Oceanological and Hydrobiological Studies
International Journal of Oceanography and Hydrobiology

Volume 42, Issue 2

ISSN 1730-413X
eISSN 1897-3191

(209–215)
2013

VERSITA

DOI: 10.2478/s13545-013-0077-8
Short communication

Received: *March 21, 2012*
Accepted: *December 05, 2012*

Ulva flexuosa subsp. *pilifera* (Chlorophyta, Ulvophyceae) from the Wielkopolska region (West Poland): a new observation on the ultrastructure of vegetative cells

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Key words: *Ulva flexuosa* subsp. *pilifera*, Malta Reservoir, morphology, ultrastructure

Abstract

Ulva flexuosa subsp. *pilifera* previously known from northern Poland, from the channel near Szczecin and ponds near Łódź, has recently been found in the Malta Reservoir in the Wielkopolska (West Poland) region. Specimens collected in the Wielkopolska region were examined in detail, also under a transmission electron microscope (TEM). The morphometric analysis of *Ulva* thalli (both young and mature specimens) was performed in order to study the differences in the ultrastructure of vegetative cells. Rectangular cells in young thalli measured from 32.21 - 55.81 µm to 20.24 – 35.12 µm, and they formed clear longitudinal rows, while cells in the mature specimens ranged from 25.09 – 47.66 µm to 18.90 – 31.56 µm. This study indicates that vegetative cells of the mature thalli show tendency towards distortions of both the longitudinal and transverse cells arrangement. This distortion is determined by the development of possible carbonate calcium crystals on the thalli surface. The ultrastructural analysis (TEM) confirmed that the structure and placement of thylakoids is genus/species specific.

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INTRODUCTION

Chlorophytes, generally known as green algae (the name comes from chlorophyll *a* and *b*), are a morphologically and ecologically diverse group with over 80% of the species occurring in freshwater habitats (John 2003). Hoek et al. (1995) divided chlorophytes into 12 classes, based mainly on ultrastructural studies of vegetative cells. The class Ulvophyceae contains unicellular, multicellular and siphonocladus non-flagellate green algae. Each cell contains one parietal chloroplast with one or several pyrenoids, which are cup-shaped. The cell walls consists of a structural fraction, made of microfibrils, embedded in the amorphous matrix. The microfibrils are usually arranged irregularly, forming a felt-like network (Hoek et al. 1995). The order Ulvales contains 24 genera and 175 species (John 2003, Leskinen et al. 2004), almost all being marine, with just a few freshwater species. The genus Ulva was one of the first described by Linnaeus (1753) and initially included many unrelated algae. The name *Ulva* was retained for green algae with distromatic blades, and *Enteromorpha* Link was assigned to tubular green algae (Link 1820). Recently, *Enteromorpha* has been regarded as synonymous with *Ulva* based on molecular data (Hayden et al. 2003). Many species are adapted to a wide range of salinity, temperature and water quality, and grow rapidly in nutrient-rich habitats causing green tides and marine fouling (Fletcher 1996). *Ulva* species are also often used in

experimental systems as model organisms for studies of algal physiology (Johnston 1991, Larsson and Axelsson 1999), spore adhesion (Dillon et al. 1989, Fletcher and Callow 1992) and as bioindicators of marine pollution (Favero et al. 1996).

The marine forms of *Ulva* genera occurring in the inland waters of Poland have been observed since the nineteenth century at more than 70 freshwater sampling sites (Messyasz and Rybak 2009). Most of them are in the northwest and central territory of Poland. *Ulva* (syn. *Enteromorpha*) *flexuosa* subsp. *pilifera* (Kütz.) Bliding 1963 (=*Enteromorpha pilifera* Kützing 1845) has been identified at four sampling sites in Poland, between June and October 1970 in the Międzyodrze channel (Szczecin) near the A2 expressway (Kowalski 1975). According to Sitkowska (1999), this species occurred in the period of 1984-1987 in fish ponds in Piotrowice and in a pond in Kuciny in 1994-1995. In 2009, *Ulva flexuosa* subsp. *pilifera* was found in the Malta Reservoir in Poznań (Rybak and Messyasz 2011). Late spring and summer are the seasons when this *Ulva* taxon most consistently proliferate in the Polish inland waters. In the studies reported from around the world, *Ulva flexuosa* subsp. *pilifera* has been identified at many inland sampling sites (Skácelová 2004, Mareš 2009, Kaštovský et al. 2010, Mareš et al. 2011). Marine and freshwater *Ulva* thalli are different. Freshwater forms of *Ulva* occur only as monostromatic tubular thalli (e.g. *Ulva intestinalis*, *Ulva compressa*, *Ulva flexuosa*). *Ulva* with distromatic frondose thalli, however, has not been reported in freshwater ecosystems (Messyasz and Rybak 2009, Rybak and Messyasz 2011). In the case of marine forms, young thalli of *Ulva* are always attached to substrate, while thalli of freshwater species can develop unattached as free-floating mats (Bilding 1968, Starmach 1972). The thallus of *Ulva flexuosa* subsp. *pilifera* can be up to 1 meter long. According to Starmach (1972) and Pliński (1988), cells of this species are square or rectangular (22-30 x 12 μm) and form longitudinal and crosswise rows. Chloroplasts have from 2 to 4 pyrenoids, but sometimes up to 6 pyrenoids. The zoospores are up 10 μm long and 5 μm wide. Male gametes (6.3 × 2.7 μm) are slightly smaller than female ones (6.7 × 3.4 μm). As generally stated, the gametes can germinate without the inseminating process. Occasionally, the number of pyrenoids can change during the lifespan of the alga, since they may be numerous in cells of sporelings but less numerous in cells of the mature thallus. Griffiths (1970) suggested that it is difficult to correlate the presence or absence of a pyrenoid with any of the major classification criteria. It seems that there is more data about marine *Ulvales* species than about the freshwater variety, and this is also true for TEM. In the 1970s, ultrastructural studies led to reclassification of this group (Melkonian 1979, McArthur and Moss 1973). Most studies of *Ulva* marine species focused on the ultrastructure aspects of the rhizoid cell morphology (Bråten 1975), the mitosis process (Lovlie and Bråten 1970), zoospores (Callow et al. 2001), and their flagellar apparatus (Melkonian 1979), and on differences in the thickness of the thylakoid membrane (Bond et al. 1997). Moreover, as evidenced by the study of marine *Ulva flexuosa*, high Ca^{2+} concentration resulted in thickening and smoothing of the cell wall internal layers, an increase in the number of starch granules, and an increase in the dimension and the number of cytoplasmic lipid droplets (Andrade et al. 2004). It is also evident that in marine forms of *Ulva*, the chloroplast takes up most of the cell volume.

MATERIALS AND METHODS

Study area

Samples of *Ulva flexuosa* subsp. *pilifera* (Kütz.) Bliding 1963 were taken from the surface of the Malta Reservoir in Poznań (52°24´14,0˝N; 16°57´21,9˝E to 52°24´0,2˝N; 16°59´13,1˝E). This is an artificial reservoir constructed in 1952 for recreation by damming the waters of the Cybina River. International rowing and canoeing regattas have been held here (Mikuła 1996). The water quality has deteriorated consistently since the reservoir's construction. Attempts to improve the water quality have taken different forms, including draining of the water (first in 1980, then regularly every 4 years), deepening of the reservoir (1981), elimination of all sources of sewage discharge into the reservoir and the river, restoration of ponds located in estuary of the river and the water flow regulation (1983), bioremediation (since 1993) and application of an aerator to activate phosphorus in the waters by ferric sulfate (since 2005) (Mikuła 1996, Kozak et al. 2007, Goldyn et al. 2010). *Ulva* was observed in various habitats of the Malta reservoir since 2009 (Messyasz and Rybak 2009). The material for ultrastructure analysis was collected near the Cybina River inflow into the reservoir, which is a shallow place with rocky bottom and reduced wave motion. In general, *Ulva* covers a significant part of the reservoir.

Sample preparations

The research was carried out in June 2011 when the freshwater population of *Ulva* was in the optimal phase of its development. Samples of young and mature thalli of *Ulva* were collected at two sites of the Malta Reservoir (Fig. 1). The thalli samples were collected into a plastic box and transported in a refrigerated container (at 4°C) to a laboratory. Next, the thalli were rinsed repeatedly with distilled water in order to remove any other algae, vascular plants (Lemnoideae), and small snails attached to them. Laboratory analysis included measurements of thalli (length, width, the presence of proliferations) and examination of the cell morphology (the size of cells, the number of pyrenoids, the shape and arrangements of cells) using a light microscope (40×) and the program ProCap. Chemical and physical parameters of the water (temperature, electrolytic conductivity, oxygen concentration, and anions: NO_3^-, NH_4^+, $P\text{-}PO_4^{3-}$, PO_4^{3-}, Cl^-, and pH) were measured using the *YSI Professional Plus* handheld multiparameter meter. Parts of each sample were used for cultivation on agar plates in the laboratory. Cultures of *Ulva* species were incubated in the standard Bristol agar medium (Stein 1979) at a temperature of 20°C and in the diurnal regime (12/12 h light/dark cycle, 3000 $\mu Em^{-2}\ s^{-1}$, using 40 watt cool fluorescent tubes). In order to obtain the monocultures, the cultures were checked for contaminations every 12 days. For transmission electron microscopy (TEM), cells were fixed as previously described (Massalski et al. 1995). Ultra-thin sections were prepared, and double stained with uranyl acetate and lead citrate. TEM was performed under a TESLA BS 500 transmission electron microscope. Fresh specimens of *Ulva* were photographed. Identification of *Ulva* species was based on the following literature: Starmach (1972), Pliński (1988), Blomster et al. (1998), Hayden et al. (2003) and Wynne (2005).

Results and Discussion

Ulva flexuosa subsp. *pilifera* is one of the five *Ulva* species which have been recorded in the Polish inland waters, and it is the most rare taxon, observed at four locations (Messyasz and Rybak 2009). In the Wielkopolska region, this species was observed for the first time in the Malta Reservoir in 2009. The abundant *Ulva* development in this flow-through water reservoir was connected with great availability

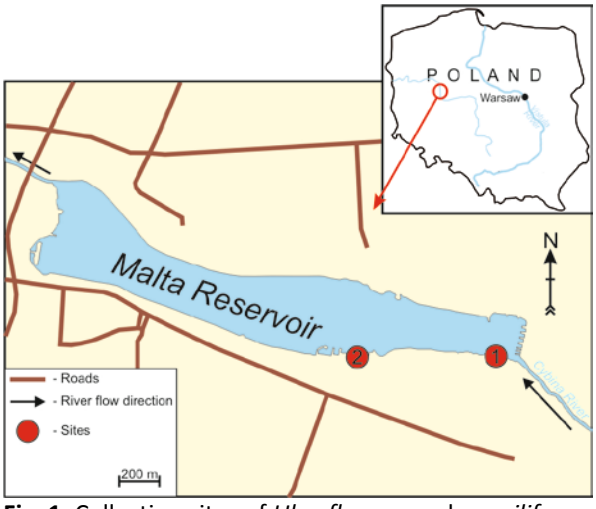

Fig. 1. Collection sites of *Ulva flexuosa* subsp. *pilifera* specimens in the Malta Reservoir.

of nutrients in the water, particularly N (Rybak and Messyasz 2011). In June 2011, the water level in the littoral zone of the Malta Reservoir was approximately 1.5 m and the water pH was 7.84 in the area with *Ulva flexuosa* subsp. *pilifera*. The value of electrolytic conductivity, however, fluctuated around 680 $\mu S\ cm^{-1}$. Oxygen concentrations were variable: 7.0 mg l^{-1} at the first site, and 6.52 mg l^{-1} at the second one. Average concentrations of NO_3^-, NH_4^+, $P\text{-}PO_4^{3-}$, PO_4^{3-}, NaCl and Cl^- were: 0.02 mg l^{-1}, 0.10 mg l^{-1}, 0.10 mg l^{-1}, 0.03 mg l^{-1}, 120 mg l^{-1} and 73 mg l^{-1}, respectively. These findings were consistent with many other studies previously published (Kowalski 1975, Sitkowska 1999, Mareš 2009, Rybak and Messyasz 2011, Mareš et al. 2011). It is interesting that the highest values (twice as high as in 2011) of nutrient concentrations in the water were recorded in 2009 when *Ulva flexuosa* subsp. *pilifera* was observed for the first time in the Malta Reservoir (Rybak and Messyasz 2011). *Ulva flexuosa* subsp. *pilifera* thalli collected in the present study from the surface of the Malta Reservoir on 16 June 2011 were green, varied in size and had many proliferations. Thalli of the submerged form (young specimens) were from 10.1 cm to 65.1 cm long, and from 0.2 cm to 1.5 cm wide. Free floating thalli (mature and dying specimens) were from 14.9 to 55.1 cm long and from 0.5 cm to 2.0 cm wide (Table 1). Vegetative cells contain one parietal, perforated chloroplast with ulvophycean pyrenoids and transverse cell walls without plasmodesmata. The cell walls are made of two distinct electron-dense and fuzzy layers containing the contiguous electron-dense, fibrous material. In the case of cell measurements, all

material was divided into young and mature thalli groups, and analyzed separately. The results of the measurements showed that (rectangular) cells in young thalli were 32.21 – 55.81 μm × 20.24 – 35.12 μm, and they formed clear longitudinal rows (Fig. 2, Table 1). While cells in mature thalli (both the shape and the arrangement of rows are less regular than in young thalli) were smaller and measured 25.09 – 47.66 μm × 18.90 – 31.56 μm. The presence of numerous possible calcium carbonate crystals on the surface of mature thalli, and their impact on the arrangement of cells was an interesting finding. The cells are arranged radially around the crystals (Figs 3-6, Table 1). The crystals were observed under a polarizing microscope, and a large amount of Ca^{+2} ions strongly indicates that the crystals are made of calcium carbonate. Detailed chemical analysis of these crystals will be the subject of a separate study.

Fig. 2. *Ulva flexuosa* subsp. *pilifera* – macroscopic view of marine thalli – LM.

Fig. 3. *Ulva flexuosa* subsp. *pilifera* - macroscopic view of freshwater forms – LM.

Table 1

The mean (standard deviation), maximum and minimum values of morphometric parameters of *Ulva flexuosa* subsp. *pilifera* from the Malta Reservoir.

	thallus (N=120)		young thallus (N=60)		mature thallus (N=60)	
	Length (cm)	Width (cm)	Length (μm)	Width (μm)	Length (μm)	Width (μm)
Min.	10.10	0.20	32.21	20.24	25.09	18.90
Max.	65.10	2.00	55.81	35.12	47.66	31.56
Average	29.09	0.72	41.99	26.40	35.89	24.66
Standard deviation	10.42	0.42	6.06	3.94	5.01	3.55

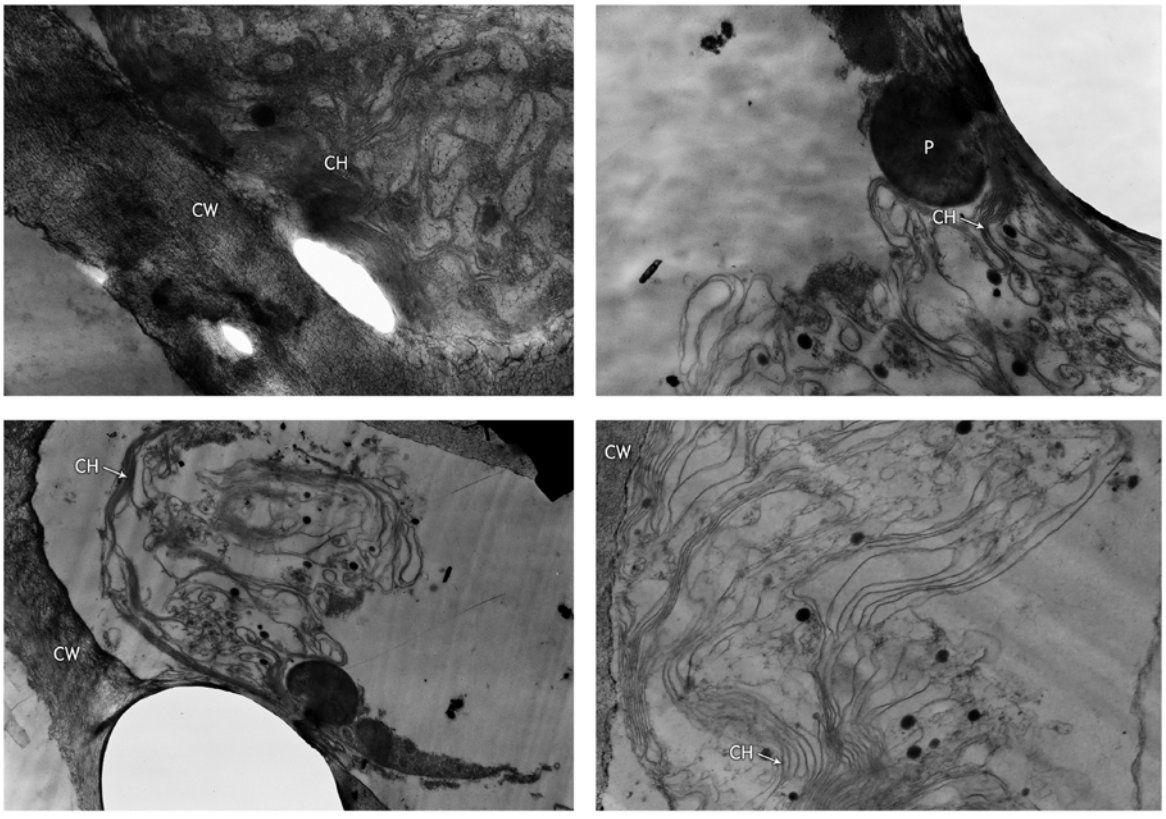

Fig. 4. Longitudinal sections of *Ulva flexuosa* subsp. *pilifera* showing the peripheral chloroplast (CH), arrangement of thylakoids (T), nucleus (N) and vacuoles (V). Note the oval pyrenoid (P) - TEM.

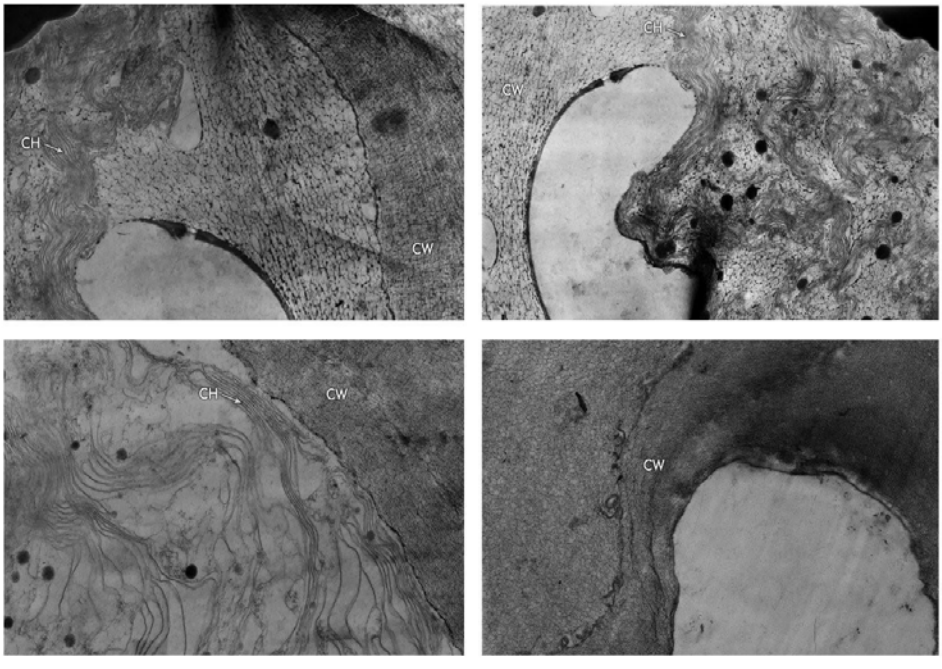

Fig. 5. Longitudinal and transverse section of *Ulva flexuosa* subsp. *pilifera* showing the number and arrangement of thylakoids (T) - TEM.

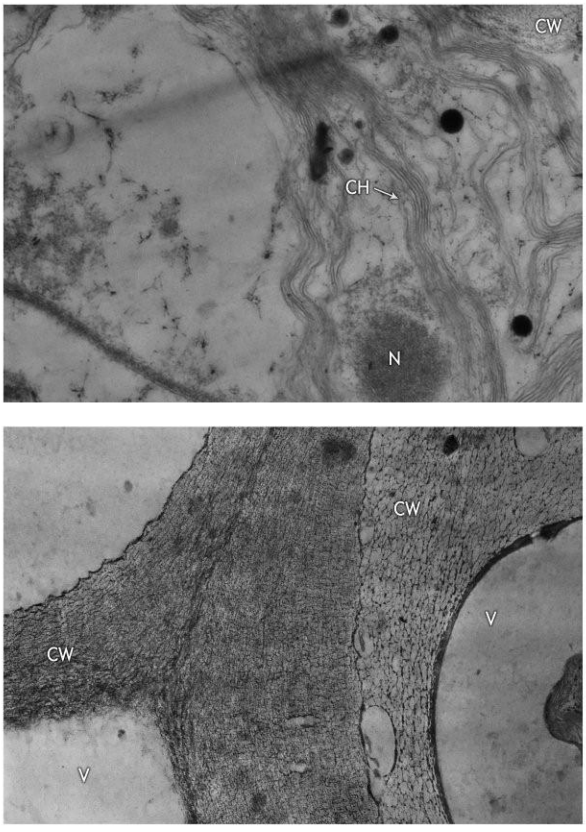

Fig. 6. Transverse section of *Ulva flexuosa* subsp. *pilifera* vegetative cell with a thick cell wall – TEM.

Cells of the *Ulva* thalli are arranged in longitudinal and transverse rows, which is in agreement with the results presented by Kowalski (1975) and Sitkowska (1999). Assuming that the size of cells is related to age, it is interesting that cells from the Malta Reservoir were shorter (the lower limit of the length range) in comparison to those observed by Sitkowska (1999) from the fish pond in Kuciny, and slightly narrower (the lower limit of the width range) compared to cells of thalli from other locations. Chloroplasts in *Ulva* cells from the Malta Reservoir, containing up to 4 pyrenoids, are similar to thalli described from other freshwater locations. Griffiths (1970) and Teng et al. (2011), paid particular attention to the number of pyrenoids per cell as an identification feature. It appears that the number of pyrenoids as a criterion for the classification of algae is of rather limited value due to difficulties with effective examination of pyrenoids under a light microscope (apex cells are often too small to observe the presence or absence of pyrenoids). Pyrenoids in our material consisting of *Ulva flexuosa* subsp. *pilifera* thalli collected from the freshwater reservoir, were more distinct in the upper thalli parts than those in the lower (basal) parts.

Our studies indicate that morphological and ultrastructural research on diversity and distribution of the species *Ulva flexuosa* subsp. *pilifera*, in the Wielkopolska region should be continued.

CONCLUSIONS

Applying the morphological and ultrastructural analysis, we were able to identify *Ulva flexuosa* subsp. *pilifera* found in the Malta Reservoir in Poznań. As evidenced by the study, the species shows morphological affinity with the species previously described by other authors. There are some interesting morphological and ultrastructural details, however, which distinguish *Ulva flexuosa* subsp. *pilifera* from the marine species. The results of this study indicate that morphological and ultrastructural characteristics of algae cells from freshwater *Ulva* forms, including the differences in different parts of thalli and different developmental phases of thalli (young, mature), require parallel examinations under a light microscope and a transmission electron microscope. No significant differences were observed in the size of *Ulva* thalli between young and mature specimens.

ACKNOWLEDGMENTS

We are very grateful to Dr. hab. Andrzej Massalski for valuable comments and critical reading of the manuscript. This work was supported by the grant NN 304 013 437.

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