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<https://doi.org/10.1016/j.ijbiomac.2017.01.141>

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Isolation and identification of chitin from heavy mineralized skeleton of *Suberea clavata* (Verongida: Demospongiae: Porifera) marine demosponge



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ARTICLE INFO

Article history:

Received 30 November 2016

Received in revised form 8 January 2017

Accepted 31 January 2017

Available online 7 February 2017

Keywords:

Chitin
sponges
Suberea clavata

ABSTRACT

Since the discovery of chitin in skeletal structures of sponges (Porifera) in 2007, studies on search of novel species which possess this structural aminopolysaccharide continue up today. The most potential source of chitin is suggested to be localized in the four families of sponges related to the order Verongida (Demospongiae) which nevertheless require further clarification. Here, we report for the first time the isolation and identification of α -chitin from the *Suberea clavata* demosponge (Aplysinidae: Verongida). Raman spectroscopy, Calcofluor White staining, chitinase test and ESI-MS techniques were used to identify chitin. We suggest that the presence of chitin within fibrous skeletons of diverse species of Verongida order, and, especially in all species of the Aplysinidae family, may be useful for the identification of novel, previously unidentified marine demosponges.

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1. Introduction

Marine demosponges of the Verongida order, because of their ability to grow under marine ranching conditions [1,2], represent an extraordinarily renewable source of both biologically active secondary metabolites and naturally structured, tubular, three-dimensional chitin matrix which closely resembles the shape of the sponge skeleton. Nowadays, chitin of poriferan origin attracts a great attention as a unique scaffolding biomaterial for a variety of applications in biomedicine and modern materials science [3–6]. Recently, chitin have been identified as a skeletal component within numerous representatives of Aplysinidae family such as *Aplysina aerophoba*, *A. cavernicola*, *A. cauliformis*, *A. fistularis*, *A. fulva*, *A. clathrata*, *A. revillagigedi* and *A. gerardogreeni*, as well

as *Aiolochoria crassa* and *Verongula gigantea* (for overview, see [7,8]). We suggest that the presence of chitin within fibrous skeletons of diverse species of Verongida order, and, especially in all species of the Aplysinidae family, may be useful for the identification of novel, previously unidentified keratosan demosponges. Consequently, since the discovery of chitin in marine demosponges in 2007 [9], we are still busy with isolation and identification of this structural aminopolysaccharide in not yet examined sponges, for example, in *Suberea* species. Among the 14 species described today, over half of the *Suberea* species are reported from the Pacific Ocean, 3 from the Red Sea, 2 from Atlantic (one of which is under revision at the genus level) and a single species from the Indian Ocean. Most of them are coral reef-associated species, but one has been found on a deep sea mount [10]. Previously, most scientific attention have been paid to brominated tyrosine-related compounds extracted from *S. ianthelliformis* [11,12], *S. molis* [13–17], *S. creba* [18], *S. praetensa* [19,20] and a variety of unidentified

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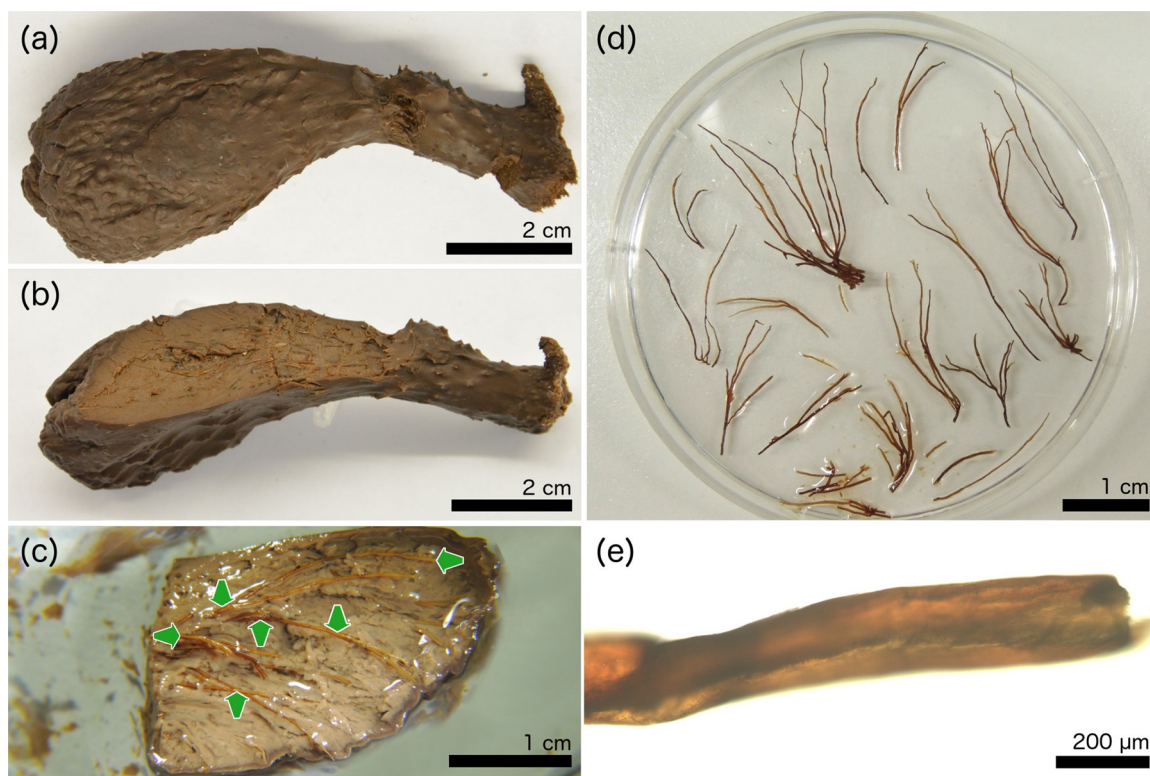


Figure 1. The surface of the massive body of *S. clavata* demosponge is smooth (a). Dense collagenous matrix rendering the sponge hard to just compressible (b). Skeletal fibers become visible (c, green arrows) after mechanical cutting of the sponge body. These very rigid fibers can be easily isolated from the sponge body manually (d, e) and used for further analytical investigations.

Suberea species [21–27]. In this study, we focused our attention on *Suberea clavata* (Aplysinidae: Verongida) demosponge (Fig. 1) that is known as a producer of bromine-containing clavatadines [28], but poorly investigated in its skeleton chemistry. In contrast to *Aplysina* species which can be characterized by their reticulated skeletons, representatives of *Suberea* mostly have dendritic skeleton [29] with only a few examples (*Suberea* species as *S. clavata*, *S. ianthelliformis* and *S. pedunculata*) containing some reticulated skeleton features [30].

The aim of this study was to isolate the skeletal fibers from *S. clavata* and to examine their chemical nature using modern bio-analytical methods. This study provides a detailed methodology for identification and characterization of chitin matter in marine demosponges.

2. Material and Methods

2.1. Sample collection and preparation

The specimen of *S. clavata* Pulitzer-Finali, 1982, originate from Bali, Indonesia. It was collected on April 19, 2001 during the expedition on Bali Lombok Strait. Geographical coordinates of the locality: N side of Nusa Nembongan, Tanjung Taal (=Tanjung Ental=“Blue”), lat: 08°39'33"S long: 115°26'37"E; Field#: BAL.30/190401/237. The specimen is originally stored in ethanol in Naturalis Biodiversity Center, Leiden, the Netherlands, under Nr.RMNH.POR.1578.

2.2. Isolation of chitin from *S. clavata*

The isolation of the chitin-based skeletal fibers from *S. clavata* was realized by a modified method, described by us previously (for review, see [8]). In brief, isolation was performed in the following steps (Fig. 2).

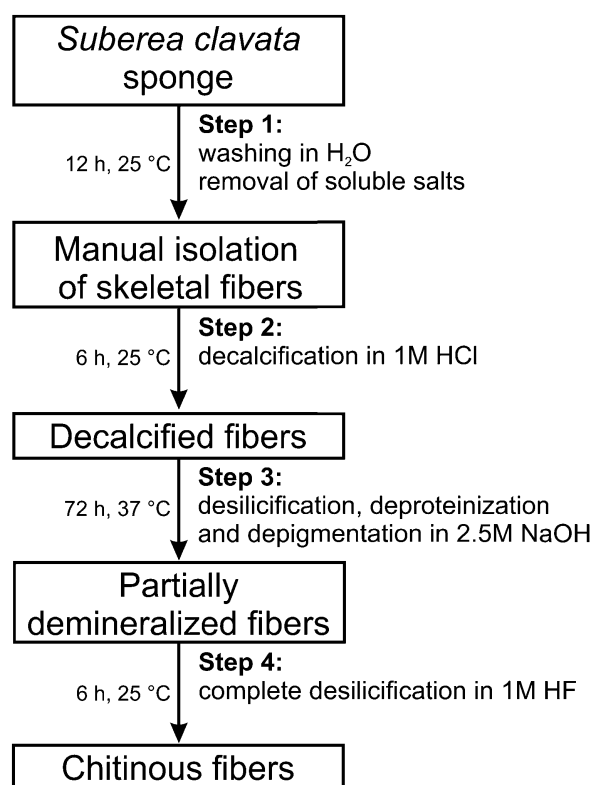


Figure 2. Step-by-step scheme of chitinous fibers isolation procedure from the skeletal fibers of marine demosponge *S. clavata*.

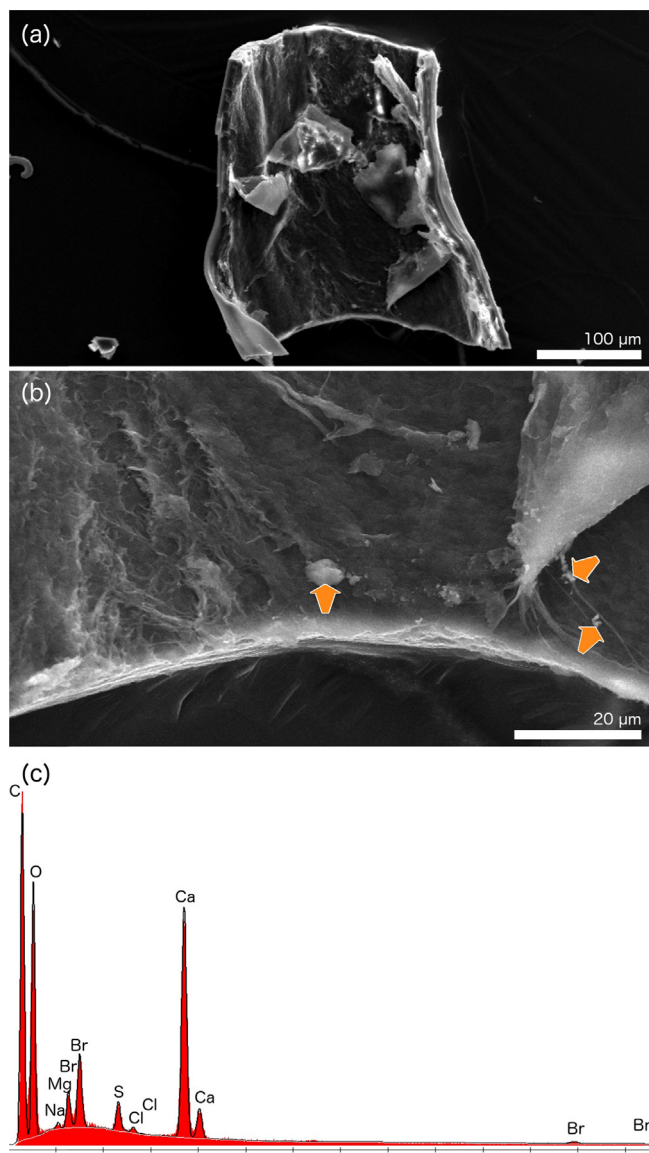


Figure 3. Identification of calcium carbonate-based mineral on the inner surface (a) of the skeletal fibers isolated from *S. clavata* after gentle disruption in liquid nitrogen. This surface remains microfibrillar in its morphology with numerous crystalline microparticles (arrows) visible using SEM (b). The results of EDX analysis (c) show with strong evidence the domination of Ca and Mg within these structures. The presence of Br and S is characteristic for naturally occurring skeletons of verongioid sponges.

Selected *S. clavata* sponge originally stored in 72% ethanol skeletons (Fig. 1a and b) was washed three times with distilled water for removal of residual water-soluble compounds like salts. The washed samples were placed into plastic boxes and cut into 1.5 × 3 cm large fragments (Fig. 1c). Well visible brownish-colored and mechanically rigid skeletal fibers (Fig. 1d and e) have been manually isolated and placed into Petri dish with distilled water (Fig. 2, Step 1). Selected specimens of manually extracted skeletal fibers have been decalcified using 1 M HCl and rinsed in distilled water up to pH 6.8 (Fig. 2, Step 2). Decalcified skeletal fibers have been treated with 2.5 M NaOH (Th. Geyer GmbH & Co. KG, Germany) at 37 °C for 72 h (Memmert Incubator, Germany) to carry out depigmentation, deproteinization as well as partial desilicification. After the removal of residual pigmentation using multiple washing in distilled water, corresponding colorless fibers have been obtained (Fig. 2, Step 3). Finally, colorless skeletal fibers have been treated

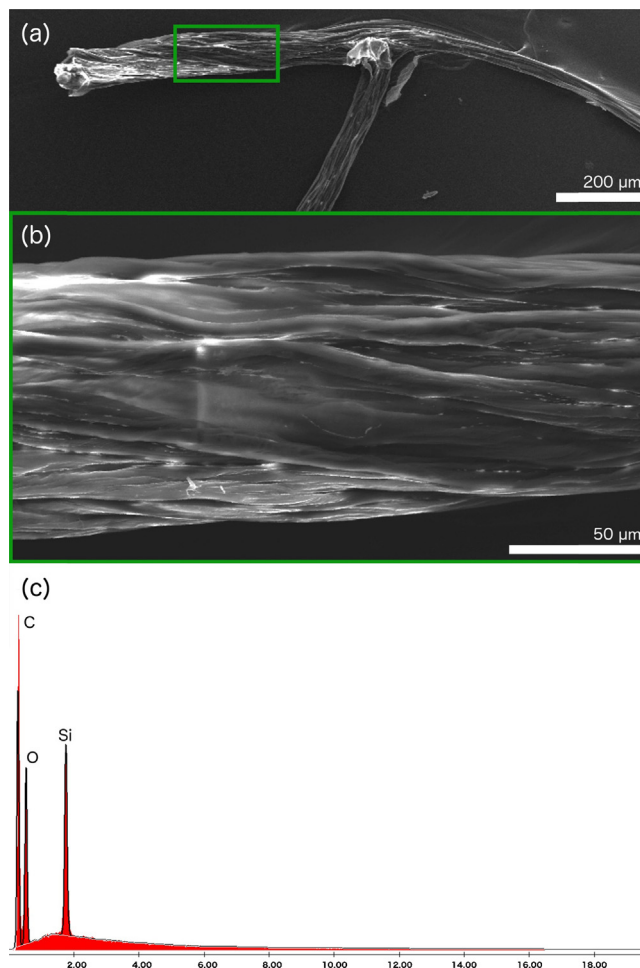


Figure 4. Decalcification of skeletal fibers of *S. clavata* using 1 M HCl as well as partial desilicification, deproteinization and depigmentation using 2.5 M NaOH lead to isolation of shrunken fibers with still high amount of silica embedded within surface layers of the organic matrix. Such fibers are still rigid and resistant to chitinase treatment.

with 1 M HF for complete desilicification. Afterwards, the samples were isolated from the plastic boxes and rinsed with distilled water up to pH 6.8. Obtained fibrous structures (Fig. 5a) were placed into 50 ml glass bottles and stored in deionized water at 4 °C till their use for analytical investigations with respect to chitin identification as reported below (Fig. 2, Step 4).

2.3. Light and fluorescent microscopy analysis and imaging

Collected sponge samples and isolated skeletal as well as purified chitinous fibers have been observed using stereomicroscope Di-Li (Germany), BZ-9000 microscope (Keyence) in light as well as in fluorescent microscopy modus.

Photos and macroscopic close-up pictures were made using camera Nikon D-7100 with objective lenses Nikon AF-S DX 18–105 mm f/3.5–5.6 G or Nikon AF-S VR Micro-Nikkor 105 mm f/2.8G IF-ED.

Figures were prepared using GNU Image Manipulation Program “GIMP 2.8”.

2.4. Calcofluor white staining test

We used calcofluor white (CFW) (Fluorescent Brightener M2R, Sigma-Aldrich), which shows enhanced fluorescence when it binds to chitin. The pieces of natural sponge skeleton samples and those

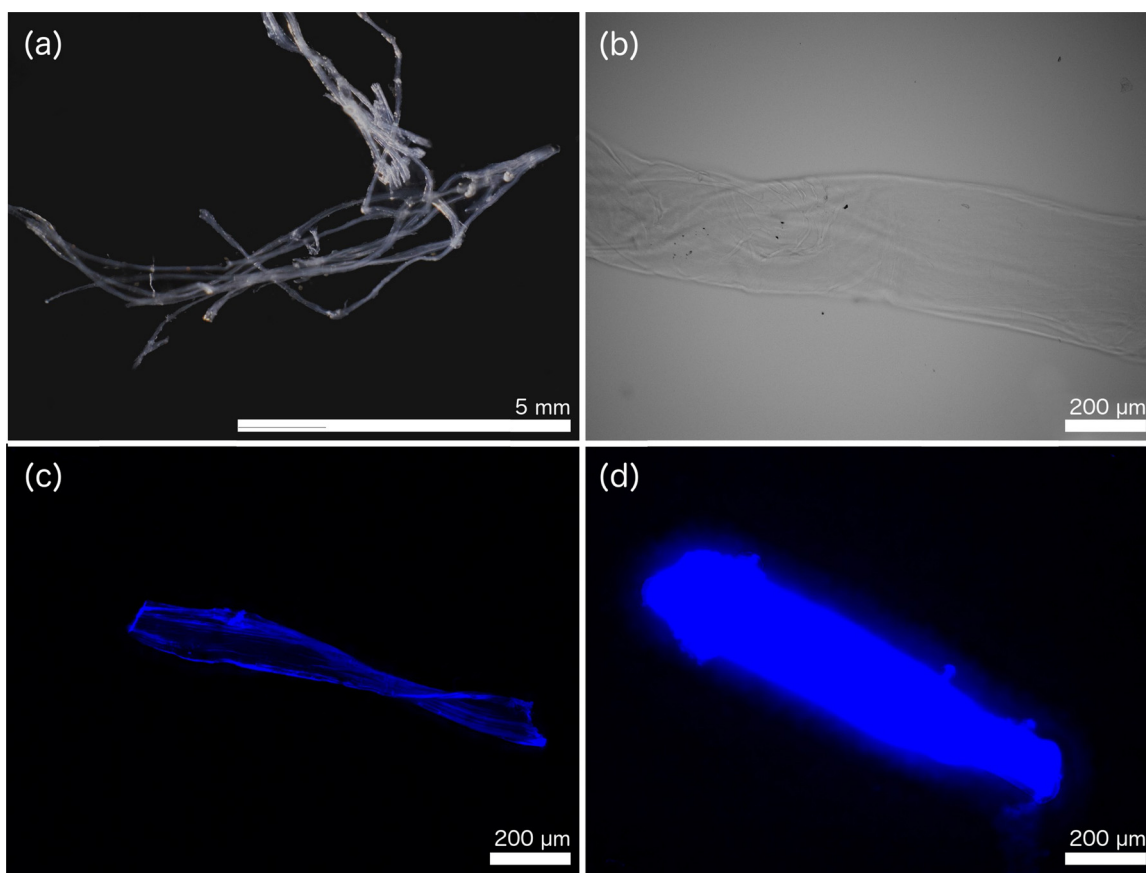


Figure 5. Photograph (a) and light microscopy image (b) of the completely demineralized, HF-treated and purified skeletal fibers of *S. clavata* (see Fig. 1 d and e). These fibers show characteristic for sponge chitin blue autofluorescence (c) as well as strong blue fluorescence (d) after staining of chitin using CFW (expose time 1/50 s).

which were subjected to demineralization (see Fig. 2) were placed in 0.1 M Tris-HCl, pH 8.5, for 30 min. After this procedure, they were stained using 0.1% calcofluor white solution for 30 min in darkness, rinsed three times with distilled water, dried at room temperature and finally investigated using fluorescent microscopy.

2.5. Scanning electron microscopy (SEM) analysis

The surface morphology, microstructure and EDX analysis of the isolated *S. clavata* skeletal fibers were examined on the basis of the SEM images. The samples were fixed in a sample holder and covered with a carbon layer for 1 min using an Edwards S150B sputter coater. The samples were then placed in an ESEM XL 30 Philips SEM.

2.6. Chitinase digestion test

Chitinase (EC 3.2.1.14, No. C-8241, Sigma-Aldrich, Germany) from the fungus *Trichoderma viride* was used. One unit of this chitinase releases 1.0 mg of *N*-acetyl-D-glucosamine from chitin per hour at pH 6.0 at 25 °C. Fiber portions of selected, completely demineralized fibers of *S. clavata* were incubated in chitinase, dissolved in 0.2 M citrate phosphate buffer at pH 4.5 at 25 °C for 12 h. Enzyme solutions were made in the same buffer at a concentration of 0.5 mg/mL. The effectiveness of the enzymatic digestion was monitored using optical microscopy (Keyence).

2.7. Raman spectroscopy

Raman spectra were recorded using a Raman spectrometer (Raman Rxn1TM, Kaiser Optical Systems Inc., Ann Arbor, USA) coupled to a light microscope (DM2500 P, Leica Microsystems GmbH,

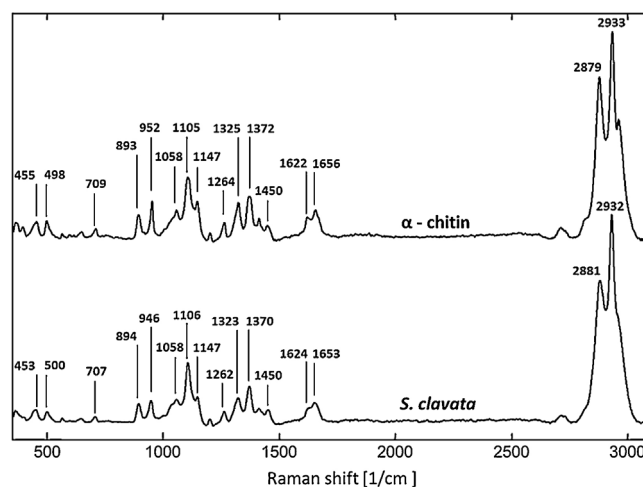


Figure 6. Raman spectra of the organic material isolated from skeletal fibers of *S. clavata* according to the procedure represented in Fig. 2 shows similar features to the α -chitin standard.

Wetzlar, Germany). For details, please see [8]. α -Chitin standard was purchased by INTIB GmbH, Freiberg, Germany.

2.8. Estimation of *N*-acetyl-D-glucosamine (NAG) contents and electro spray ionization mass spectrometry (ESI-MS)

The Morgan-Elson assay was used to quantify *N*-acetyl-D-glucosamine released after chitinase treatment. Dried, purified

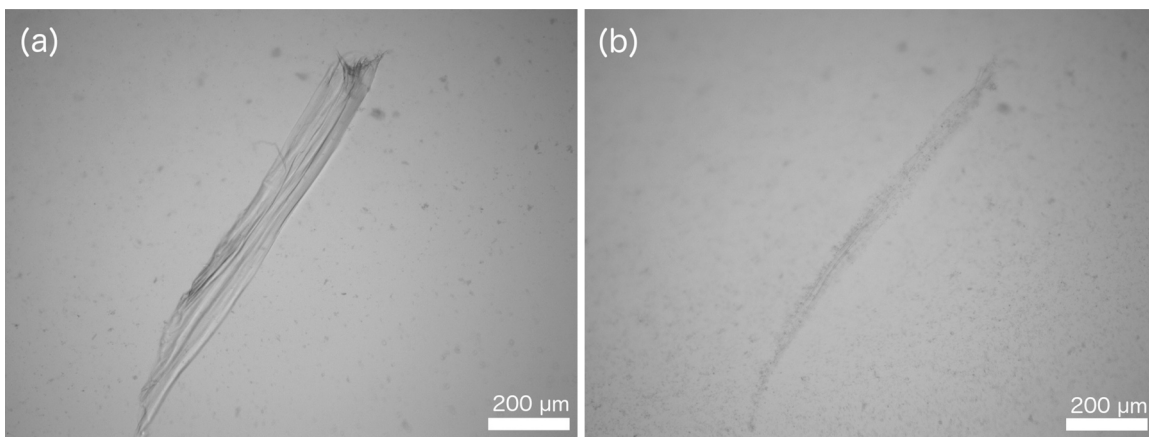


Figure 7. Chitinase digestion of purified and completely demineralized skeletal fiber isolated from *S. clavata*. Initial stage (a) and the same fragment after 17 h treatment with chitinase (b).

chitinous fibers of *S. clavata* (6 mg) were pulverized to a fine powder in anagate mortar. For details, please see [8].

Sample preparation for ESI-MS: specimens obtained before (Fig. 1e) and after HF treatment (Fig. 5a and b) were hydrolyzed in 6 M HCl for 24 h at 50 °C. The samples after the HCl hydrolysis were filtrated with 0.4 μm filter and freeze-dried in order to remove the excess of HCl. The solid remain was dissolved in water for ESI-MS analysis. The standard d-glucosamine as a control was purchased from Sigma (USA). All ESI-MS measurements were performed on Waters TQ Detector ACQUITYuplc mass spectrometer (Waters, USA) equipped with ACQUITYuplc pump (Waters, USA) and BEHC18 1.7 mm 2.1 × 50 mm UPLC column. Nitrogen was used as nebulizing and desolvation gas. Graphs were generated using Origin 8.5 for PC.

3. Results and Discussion

Skeletal fibers shown in Fig. 1d and e have been isolated manually from the sponge body using pincers. Because of their unusual hardness, it was suggested that some mineral compounds could be localized within them. This suggestion was based on the previously studied representatives of Aplysinidae family, *V. gigantea*, *A. cavernicola* and *A. cauliformis* in which skeletal chitin was identified as a specific template for two minerals (silica and aragonite in amorphous and crystalline forms) which are formed around the chitin microfibrils [31]. In the similar way, we disrupted skeletal fibers of *S. clavata* (Fig. 1d and e) in liquid nitrogen and studied the inner surface of selected fibers (Fig. 3a). Crystalline microparticles observed on the inner surface of the microfibrils (Fig. 3b) contain Ca and Mg (Fig. 3c) which suggests the presence of Mg-bearing calcite. In order to obtain chitin in purified form, we carried out the decalcification of the skeletal fibers using acidic treatment (Fig. 2). Surprisingly, our attempts to digest the decalcified fibers with chitinase were unsuccessful which suggested that other, acid-resistant, mineral phase was still localized within fibers. EDX analysis of them showed the presence of silica (Fig. 4). To dissolve residual silica from the organic matter, we used the HF treatment (Fig. 2) which lead to purified chitin (Fig. 5).

Characterization of chitin in purified skeletal fibers of *S. clavata* has been carried out using well-established analytical methods. The results of Raman spectroscopy investigations (Fig. 6) showed that *S. clavata* possess α-chitin. This is similar to the data obtained for chitin of demosponge origin reported previously [7,32–34]. Also, similar results have been obtained with chitinase treatment (Fig. 7). However, it must be noted that in this work we use the HF treatment for desilicification of the verongioid sponges skeleton. Usually, the

alkaline treatment of 2.5 M NaOH was enough to obtain purified chitinous fibers from other demosponges samples [7].

Additionally, the ESI-MS measurements were used to identify the presence of chitin. d-glucosamine (dGlcN) standard revealed in ESI-MS spectra (Fig. 8) two main signals with $M_{w/z} = 161.85$ and 179.9. The signal at $M_{w/z} = 179.9$ corresponds to a $[M+H^+]$ species with molecular weight of 178.9 which is dGlcN molecule (calculated: 179.1). The signal at $M_{w/z} = 161.85$ corresponds to a $[M+H^+]$ species with a molecular weight of 160.85 that is dGlcN ion $[M - H_2O + H^+]$ without one water molecule (calculated: 161.1) which is very common for this type of molecules [35]. There are also weak signals at $M_{w/z} = 358.93$ and 380.91 corresponding to $[2M+H^+]$ and $[2M+K^+]$ species which are proton- or potassium-bound dGlcN noncovalent dimer [36]. ESI-MS spectra of the *S. clavata* samples have revealed very similar to d-glucosamine standard signal pattern. Both the untreated (Fig. 1c) and completely demineralized (Fig. 5a and b) samples revealed strongest signals at $M_{w/z} = 161.91$ and 179.87 and weak signals at 358.92 and 381.05. These signals clearly point out the presence of dGlcN molecules in the sample which indicates the presence of chitin.

Thus, the discovery of chitin within skeletal fibers of *S. clavata* confirms our suggestion that chitin is a characteristic feature of the Aplysinidae family. More than 70% of the worldwide species has chitin in their skeletons and only less than 17% of them have cellulose [37]. With more than 7500 species described up to now, the phylum Porifera can represent an important group for chitin source. Chitin synthase genes have been reported for numerous sponge species (for review, see [38,39]), however it has not necessarily granted the location of chitin within their skeletal structures. For example, in some cases, chitin was identified within sponge holdfast and not in the exoskeleton [40] which suggested the importance of the chitin at the different stages of sponges development. It also has to be noted that the localization of chitin in sponges plays the crucial role for their practical application only in the form of naturally occurring and prestructured 3D scaffolds.

4. Conclusions

Here, we show for the first time that *S. clavata* represents the next demosponge species from the Verongida order that use chitin as structural component of its fibrous skeleton. Chitin has been identified unambiguously using Raman and ESI-MS spectroscopies, as well as with chitinase test. Phenomenon of multiphase biomineralization is well observable in this demosponge. In contrast to other sponges species reported previously, this organism possesses especially heavy mineralized skeletal fibers which contain both calcium

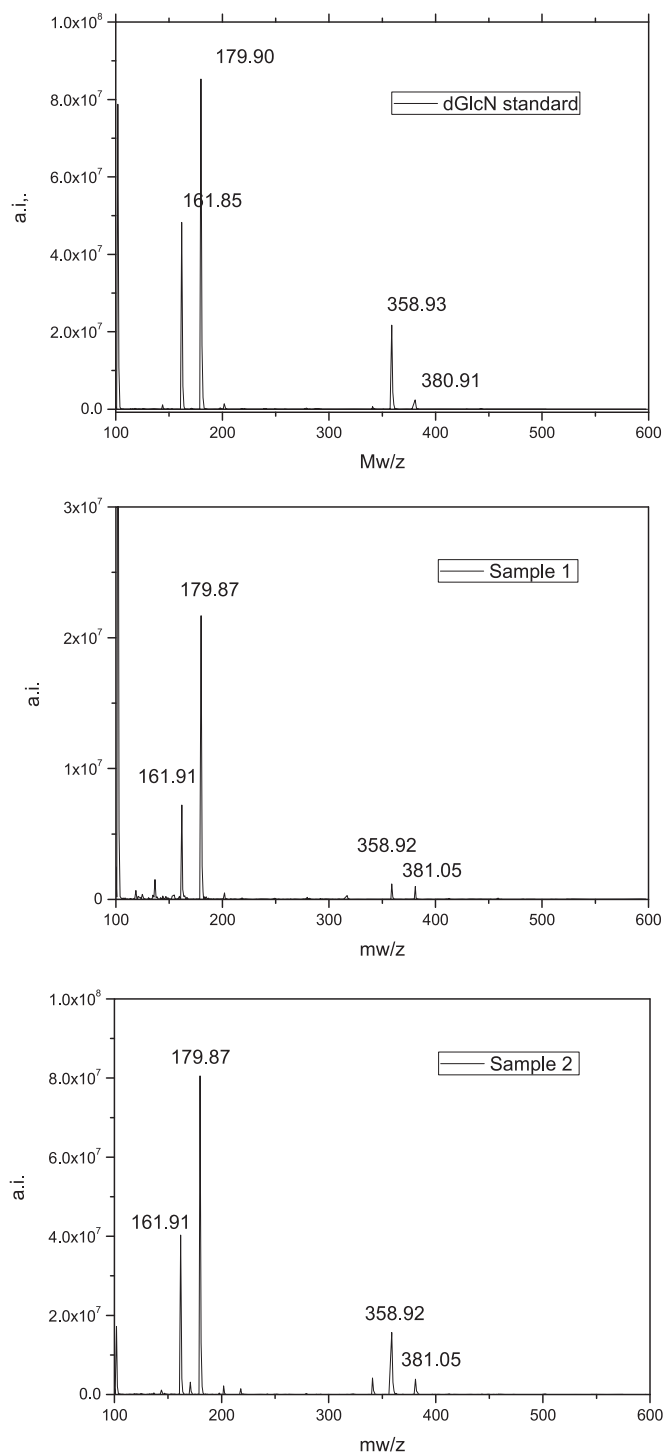


Figure 8. ESI-MS investigation of the chitin isolated from the skeletal fibers (Sample 1) as well as from purified chitinous fibers (Sample 2) of *S. clavata*.

carbonate and silica. This silica has been shown to be embedded within chitinous matrix and can be dissolved only after hydrofluoric acid treatment. The aim of the future studies is focused on comparative investigations of the presence and peculiarities of chitin in other species of the *Suberea* genus.

Acknowledgments

This work was supported by DFG Grant EH 394-3 (Germany) and PUT research grant 03/32/DSPB/0706/2017 (Poland). The authors

thank Prof. Dr. Yixin Zhang for the use of the ESI-MS instrument and Rob van Soest for his support and comments.

References

- [1] R. Manconi, F.D. Ledda, M.F. Ortu, A. Serusi, G.A. Stocchino, G. Corso, R. Pronzato, Spongicoltura integrata nell'AMP "Capo Caccia - Isola Piana": il caso di *Aplysina aerophoba* (Aplysinidae, Verongida), *Biol. Mar. Medit.* 12 (2005) 191–194.
- [2] H. Ehrlich, V.V. Bazhenov, S. Meschke, M. Bürger, A. Ehrlich, S. Petovic, M. Djurovic (2016) Marine invertebrates of Boka Kotorska Bay unique sources for bioinspired materials science, in: A. Joksimović, A. Djurović, M. Semenov, A.V. Zonn, I.S. Kostianoy, A.G. (Eds.), *The Boka Kotorska Bay Environment*, Series: *The Handbook of Environmental Chemistry*, Springer International Publishing AG, Berlin, Heidelberg, 2016, pp. 1–22.
- [3] H. Ehrlich, E. Steck, M. Ilan, M. Maldonado, G. Muricy, G. Bavestrello, Z. Kljajic, J.L. Carballo, S. Shiaparelli, A. Ereskovsky, P. Schupp, R. Born, H. Worch, V.V. Bazhenov, D. Kurek, V. Varlamow, D. Vyaliikh, K. Kummer, V.V. Sivkov, S.L. Molodtsov, H. Meissner, G. Richter, S. Hunoldt, M. Kammer, S. Paasch, V. Krasokhin, G. Patzke, E. Brunner, W. Richter, Three dimensional chitin-based scaffolds from Verongida sponges (Demospongiae: Porifera). Part II. Biomimetic potential and applications, *Int. J. Biol. Macromol.* 47 (2010) 141–147.
- [4] H. Ehrlich, Biomimetic potential of chitin-based composite biomaterials of poriferan origin, in: A.J. Ruys (Ed.), *Biomimetic biomaterials: structure and applications*, Woodhead Publishing, Sawston, Cambridge, UK, 2013, pp. 47–67.
- [5] M. Wysokowski, I. Petrenko, A.L. Stelling, D. Stawski, T. Jesionowski, H. Ehrlich, Chitin as a versatile template for extreme biomimetics, *Polymers* 7 (2015) 235–265.
- [6] I. Stepniak, M. Galinski, K. Nowacki, M. Wysokowski, P. Jakubowska, V.V. Bazhenov, T. Leisegang, H. Ehrlich, T. Jesionowski, A novel chitosan/sponge chitin origin material as a membrane for supercapacitors—preparation and characterization, *RSC Adv.* 6 (2016) 4007–4013.
- [7] H. Ehrlich, M. Ilan, M. Maldonado, G. Muricy, G. Bavestrello, Z. Kljajic, J.L. Carballo, S. Shiaparelli, A. Ereskovsky, P. Schupp, R. Born, H. Worch, V.V. Bazhenov, D. Kurek, V. Varlamow, D. Vyaliikh, K. Kummer, V.V. Sivkov, S.L. Molodtsov, H. Meissner, G. Richter, E. Steck, W. Richter, S. Hunoldt, M. Kammer, S. Paasch, V. Krasokhin, G. Patzke, E. Brunner, Three dimensional chitin-based scaffolds from Verongida sponges (Demospongiae: Porifera). Part I. Isolation and identification of chitin, *Int. J. Biol. Macromol.* 47 (2010) 132–140.
- [8] M. Wysokowski, V.V. Bazhenov, M.V. Tsurkan, R. Galli, A.L. Stelling, H. Stöcker, S. Kaiser, E. Niederschlag, G. Gärtner, T. Behm, M. Ilan, A.Y. Petrenko, T. Jesionowski, H. Ehrlich, Isolation and identification of chitin in three-dimensional skeleton of *Aplysina fistularis* marine sponge, *Int. J. Biol. Macromol.* 62 (2013) 94–100.
- [9] H. Ehrlich, M. Maldonado, K.D. Spindler, C. Eckert, T. Hanke, R. Born, C. Goebel, P. Simon, S. Heinemann, H. Worch, First evidence of chitin as a component of the skeletal fibers of marine sponges. Part I. Verongidae (Demospongia: Porifera), *J. Exp. Zool.* 308B (2007) 347–356.
- [10] R.W.M. Van Soest; N. Boury-Esnault, J.N.A. Hooper, K. Rützler, N.J. de Voogd, B. Alvarez de Glasby, E. Hajdu, A.B. Pisera, R. Manconi, C. Schoenberg, M. Klautau, B. Picton, M. Kelly, J. Vacelet, M. Dohrmann, M.-C. Díaz, P. Cárdenas, J.L. Carballo, *World Porifera database* (2016). Accessed at <http://www.marinespecies.org/porifera> on 2016-11-29/.
- [11] L. Mani, V. Jullian, B. Mourkaze, A. Valentin, J. Dubois, T. Cresteil, E. Folcher, J.N. Hooper, D. Erpenbeck, W. Aalbersberg, C. Debitus, New antiplasmodial bromotyrosine derivatives from *Suberea ianthelliformis* Lendenfeld, 1888, *Chem. Biodivers.* 9 (2012) 1436–1451.
- [12] M. Xu, R.A. Davis, Y.J. Feng, M. Sykes, T. Shelper, V.M. Avery, D.B. Camp, R.J. Quinn, Ianthelliformisamines A–C, antibacterial bromotyrosine-derived metabolites from the marine sponge *Suberea ianthelliformis*, *J. Nat. Prod.* 75 (2012) 1001–1005.
- [13] M.I. Abou-Shoer, L.A. Shaala, D.T.A. Youssef, J.M. Badr, A.M. Habib, Bioactive brominated metabolites from the Red Sea sponge *Suberea mollis*, *J. Nat. Prod.* 71 (2008) 1464–1467.
- [14] L.A. Shaala, S.I. Khalifa, M.K. Mesbah, R.W.M. van Soest, D.T.A. Youssef, Subereaphenol A, a new cytotoxic and antimicrobial dibrominated phenol from the Red Sea sponge *Suberea mollis*, *Nat. Prod. Commun.* 3 (2008) 219–222.
- [15] L.A. Shaala, F.H. Bamane, J.M. Badr, D.T.A. Youssef, Brominated arginine-derived alkaloids from the Red Sea sponge *Suberea mollis*, *J. Nat. Prod.* 74 (2011) 1517–1520.
- [16] L.A. Shaala, D.T. Youssef, J.M. Badr, M. Sulaiman, A. Khedr, Bioactive secondary metabolites from the Red Sea marine verongid sponge *Suberea* species, *Mar. Drugs* 13 (2015) 1621–1631.
- [17] A.T. Abbas, N.A. El-Shitany, L.A. Shaala, S.S. Ali, E.I. Azhar, U.A. Abdel-Dayem, D.T.A. Youssef, Red Sea *Suberea mollis* sponge extract protects against CCl₄-induced acute liver injury in rats via an antioxidant mechanism. *Evid. Based. Complement. Alternat. Med.* Article ID 745606 (2014) doi:10.1155/2014/745606.

- [18] C. Debitus, G. Guella, I. Mancini, J. Waikedre, J.-P. Guemas, J.L. Nicolas, F. Pietra, Quinolones from a bacterium and tyrosine metabolites from its host sponge, *Suberea creba* from the Coral Sea, *J. Mar. Biotechnol.* 6 (1998) 136–141.
- [19] A. Kijjoo, R. Watanadilok, P. Sonchaeng, P. Sawangwong, M. Pedro, M.S.J. Nascimento, A.M.S. Silva, G. Eaton, W. Herz, Further halotyrosine derivatives from the marine sponge *Suberea* aff. *praetensa*, *Z. Naturforsch.* 57 (2002) 732–738.
- [20] A. Kijjoo, R. Watanadilok, P. Sonchaeng, A.M. Silva, G. Eaton, W. Herz, 11,17-dideoxyagelarin A and B, new bromotyrosine derivatives and analogs from the marine sponge *Suberea* aff. *praetensa*, *Z. Naturforsch. C* 56 (2001) 1116–1119.
- [21] K. Hirano, T. Kubota, M. Tsuda, K. Watanabe, J. Fromont, I. Kobayashi, Ma'edamines, A and B, cytotoxic bromotyrosine alkaloids with a unique 2(1H)pyrazinone ring from sponge *Suberea* sp., *Tetrahedron* 56 (2000) 8107–8110.
- [22] M. Tsuda, Y. Sakuma, J. Kobayashi, Suberedamines A and B, new bromotyrosine alkaloids from a sponge *Suberea* species, *J. Nat. Prod.* 64 (2001) 980–982.
- [23] J. Carroll, E.N. Jonsson, R. Ebel, M.S. Hartman, T.R. Holman, P. Crews, Probing sponge-derived terpenoids for human 15-lipoxygenase inhibitors, *J. Org. Chem.* 66 (21) (2001) 6847–6851.
- [24] B.F. Bowden, B.J. McCool, R.H. Willis, Lihoudine, a novel spiro polycyclic aromatic alkaloid from the marine sponge *Suberea* n. sp. (Aplysinellidae, Verongida), *J. Org. Chem.* 69 (2004) 7791–7793.
- [25] K.H. Shaker, H. Zinecker, M.A. Ghani, J.F. Imhoff, B. Schneider, Bioactive metabolites from the sponge *Suberea* sp., *Chem. Biodivers.* 7 (2010) 2880–2887.
- [26] Y.L. Lee, S. Han, H.S. Lee, J.S. Kang, J. Yun, C.J. Sim, H.J. Shin, J.S. Lee, Cytotoxic psammaphysin analogues from a *Suberea* sp. marine sponge and the role of the spirooxepinisoxazoline in their activity, *J. Nat. Prod.* 76 (2013) 1731–1736.
- [27] A.D. Wright, P.J. Schupp, J.P. Schrör, A. Engemann, S. Rohde, D. Kelman, N. de Voogd, A. Carroll, C.A. Motti, Twilight zone sponges from Guam yield theonellin isocyanate and psammaphysins I and J, *J. Nat. Prod.* 75 (2012) 502–506.
- [28] M.S. Buchanan, A.R. Carroll, D. Wessling, M. Jobling, V.M. Avery, R.A. Davis, Y. Feng, Y. Xue, L. Oster, T. Fex, J. Deinum, J.N. Hooper, R.J. Quinn, Clavatadine A, a natural product with selective recognition and irreversible inhibition of factor XIa, *J. Med. Chem.* 51 (2008) 3583–3587.
- [29] M. Kelly, S. Amirapu, S. Mills, M. Page, H. Reisinger, Kermadec Islands sponge biodiversity: a review and description of a new species, *Suberea meandrina* sp. nov. (Demospongiae, Verongiida, Aplysinellidae), *Bull. Auckland Museum* 20 (2011) 311–324.
- [30] J. Gugel, M. Wagler, F. Brümmer, Porifera, one new species *Suberea purpureaflava* n. sp. (Demospongiae, Verongida, Aplysinellidae) from northern Red Sea coral reefs, with short descriptions of Red Sea Verongida and known *Suberea* species, *Zootaxa* 2994 (2011) 60–68.
- [31] H. Ehrlich, P. Simon, W. Carrillo-Cabrera, V.V. Bazhenov, J. Botting, M. Ilan, A.V. Ereskovsky, G. Muricy, H. Worch, A. Mensch, R. Born, A. Springer, K. Kummer, D.V. Vyalikh, S.L. Molodtsov, D.V. Kurek, M. Kammer, S. Paasch, E. Brunner, Insights into chemistry of biological materials: newly discovered silica-aragonite-chitin biocomposites in demosponges, *Chem. Mater.* 22 (2010) 1462–1471.
- [32] H. Ehrlich, M. Krautter, T. Hanke, P. Simon, C. Knieb, S. Heinemann, H. Worch, First evidence of the presence of chitin in skeletons of marine sponges. Part II. Glass sponges (Hexactinellida: Porifera), *J. Exp. Zool.* 308B (2007) 473–483.
- [33] H. Ehrlich, Chitin and collagen as universal and alternative templates in biomineralization, *Int. Geol. Rev.* 52 (2010) 661–699.
- [34] E. Brunner, H. Ehrlich, P. Schupp, R. Hedrich, S. Hunold, M. Kammer, S. Machill, S. Paasch, V.V. Bazhenov, D.V. Kurek, T. Arnold, S. Brockmann, M. Ruhnow, R. Born, Chitin-based scaffolds are an integral part of the skeleton of the marine demosponge *lanthella basta*, *J. Struct. Biol.* 168 (2009) 539–547.
- [35] J. Banoub, P. Boullanger, D. Lafont, A. Cohen, A. El Aneel, E. Rowlands, *In situ* formation of C-glycosides during electrospray ionization tandem mass spectrometry of a series of synthetic amphiphilic cholesteryl polyethoxy neoglycolipids containing N-acetyl-D-glucosamine, *J. Am. Soc. Mass Spectrom.* 16 (2005) 565–570.
- [36] J. Hsu, S.J. Chang, A.H. Franz, MALDI-TOF and ESI-MS analysis of oligosaccharides labeled with a new multifunctional oligosaccharide tag, *J. Am. Soc. Mass Spectrom.* 17 (2006) 194–204.
- [37] A. Chapman, Numbers of Living Species in Australia and the World, 2nd edition, Australian Biodiversity Information Services, Toowoomba, Australia, 2009.
- [38] H. Ehrlich, J.K. Rigby, J. Botting, M. Tsurkan, C. Werner, P. Schwillie, Z. Petrasek, A. Piseri, P. Simon, V. Sivkov, D. Vyalikh, S.L. Molodtsov, D. Kurek, M. Kammer, S. Hunoldt, R. Bor, D. Stawski, A. Steinhof, V.V. Bazhenov, T. Geisler, Discovery of 505-million-year old chitin in the basal demosponge *Vauxia gracilentia*, *Sci. Rep.* 3 (2013) 3497.
- [39] H. Ehrlich, O.V. Kaluzhnaya, E. Brunner, M.V. Tsurkan, A. Ereskovsky, M. Ilan, K.R. Tabachnick, V.V. Bazhenov, S. Paasch, M. Kammer, R. Born, A. Stelling, R. Galli, S. Belikov, O.V. Petrova, V.V. Sivkov, D. Vyalikh, S. Hunoldt, G. Wörheide, Identification and first insights into the structure and biosynthesis of chitin from the freshwater sponge *Spongilla lacustris*, *J. Struct. Biol.* 183 (2013) 474–483.
- [40] H. Ehrlich, O.V. Kaluzhnaya, M.V. Tsurkan, A. Ereskovsky, K.R. Tabachnick, M. Ilan, A. Stelling, R. Galli, O.V. Petrova, S.V. Nekipelov, V. Belikov, D. Janussen, V.V. Bazhenov, G. Wörheide, First report on chitinous holdfast in sponges (Porifera), *Proc. R. Soc. B* 280 (2013) 20130339.