

MORPHOLOGICAL CHARACTERIZATION OF *SKELETONEMA GREVILLEI* (BACILLARIOPHYTA, THALASSIOSIRALES) IN THE EASTERN ADRIATIC SEA

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ABSTRACT. – The present study shows the concise morphological description of *Skeletonema grevillei* Sarno & Zingone in the Šibenik Bay on the eastern coast of the Adriatic Sea. Increased abundance of the species was observed during January 2016. The scanning electron microscope (SEM) was used to detect the ultrastructural morphological features that distinguished the investigated species *S. grevillei* from the other species in the genus. The average diameter of the cell was 4.1 µm and the perivalvar axis of the valve was 2.2 µm. Intercalary fulcportula processes connecting two adjacent cells, formed a knuckle like junction that interlocked with one (1:1) and occasionally two (1:2) processes from the adjacent cell. Terminal fulcportula processes were open tubes, split along the entire length of the process, with irregularly truncated tips, which bore one or two small lateral spines. External processes of rimoportulae were short in the intercalary valves, but long in the terminal valves of the colony. The terminal rimoportula process had a truncated tip with a teapot spout-like end. The cingular band was composed of numerous copulae that had thin, transverse branching ribs, interspersed with 11-13 hyaline areas in 1 µm.

INTRODUCTION

Diatoms from the genus *Skeletonema* Greville (1865) regularly occur in the plankton community of coastal waters worldwide as important primary producers.

The genus *Skeletonema* was established by Greville (1865) for a single, now extinct species *Skeletonema barbadense*, based on light microscopy (LM). Greville (1866) identified a new species and described it as *Melosira costata*. Cleve (1873) recognized its similarity to *S. barbadensis* and transferred the former species to the genus *Skeletonema*, as *Skeletonema costatum* (Greville) Cleve (1873). Hasle (1973) described a rather wide range of morphological variability for *S. costatum* and some allied species. *Skeletonema costatum* once considered to be ubiquitous, has now been distinguished into several new species (Hasle *et al.* 1975, 1976, Medlin *et al.* 1991, Zingone *et al.* 2005, Sarno *et al.* 2005, 2007). Description of the genus was emended by Sarno & Zingone (Sarno *et al.* 2005). Several species of *Skeletonema* have been described since then.

Previous research has revealed both morphologic and genetic differences between *Skeletonema* species. Today they are grouped into four morphologically distinct groups (Sarno *et al.* 2007, Kooistra *et al.* 2008) and, according to AlgaBase (2017), 20 species are flagged as being currently accepted taxonomically. This new classification raises new questions about the diversity and seasonality of this genus, its relationship with other species in the phytoplankton community and the ecological

significance of the genus. These questions are yet to be explored.

Skeletonema is often dominant in coastal waters that are characterized by salinity fluctuations (Rijstenbil *et al.* 1987). Regarding the response of the genus to nutrient enrichment, previous studies have suggested that species belonging to the *Skeletonema costatum sensu lato* group induce intense blooming in areas rich in phosphates (Rodriguez *et al.* 2000) and ammonium nutrients (DeManche *et al.* 1979). This makes it a better competitor in eutrophic conditions in comparison to many other diatoms (Collos *et al.* 1997, Lagus *et al.* 2004). Since the increase in the levels of phosphate and ammonium is generally used as an indicator of anthropogenic impacts, this genus can also be considered as one of the indicator species of increased anthropogenic eutrophication (Ninčević *et al.* 2015). This is also supported by the findings of Collos *et al.* (2009) in Thau lagoon, in southern France, and by Šolić *et al.* (2010) in Kaštela Bay, in the eastern Adriatic Sea, where the oligotrophication process was accompanied by a reduction in the abundance of *S. costatum sensu lato*.

Previous studies (Bužančić *et al.* 2012, 2016) of the phytoplankton community in the investigated area of the Šibenik Bay show that *Skeletonema* represents a significant part of the diatom group. Previous studies of diatoms in the Adriatic Sea report *Skeletonema costatum* (Caroppo *et al.* 1999, Viličić *et al.* 2002, Bernardi Aubry *et al.* 2004) as a typical species of the winter Adriatic phytoplankton. However, Bužančić *et al.* (2012) and Skejić *et al.* (2014) report elevated abundances during stratified condi-

tions. In recent studies, some authors (Bernardi Aubry *et al.* 2006, Viličić *et al.* 2009, Godrijan *et al.* 2013) have reported *Skeletonema marinoi* Sarno & Zingone in the North Adriatic and have validated it by morphological analyses and molecular data (Sarno *et al.* 2005, Kooistra *et al.* 2008; Pfannkuchen *et al.* 2010). This indicates the existence of several *Skeletonema* species in the Adriatic Sea, and the need to differentiate them. The most recent finding of Marić Pfannkuchen *et al.* (2017) has reported three species of the genus in the North Adriatic, *Skeletonema menzelii* Guillard, Carpenter & Reimann, *S. marinoi* and *S. grevillei*. While *S. menzelii* is observed only sporadically, *S. marinoi* appears throughout the year with expressed blooms in late winter and early spring. The species *S. grevillei* is recorded in autumn and early winter, although some authors (Kooistra *et al.* 2008, Sarno *et al.* 2005) characterized it as a summer/autumn species, but based on a limited number of data.

Overall, the available data of the species composition of this genus in the Adriatic Sea are very scarce. These findings indicate a need for further studies of this genus, which is of a great ecological significance. For this purpose, the use of the scanning electron microscope (SEM) is irreplaceable. Species of the genus *Skeletonema*, like all other diatoms, have ornamented cell walls of opaline silica, which have important characteristics for morphological differentiation. In recent times, SEM data, along with the molecular analyses, have been foundational to nearly all levels of the diatom classification system (Round *et al.* 1990, Cox 2009).

The aim of this paper is to identify and describe in detail the morphology of *Skeletonema* species using SEM and to contribute to the understanding of the genus diversity and distribution.

MATERIAL AND METHODS

The sampling was performed during the winter bloom, in January 2016 at the station Strmica in the Šibenik Bay, located in the middle part of the eastern Adriatic coast (Fig. 1).

Strmica (43.776320 N; 15.848007 E) is situated in a highly stratified estuary of the karstic river Krka, with small tidal amplitudes and permanently brackish surface water (Svensen *et al.* 2007). The Krka River estuary is 25 km long and relatively narrow except for two wider parts, Prokljan Lake and Šibenik Harbor. The depth gradually increases from 5 m to 43 m at the mouth. The National Park Krka is upstream from the investigated area. The Krka River is one of the most pristine European rivers, characterized by low concentrations of nutrients and extremely low input of terrigenous material (Legović *et al.* 1994). Eutrophication in this area can be of natural or anthropogenic origin. Decomposition of freshwater phytoplankton greatly contributes to natural eutrophication and the regeneration of nutrients in the upper reaches of the estuary, whereas in the lower parts, the eutrophication favours anthropogenic sources (Legović *et al.* 1994, Svensen *et al.* 2007, Viličić *et al.* 1989). Previous studies of this area show that the main source of nitrates and orthosilicates is the Krka River, while the total phosphorus is mainly of anthropogenic origin from the Šibenik urban area (Legović *et al.* 1994). The investigated area of the Krka River is intended for the cultivation of shellfish, which emphasizes the need for constant monitoring with special attention to indicator species of increased eutrophication.

Sampling of *Skeletonema* diatoms was conducted using Water bottles and plankton nets according to the standard oceanographic methods (Strickland & Parsons 1972). Water samples (250 ml) for the light microscopy were collected with the sampling bottles and preserved with formaldehyde solution (2 % solution). For the SEM analysis, sampling was done using a plankton net (mesh size of 20 µm) and preserved with a Lugol solution (250 ml). Sampling was performed on the whole water column.

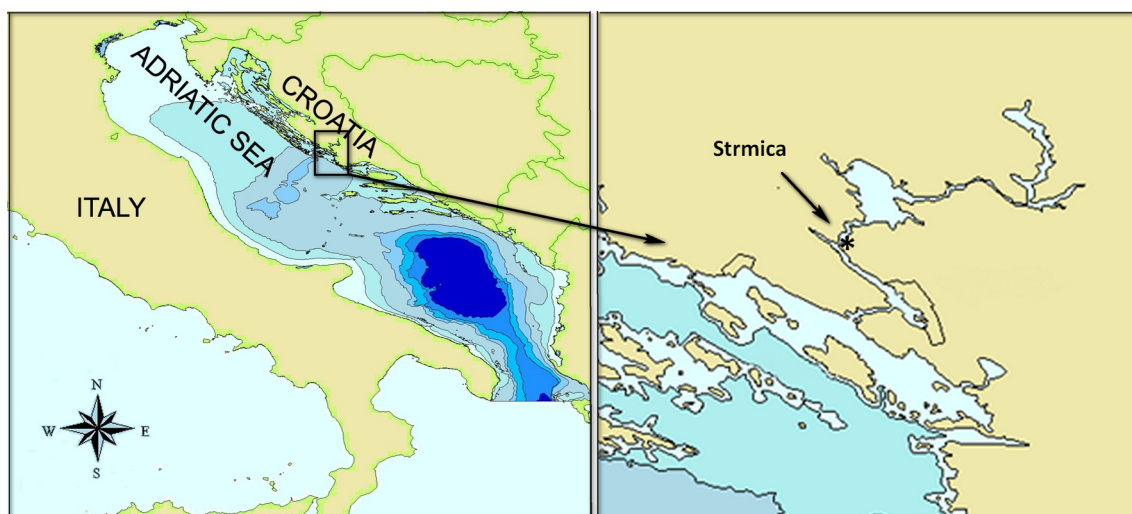


Fig. 1. – Study area with sampling station.

Morphological features of the genus were assessed by light microscopy (Leica DMI4000B) and SEM (Tescan MIRA3 FE SEM – field emission scanning electron microscope). For the light microscopy, subsamples of 25 ml were settled in counting chambers for at least 24 hours (h) (Utermöhl 1958). Sample processing was performed using an inverted microscope (Leica 4000B) at 400× magnification. For the SEM, net samples fixed with Lugol solution were prepared separately. Organic material from the cells was removed by adding 1 ml of 10 % hydrochloric acid (HCl), 2 ml of 30 % sulfuric acid (H₂SO₄) and 10 ml of saturated solution of potassium permanganate (KMnO₄) in 10 ml of net sample. After 24 h, approximately 10 ml of saturated oxalic acid (COOH)₂ was added slowly and afterwards rinsed three times in distilled water. The cleaned samples were then filtered on polycarbonate membrane filters with a pore size of 2 µm (Nucleopore, Whatman) and left to dry in a desiccator for at least 24 h. Filters were mounted on aluminium stubs, gold coated on a sputter coater (Quorum, Q150R ES) and observed with SEM. The accelerating voltage for the electrons (HV) was 5.0 kV and the SE detector was used to enhance topographic contrast of the cells.

Taxonomically important ultrastructural features were obtained by detailed examination of 180 cells. The intercalary and terminal cell measurements were expressed separately. Morphometric characters measured were the diameter of the cell, the length of perivalvar axis of the valve, the number of fulcra processes (FPPs) per cell, the distance between FPPs, the FPP

length, the FPP width and the position and the length of intercalary and terminal rimoportula processes (RPPs). The results were expressed on a µm scale with their minimum, maximum, average and standard deviations. In addition, the shapes of the valve and the terminal fulcra process (TFPP) and terminal rimoportula process (TRPP) tips were observed, as well as the linkage between intercalary fulcra processes (IFPPs).

RESULTS

The increased abundance of the species was determined by qualitative analysis of the sample under the LM. It has been established that the cells were forming short colonies (Fig. 2). However, to study detailed ultrastructural features of the cells, the collected samples have been processed with SEM (Fig.2). Morphometric characteristics obtained by detailed examination of the cells are shown in Table I.

Skeletonema grevillei forms chains of cells joined in the colony by elongate tubular processes that run perpendicular to the valve face (Fig. 3). A single ring of hollow processes emerges marginally around the top of the mantle. At the base of these processes, the short tubes or fulcra are located. On the internal face of the valve the fulcra is easily observed as a tubular process surrounded by three satellite pores (Fig. 4). Externally, an

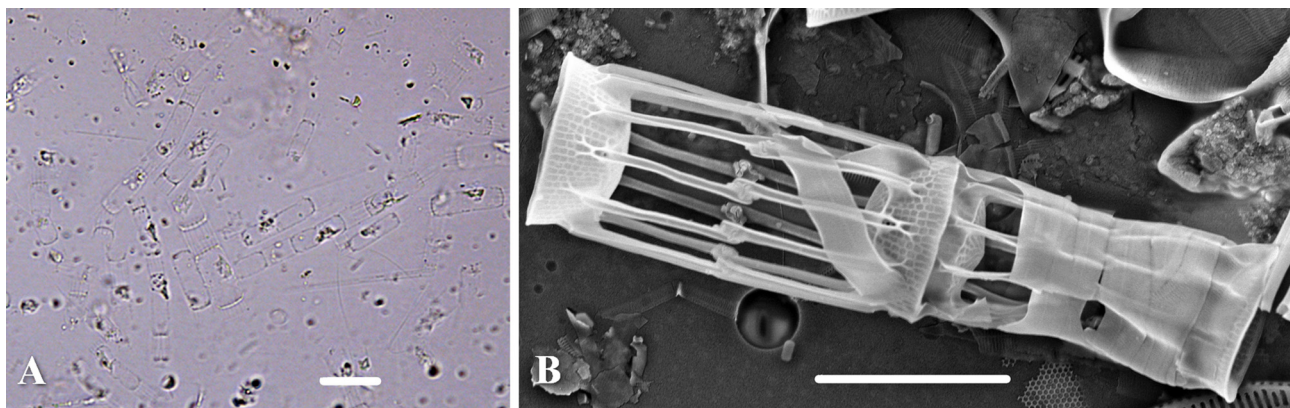


Fig. 2. – Micrographs of the diatom *Skeletonema grevillei* under LM (A) and SEM (B). Scale bars: A = 20 µm; B = 5 µm.

Table I. – Morphometric characteristics (minimum, maximum, average and standard deviation) associated with terminal and intercalary cells of *Skeletonema grevillei* Sarno & Zingone used in the present study. FPP, fulcra process; RPP, rimoportula process.

<i>Skeletonema grevillei</i>	Terminal cells (n = 60)				Intercalary cells (n = 120)			
	min	max	avg	st dev	min	max	avg	st dev
Diameter of the cell (µm)	4.1	11.8	6.4	1.3	4.2	15.9	7.5	2.8
Length of perivalvar axis of the valve (µm)	1.3	3.0	2.1	0.4	1.7	4.7	2.4	0.5
FPP per cell	9	21	12	2.1	6	20	12.4	3.1
Distance between FPP (µm)	0.8	1.7	1.3	0.2	1.0	2.6	1.6	0.3
FPP length (µm)	2.6	6.1	4.0	0.7	3.1	9.0	5.5	1.3
FPP width - BASAL (µm)	0.3	0.5	0.3	0.1	0.2	0.4	0.3	0.1
FPP width - DISTAL (µm)	0.4	1.1	0.7	0.1	0.2	0.4	0.3	0.1
RPP length (µm)	2.5	5.7	3.8	0.7	0.1	0.5	0.2	0.1

Fig 3. – Intercalary valves. **A:** Sibling valves with IFPPs open throughout the length, linked with 1:1 junctions (a) and a single 1:2 junction (b); short IRPP (c); **B:** 1:1 and 1:2 knuckle-like junctions of IFPPs; **C:** Short IRPP on intercalary valve (a) and siliceous ridges joining bases of the processes with mantle of the valve (b); Scale bars: A, B = 2 μ m; C = 1 μ m.

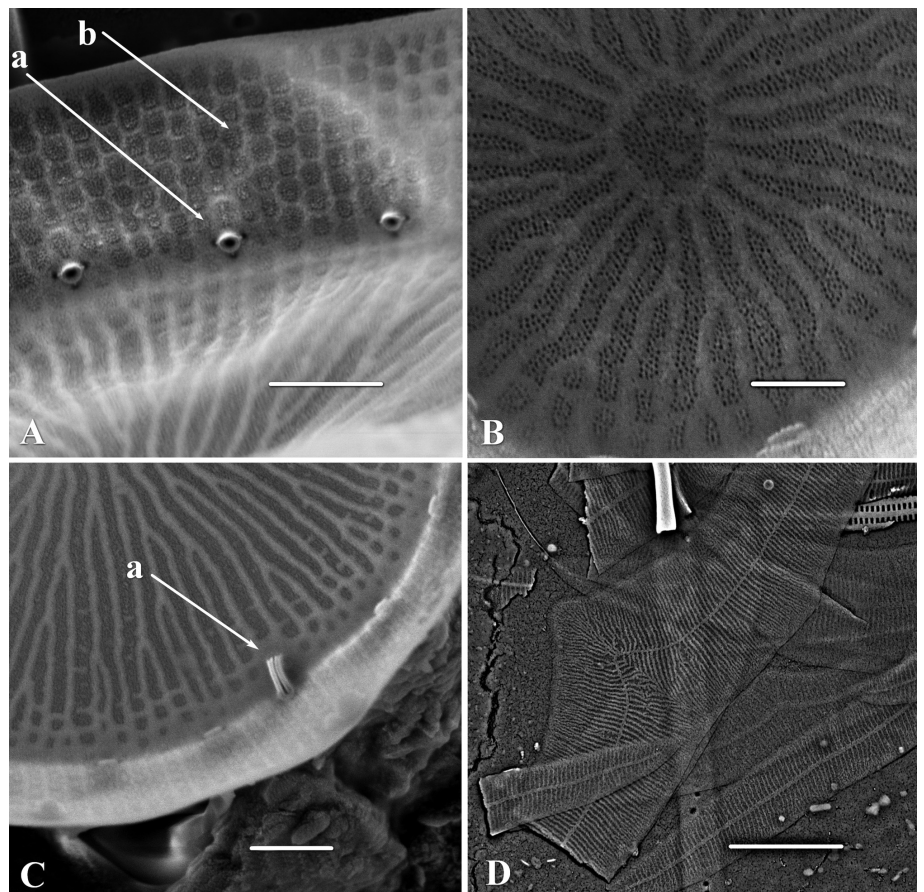
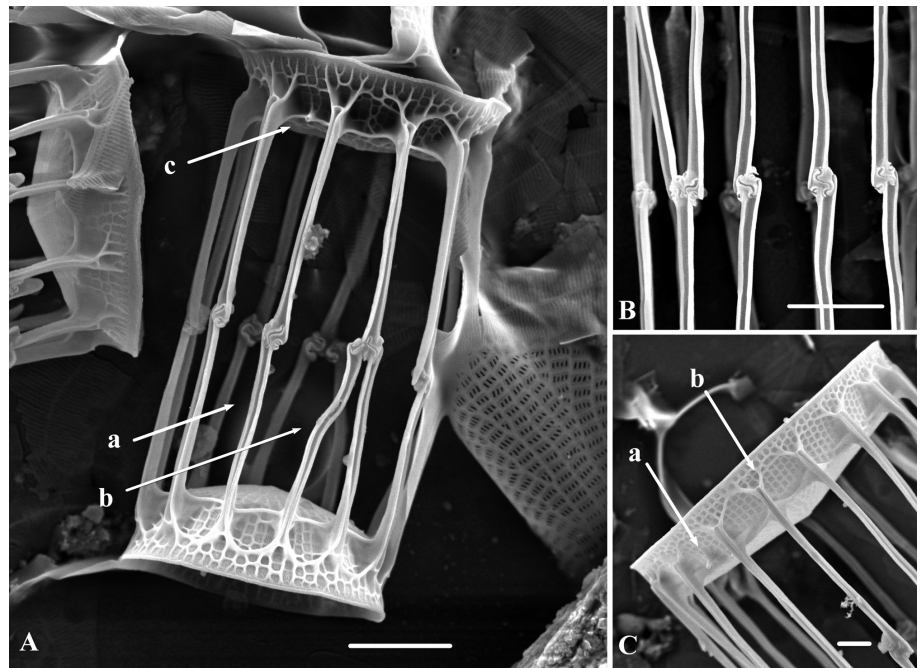


Fig. 4. – Cell details. **A:** Internal valve face with tubular process of fultoportulae surrounded by three satellite pores (a), loculate areole covered with the vellum, a siliceous layer with smaller pores (b); **B:** Internal valve face with the central annulus and distinct cribrum, a porous plate of silica covering the internal opening of an areola; **C:** Internal view of marginal rimoportula showing labiate process (a); **D:** Cingular band ultrastructure with thin transverse ribs interspersed by hyaline areas; Scale bars: A, C = 1 μ m; B = 0.5 μ m; D = 2 μ m.

extension of a fultoportula on the valve face appears as a simple tube, open along its length (Fig. 3).

The processes that connect two adjacent cells expand at their apices to form a knuckle-like junction that inter-

locks with the processes from the adjacent cell (Fig. 3). These are the intercalary fultoportulae processes (IFPPs). Each IFPP is interlocked with one (1:1) and occasionally two (1:2) processes of the sibling valve (Fig. 3). The

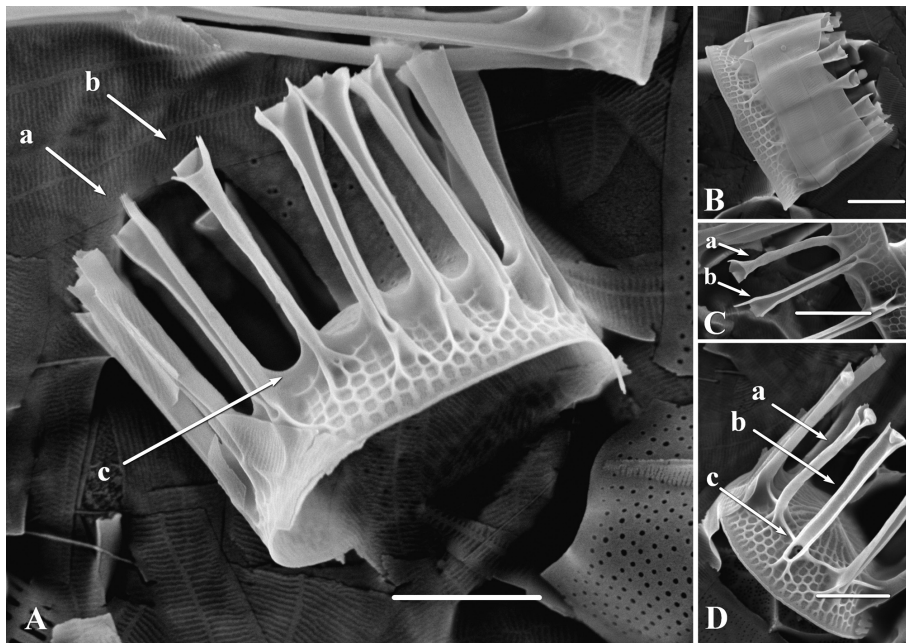


Fig. 5. – Terminal valves. **A:** Valve with a ring of irregularly truncated TFPPs (a), marginal rimoportula (b), siliceous ridges at the base of the processes joining them with the mantle and the valve (c); **B:** Valve with a newly divided cell with girdle bands still wound around TFPPs; **C:** Detail of an open tube of TFPP with a lateral spine at the tip (a), marginal TRPP with a truncated tip of the process with a teapot spout-like end (b); **D:** Detail of the valve with marginal TRPP (a), open tube TFPP with the spine at the tip (b), tubular opening of fultoportula on the valve (c); Scale bars: A-D = 2 μ m.

Table II. – Morphometric characteristics of *Skeletonema grevillei* recorded in this study (*) and comparison to morphologically similar taxa (Sarno *et al.* 2007 observation **). FPP, fultoportula process; TFPP, terminal fultoportula process; IFPP, intercalary fultoportula process; TRP, terminal rimoportula; IRP, intercalary rimoportula.

Species	Satellite pores	FPP shape	TFPP tip width	TFPP tip shape	IFPP Joints	TRP position	IRP	Cingular band ultrastructure
<i>Skeletonema grevillei</i> *	3	Split tube	Narrow	Truncated or spiny	1:1	Marginal	Short	Hyaline areas
<i>S. costatum</i> **	3	Close with a pore	Narrow	Clawlike	1:2	Marginal	Long	Rows of pores
<i>S. ardens</i> **	3	Split tube	Narrow	Spiny	1:1	Marginal	Short	Hyaline areas
<i>S. grethae</i> **	3	Split tube	Narrow	Clawlike, truncated or spiny	1:1	Central	Short	Hyaline areas
<i>S. marinoi</i> **	3	Split tube	Flared	Dentate	1:2	Central	Short	Rows of pores
<i>S. menzeli</i> **	2	Split tube	Narrow	Spiny	–	Central		Hyaline areas

terminal fultoportulae processes (TFPPs) are different from the IFPPs (Fig. 5). TFPPs are also open tubes, split through the entire length of the process, but with irregularly truncated tips, which bear one or two small lateral spines (Fig. 5).

A single rimoportula (labiate process), a tubular process through the valve of the cell, is located at the base of the FPP. On the internal valve face, the rimoportula opening has the shape of a pair of lips (Fig. 4). On the external valve face, the rimoportula opening is a tube extending out from the valve. The external process of the rimoportula is located marginally, in both intercalary and terminal valves (Fig. 3), but long in the terminal valves (Fig. 5) of the colony, which is a taxonomic feature for *Skeletonema grevillei*. TRPP has a truncated tip of the process with a teapot spout-like end (Fig. 5).

Thin siliceous ridges join the bases of the processes and valve, and can also be found on the mantle (Fig. 5).

The valve face is slightly convex and the mantle is deep. Internally, the valve has distinct cribra on the surface (Fig. 4). Epivalves and hypovalves are connected with a series of coupling siliceous bands that form the cingulum. The cingular band ultrastructure of *Skeletonema grevillei* is distinct. Each copula of the cingular band has a ligula and antiligula on the central longitudinal ridge, from which the transverse ribs are branching one each side, interspaced by smooth hyaline areas with fine perforations (Fig. 4). *Skeletonema grevillei* have 11-13 hyaline areas in 1 μ m. The main morphological characteristics distinguishing *S. grevillei* from very similar congeneric species are given in Table II.

DISCUSSION

Taxonomically important ultrastructural features that differentiate species in the genus *Skeletonema* are the location of rimoportula processes, the shape of fultoportula

processes and the way they link together to form chains, the ultrastructure of cingular band and the cell surface ornamentation. Based on these ultrastructural features, this investigation shows that *Skeletonema*, which was recorded in the Šibenik Bay, is *Skeletonema grevillei* Sarno & Zingone. One of the most important taxonomic features that points to *S. grevillei* is the siliceous ridges on the valve surface at the base of the FPPs and on the valve mantle (Sarno *et al.* 2007, Zingone *et al.* 2005). In addition, *S. grevillei* differs from most other species of *Skeletonema* by having a marginally located terminal rimportula process (TRP). This feature is shared with *Skeletonema costatum* (Greville) Cleve and *Skeletonema ardens* Sarno & Zingone, whereas in most other species it is located closer to the centre of the cell (Sarno *et al.* 2005, Sarno *et al.* 2007). The features that morphologically differentiate *S. grevillei* from *S. costatum sensu stricto* are open IFPPs, as opposed to closed tubes with a pore at the base, and a short intercalary rimportula process (IRPP) as opposed to the long IRPP of *S. costatum* (Zingone *et al.* 2005). The interlocking mode of the IFPPs differs, as *S. grevillei* has aligned 1:1 junction lines as opposed to the distinct zigzag 1:2 junctions of *S. costatum*. The cingular band ornamentation of *S. grevillei* has thin, transverse branching ribs, interspaced by smooth hyaline areas, unlike *S. costatum* which has uniseriate rows of pores (Zingone *et al.* 2005). *Skeletonema ardens* shares with *S. grevillei* the marginal position of TRPP, the split tube of IFPP, the 1:1 junctions and the delicate cingular band, however, the flame-like edges of the TFPPs differentiate it (Sarno *et al.* 2007). *Skeletonema grevillei* resembles *Skeletonema cf. grethae* Sarno & Zingone in the split tube of the IFPP, narrow irregularly truncated TFPP ends, the 1:1 knuckle-like junctions, the short IRPP and the ultrastructure of cingular bands (Sarno *et al.* 2005, Sarno *et al.* 2007), however, they differ in the TRPP position and the ornamentation of the cell. This comparison of important morphometric ultrastructural features points to *S. grevillei*.

Balzano *et al.* (2011) showed that *Skeletonema* has euryhaline responses, but noticed the differences among species and strains. Kooistra *et al.* (2008) suggested that *Skeletonema grevillei* has a wide distribution range, but the research was based on data found infrequently and at very distant sites. The most recent finding of Marić Pfannkuchen *et al.* (2017) reported *S. grevillei* in the northern Adriatic, which is also the first report for the Adriatic Sea and Mediterranean. This study confirms the presence of this species in the middle part of the eastern Adriatic coast, but raises new questions about ecology, as it was found in the estuary of the Krka River.

The distribution pattern and ecological characterization of *Skeletonema grevillei* are still poorly known. This points to the need for further investigation of this species and the genus in general, including its ecology, especially when *Skeletonema* is considered one of the indicator species of increased anthropogenic eutrophication.

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