were collected with a Pasteur pipette under an inverted light microscope (Fig. 2G). The collected cells were mounted on glass slides (Fig. 2H). To avoid crushing the radiolarians with a cover slip, white Vaseline was placed in a ridge to a height of 0.5 mm at the edge of the cover slip. After adding the Vaseline, the cover slip was attached to the slide (Fig. 2I).
8) The slide was observed with an epifluorescence microscope (AX-80, Olympus) with UV-excitation (WU: BP330–385, DM400, BA420), and images of selected cells were digitally captured with a CCD camera (DXM-1200, Nikon 150 ppi).
9) After observing the slide, the cover slip was removed with a box cutter, and the embedded cells were removed from the slide to the petri dish containing 50% chlorine bleach with a fine brush (Fig.

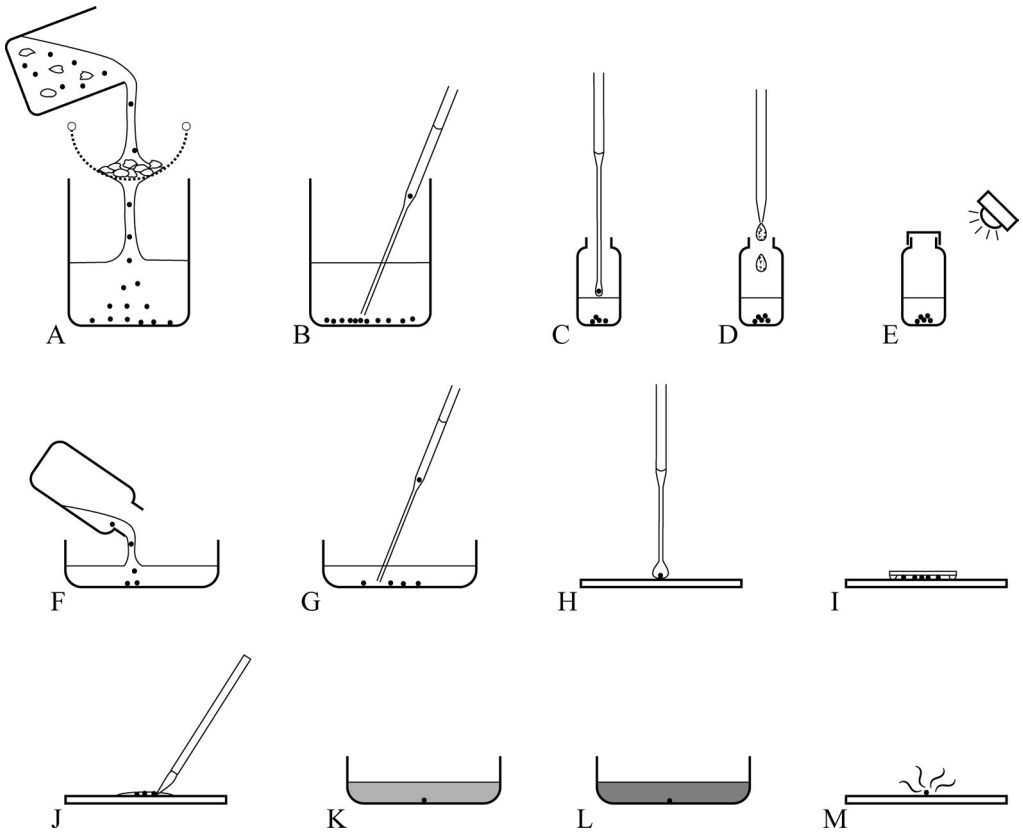

Fig. 2. The procedure for staining the radiolarian cells with PDMPO. A: filtering through a sieve with a 1-mm opening to remove 1 mm plankton; B: removing a small portion of the settled radiolarians from the bottles with a Pasteur pipette; C: transferring the radiolarian cells into a small glass tube with 16 mL of percolated seawater; D: adding PDMPO to the radiolarian tube; E: incubating the samples under an artificial day-night cycle (12 h day/12 h night) with red and blue LED lights for 24 hours at 26°C; F: moving the incubated samples into petri dishes; G: collecting the polycystines with a Pasteur pipette; H: dropping the cells on glass slides; I: attaching the cover slip to the slide; J: removing polycystines with a brush; K: immersing the cells in bleach for 1 hour, in order to dissolve the soft bodies of cells; L: immersing the cells in water for 1 hour; M: putting the cells on a tray to dry.

2J). They were immersed for 1h, to dissolve the soft bodies of the cells (Fig. 2K).

10) The cells were removed from the bleach with a fine brush to another petri dish filled with water for 1h, to remove the bleach (Fig. 2L).
11) The cells were removed from the petri dish with a fine brush, put on a tray to dry (Fig. 2M), and were observed using scanning electron microscopy.

Results

Experimental PDMPO treatment was applied to six polycystine cells from five species, *Rhizosphaera trigonacantha* Haeckel (Order Entactinaria, Family Rhizosphaeridae, based on De Wever et al. (2001)), *Acanthodesmia* sp. (Order Nassellaria, Family Acanthodesmiidae, based on De Wever et al. (2001)), *Spirocyrtis scalaris* Haeckel (Order Nassellaria, Family Artostrobiidae, based on De Wever et al. (2001)), *Spongaster tetras tetras* Ehrenberg (Order Spumellaria, Family Spongodiscidae, based on De Weber et al. (2001)), and *Rhopalastrum elegans* (Ehrenberg) (Order Spumellaria, Family Spongodiscidae, based on De Weber et al. (2001)). As a result, each of *Rz. trigonacantha*, *Acanthodesmia* sp. and *Sr. scalaris* cells emitted fluorescence. The other two cells of *Sg. t. tetras* and *Rp. elegans* did not emit green fluorescent light, although because of their stretched pseudopodia they were considered to be alive in the glass bottle.

Rhizosphaera trigonacantha Haeckel (Fig. 3A–D)

This cell has a large, spherical cortical shell (430 μm in diameter) with many short radial spines (Fig. 3A, B). The medullary shells (Fig. 1A) are hidden within the thick spherical intracapsulum (Fig. 3A, B). The cortical shell was brightly fluorescent at green wavelengths (Fig. 3C, D). The condition of the hidden skeletons within the intracapsulum was not recognizable.

Acanthodesmia sp. (Fig. 3E–I)

The skeleton consists of a large, D-shaped ring in the center of the test and a bilobate framework, but the illus-

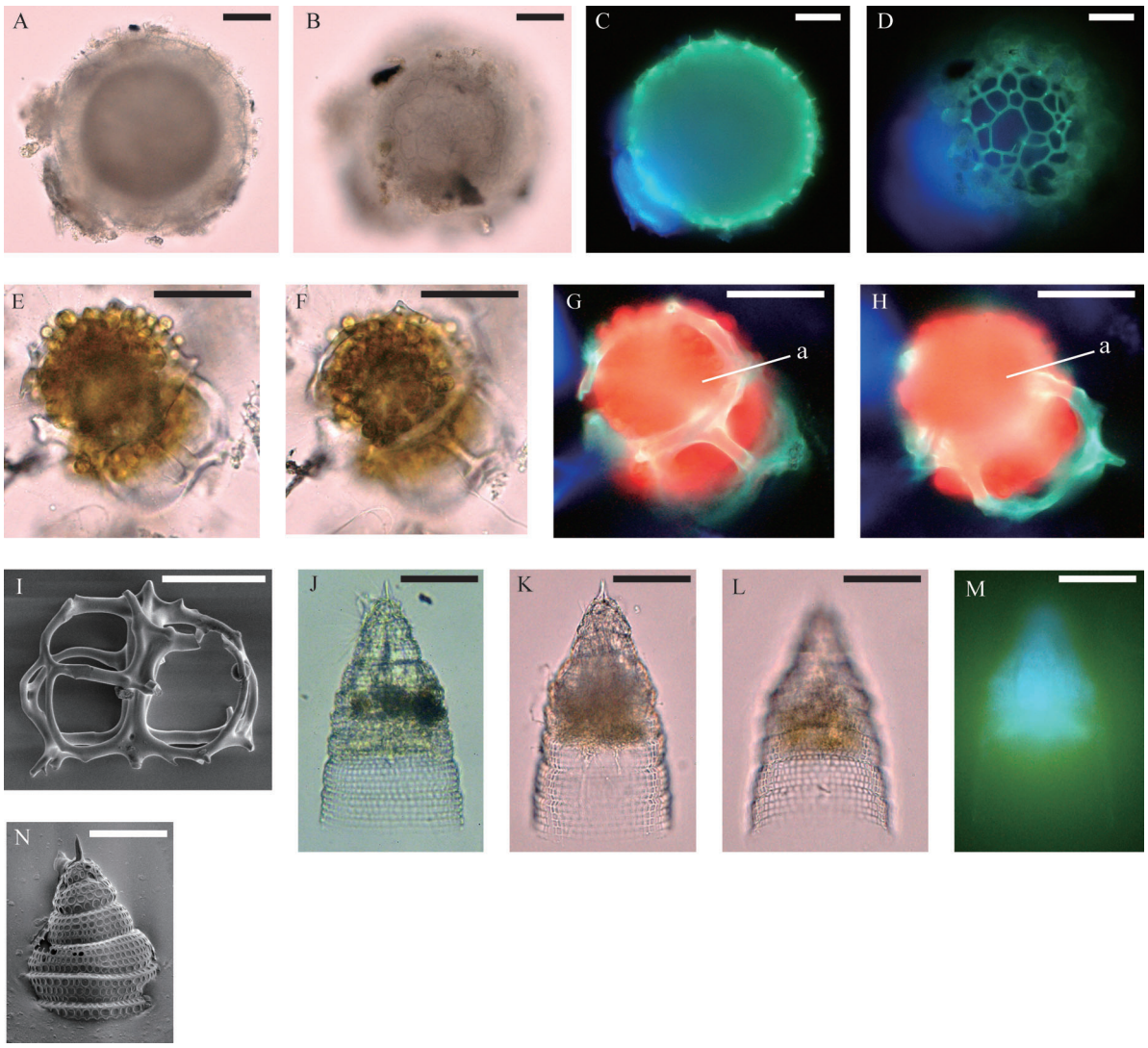

Fig. 3. A–D. *Rhizosphaera trigonacantha*. A, B: transmitted light microphotographs of a fixed cell focused in different planes; C, D: fluorescent microphotograph of a fixed cell focused in different planes. E–I. *Acanthodesmia* sp. E, F: transmitted light microphotographs of a fixed cell focused in different planes; G, H: fluorescent microphotograph of a fixed cell focused in different planes; I: SEM photograph of the skeleton. J–N: *Spirocyrtis scalaris*. J: transmitted light microphotograph of a living cell; K, L: transmitted light microphotographs of a fixed cell focused in different planes; M: fluorescent microphotograph of a fixed cell; N: SEM photograph of the skeleton. a: symbiotic algae. All scale bars = 50 μm.

trated cell faces the oblique distal end of the bilobate framework (Fig. 3E, F, I). Strong, green fluorescence is visible on the edge of the skeleton bars, while red fluorescence occurs in the symbiont algae (a in Fig. 3G, H), which appear as large, yellowish-orange spherules under normal light (Fig. 3G, H).

Spirocyrtis scalaris Haeckel (Fig. 3J–N)

This cell has a conical test with nine segments (Fig. 3J, K, L). This appearance is typical of most multi-segmented, conical Nassellaria since the early Triassic (ca. 245 million years ago). The conical intracapsulum is in the first to seventh segments and fluoresces bright bluegreen (Fig. 3M). The siliceous skeleton fluoresces green, but the light is blurred and much weaker than that in the intracapsulum (Fig. 3M).

Discussion

This study is the first to use PDMPO in polycystine radiolarians, and the results could shed light not only on the feasibility of PDMPO for polycystines, but also on its great potential in the study of silicification, because the tests of *Acanthodesmia* sp., *Rhizosphaera trigonacantha*, and *Spirocyrtis scalaris* emitted green fluorescent light to varying degrees (Fig. 3C, D, G, H, M).

The green fluorescent light emitted by polycystine shells,

similar to that seen in diatom shells (Shimizu et al. 2001), suggests that PDMPO was deposited on the polycystine skeleton. The fluorescent image of *Rz. trigonacantha*, without PDMPO, in Suzuki (2005) showed only blue auto-fluorescent light in the intracapsulum, and its test did not emit green fluorescent light. Other published fluorescent images of several polycystines without PDMPO treatment (Takahashi et al. 2003, Matsuoka 2007) showed blue, yellow, orange, and red auto-fluorescence of the soft body or symbiont, but no fluorescence from the skeleton.

The green fluorescence in PDMPO-treated polycystines suggests the presence of metabolic systems for silica polymerization with controlled pH, similar to that seen in diatoms (Shimizu et al. 2001). The PDMPO is accumulated with silica in acidic cell compartments of diatoms, and then deposited in the skeleton. The PDMPO accumulating on the polycystine skeletons suggests that the silica of the skeleton was deposited in a structure similar to the SDV. Our results suggest the presence of SDVs in polycystines, which was also suggested by Anderson (1981, 1994).

The brightness of the fluorescence varies with each species. The fluorescence of *Rz. trigonacantha* and *Acanthodesmia* sp. is bright (Fig. 3C, D, G, H), but *Sr. scalaris* was not as bright as other cells (Fig. 3M). The green fluorescent light of *Rz. trigonacantha*, *Acanthodesmia* sp., and *Sr. scalaris* suggest that these cells formed new skeletons after the addition of the PDMPO. The brightness of green fluorescent light reflects the concentration of PDMPO in the skeleton, and suggests differences in the accumulation rate of silica among cells. In contrast, *Spongaster tetras tetras* and *Rhopalastrum elegans* did not show any green fluorescent light. Both of them belong to the family Spongodiscidae. Matsuoka (1992) reported that *Rhopalastrum truncatum* Ehrenberg, which belongs to Spongodiscidae, developed its test step-by-step, and finally stopped growing. It is possible that the *Sg. t. tetras* and *Rp. elegans* we observed were not in a growth period or had stopped growing.

Further application of the PDMPO method could refine polycystine skeletogenic hypotheses and bring new insights into the physiological functions of silica assimilation. A previous study suggested that the concentric shell of the superfamily Actinommoidea develops by the addition of new spherical shells, while Cyrtellaria grows by making new segments (Hollande & Enjumet 1960, Petrushevskaya 1962, Itaki & Bjorklund 2007). This idea was verified by Suzuki (2006) who compared the various growth stages of the genus *Sphaeropyle* (Family Actinommidae). However, we found that the entire skeleton showed green fluorescence, which means that polycystine shells grow by thickening their skeleton rather than by making new segments. The further application of the PDMPO method could resolve this problem.

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References

Anderson OR (1981) Radiolarian fine structure and silica deposition. In: Silicon and Siliceous Structures in Biological Systems (eds Simpson TL, Volcani BE). Springer-Verlag, New York, pp. 347–380.

Anderson OR (1983) Radiolaria. Springer-Verlag, New York, 355 pp.

Anderson OR (1994) Cytoplasmic origin and surface deposition of siliceous structures in Sarcodina. Protoplasma 181: 61–77.

Anderson OR, Bennett P, Bryan M (1989) Experimental and observational studies of radiolarian physiological ecology: 3. Effects of temperature, salinity and light intensity on the growth and survival of *Spongaster tetras tetras* maintained in laboratory culture. Mar Micropaleontol 14: 275–282.

Anderson OR, Swanberg NR (1981) Skeletal morphogenesis in some living collosphaerid Radiolaria. Mar Micropaleontol 6: 385–396

De Wever P, Dumitrica P, Caulet JP, Nigrini C, Caridroit M (2001) Radiolarians in the Sedimentary Record. Gordon and Breach Science Publishers, Amsterdam, 533 pp.

Hazellar S, van der Strate HJ, Gieskes WWC, Vrieling EG (2005) Monitoring rapid valve formation in the pennate diatom Navicula salinarum (Bacillariophyceae). J Phycol 41: 354–358.

Hollande A, Enjumet M (1960) Cytologie, évolution et systématique des Sphaeroïdés (Radiolaires). Arch Mus Natl Hist Nat, Ser 7 7: 1–134.

Itaki T, Bjørklund, KR (2007) Conjoined radiolarian skeletons (Actinommidae) from the Japan Sea sediments. Micropaleontology 53: 371–389.

Matsuoka A (1992) Skeletal growth of a spongoise radiolarian *Dictyocoryne truncatum* in laboratory culture. Mar Micropaleontol 19: 287–297.

Matsuoka A (2002) Methods and research instruments for living radiolarian studies. Fossils 71: 19–27. (in Japanese with English abstract)

Matsuoka A (2007) Living radiolarian feeding mechanisms: new light on past marine ecosystems. Swiss J Geosci 100: 273–279.

Matsuoka A, Anderson OR (1992) Experimental and observational studies of radiolarian physiological ecology: 5. Temperature and salinity tolerance of *Dictyocoryne truncatum*. Mar Micropaleontology 19: 299–313.

Nishimura H (1990) Taxonomic study on Cenozoic Nassellaria (Radiolaria). Sci Rep Inst Geosci, Univ Tsukuba, B 11: 69–172.

Petrushevskaya MG (1962) Znacheniye rosta skeleta radiolyariy dlya ikh sistematiki. Zool Zh 41: 331–341. (in Russian with

English abstract)

Schröder HC, Perovic-Ottstadt S, Rothenberger M, Wiens M, Schwertner H, Batel R, Korzhev M, Müller IM, Muller WEG (2004) Silica transport in the demosponge *Suberites domuncula*: fluorescence emission analysis using the PDMPO probe and cloning of a potential transporter. Biochem J 381: 665–673.

Shimizu K, Del Amo Y, Brzezinski MA, Stucky GD, Morse DE (2001) A novel fluorescent silica tracer for biological silicification studies. Chem Biol 8: 1051–1060.

Sugiyama K, Anderson OR (1997) Experimental and observational studies of radiolarian physiological ecology, 6. Effects of silicate-supplemented seawater on the longevity and weight gain of spongoise radiolarians *Spongaster tetras* and *Dictyocoryne truncatum*. Mar Micropaleontol 29: 159–172.

Suzuki N (1998) Morphological terminology of spheroidal Polycystine (Radiolaria). NOM, Spec Vol 11: 251–287. (in Japanese with English abstract)

Suzuki N (2005) Physiological axopodial activity of *Rhizosphaera trigonacantha* Haeckel (a spheroidal radiolarian, Polycystina, Protista). Mar Micropaleontol 54: 141–153.

Suzuki N (2006). Ontogenetic growth and variation in the skeletal structure of two late Neogene *sphaeropyle* species (polycystina radiolarians). J Paleontol 80: 849–866.

Takahashi O, Mayama S, Matsuoka A (2003) Host-symbiont associations of polycystine Radiolaria: epifluorescence microscopic observation of living Radiolaria. Mar Micropaleontology 49: 187–194.

Thurow J, Anderson OR (1986) An interpretation of skeletal growth patterns of some Middle Cretaceous and modern radiolarians. Micropaleontology, 32: 289–302.

Vrieling EG, Poort L, Beelen TPM, Gieskes WWC (1999) Growth and silica content of the diatoms *Thalassiosira weissflogii* and *Navicula salinarum* at different salinities and enrichments with aluminium. Eur J Phycol 34: 307–316.