Oceanological and Hydrobiological Studies

International Journal of Oceanography and Hydrobiology

Volume 44, Issue 3, September 2015
pages (393-409)

ISSN 1730-413X
eISSN 1897-3191

Differentiation of inland *Ulva flexuosa* Wulfen (Ulvaceae, Chlorophyta) from Western Poland

by

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DOI: **10.1515/ohs-2015-0037**
Category: **Original research paper**
Received: **February 23, 2015**
Accepted: **April 13, 2015**

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Abstract

The macroalgae representing the cosmopolitan *Ulva flexuosa* Wulfen species have been found in inland water ecosystems of Europe since the mid-1800s. The presence of the *Ulva* genus in waters of inland salt marshes was explained by high salinity of water in such systems. However, *Ulva flexuosa* thalli were found in lakes and rivers having no supply from strongly mineralized sources, either of natural or anthropogenic origin. The study focused only on populations of *Ulva flexuosa* subsp. *pilifera* (Kűtz.) M.J. Wynne and *Ulva flexuosa* subsp. *paradoxa* (C. Agardh) M.J. Wynne occurring in inland ecosystems isolated from seawater. The differentiation of inland *U. flexuosa* subsp. *pilifera* and subspecies *paradoxa* was assessed by using classical morphological data and molecular techniques. We found that only two subspecies of *Ulva flexuosa* Wulfen occur in inland water ecosystems of Poland. On the basis of the analysis combining morphological features with the ITS region and *rbc*L gene sequences, a small degree of differentiation of the two inland taxa was demonstrated. These two subspecies have high differentiation of the habitat niches. *U. flexuosa* subsp. *paradoxa* settled in habitats featuring high salinity levels, and the second subspecies *pilifera* occurred only in fresh waters.

Key words: freshwater *Ulva*, inland *Ulva*, *Ulva flexuosa*, *Enteromorpha flexuosa*, habitat

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Introduction

In macroscopic terms, *Ulva* sp. (syn. *Enteromorpha*) species are discernible algae belonging to green algae (Chlorophyta). Their thallus structures, shapes, and colors vary considerably. As a rule, *Ulva* species representing foliaceous types of thallus structures are flat and have undulating rims (e.g. *U. lactuca*). Their thallus cross sections have two layers of accreted cells. The cells have one nucleus and one chromatophore with one to several pyrenoids. The second morphological group in the *Ulva* genus has tubular thalli (tube-like *Ulva*, e.g., *U. intestinalis*) that are empty inside, single or ramate to various extents. Their thallus cross sections have only one thick-walled cell layer (Koeman and van den Hoek 1982a, b).

Taxa of the *Ulva* genus can grow if they are fixed to the substrate by scutella or if they freely creep along a sandy or muddy sea floor. Most often, however, they overgrow any natural (rocks, stones, mollusk shells, chips of wood and vascular plant stems) or man-made substrate (breakwater, concrete and metallic seaport structures, jetties, and ship hulls) submerged in coastal regions.

In coastal regions, they most frequently form submarine meadows with diversified structures and species compositions, usually at a depth of 2 to 8 m (McAvoy & Klug 2005), although they occur even at a depth of 40 m (Chapman 1959, Adams 1994) in open waters.

High rates of ecological expansion of *Ulva* taxa are directly related to the characteristics of biological development of these macroalgae. Under favorable conditions, *Ulva intestinalis* or *U. prolifera* thalli will develop very quickly. In addition, most species of this genus feature a very high ecological tolerance to key environmental parameters that determine their occurrence (in particular, salinity and nutrient concentrations) (Sfriso et al. 1987, Sze 1998). In waters rich in nutrients, *Ulva prolifera*, *U. intestinalis* and *U. compressa* may form blooms (referred to as *green tides*) that tightly cover the water surface. Such blooms have a strong impact on the physical and chemical conditions prevailing under the mat, which determine the quantitative and qualitative compositions of the bottom fauna (Sundbäck et al. 1990, Narkko and Bonsdorff 1996, Bonsdorff et al. 1997, Valiela et al. 1997, Romano et al. 2003) and consequently determine the predators that feed on the bottom fauna (Nicholls et al. 1981, Raffaelli et al. 1989).

Most often, *Ulva* species occur in coastal ecosystems in over-fertilized waters with high nutrient levels. Therefore, *Ulva intestinalis* and *U. lactuca* were used as biofilters as early as the 1970s for the purpose of nitrogen and phosphorus removal from waters used in commercial aquaculture (fish and shrimp breeding farms) (Neori et al. 1991, Jimenez del Rio et al. 1996, Schroeder et al. 2013). Given the massive presence of *Ulva intestinalis, U. flexuosa* or *U. prolifera* in waters featuring very high concentrations of nitrogen and phosphorus, this group of macroalgae is considered in the monitoring of waters strongly contaminated by nutrients (Reed & Moffat 2003).

The presence of *Ulva flexuosa* taxa in fresh waters of Europe has always been a focus of interest because of their systematic association with coastal ecosystems. Additionally, the occurrence of *Ulva flexuosa* in Europe was noticed in mineral river source areas, inland water salt pans, salt marshes and waterways near graduation towers, which featured high chloride levels (Mareš 2009, Messyasz & Rybak 2009, Mareš et al. 2011, Messyasz & Rybak 2011). This situation was the reason why the thalli collected from such sites were compared with taxa originating from the sea, most often identified as members of such species as e.g.: *Enteromorpha intestinalis* (syn. – *Ulva intestinalis*) and *E. salina* (syn. – *U. prolifera*) (from the herbaria built by: Rabenhorst – 1849; Beck – 1852; de Bahr – 1852; Braun – 1862; Wagner – 1876; Liebetantz – 1921; Schenck – 1924, and Krist – 1934), or even *U. linza* (Nave 1863). Mass occurrences of *Ulva flexuosa* have been noted in European lakes, ponds and rivers with optimal conditions for its development, becoming an obvious dominant element on the water surface (Kaštovský et al. 2010, Mareš et al. 2011). In the inland ecosystems of Europe (especially in Germany, the Republic of Czech and Poland), which are not exposed to the impact of sea and ocean waters, the *Ulva flexuosa* subspecies have been recorded at more than 250 sites (Messyasz & Rybak 2009, Mareš 2009, Messyasz & Rybak 2011, Mareš et al. 2011).

Outside Europe, a dozen or so *Ulva* inland habitats have been reported – e.g. in North America (Taft 1964, Vinyard 1966, Pfiester & Felker 1976,

Conner et al. 1978, Catling & McKay 1980, Reinke 1981, Lougheed & Stevenson 2004) and South America (Leonardi & Caceres 1988). In those parts of the world, inland *Ulva* populations have not been studied in detail and the research focused mainly on the identification of new inland habitats of species such as *Enteromorpha intestinalis* (Taft 1964, Reinke 1981), *E. intestinalis* f. *maxima* and f. *cylindracea*, *E. prolifera* (Catling & McKay 1980), *E. prolifera* var. *tubulosa* (Vinyard 1966; Conner et al. 1978) and *E. compressa* (Pfiester & Felker 1976) in selected regions of the United States and Canada.

In this paper, we discuss the phylogenetic position of inland *Ulva flexuosa* populations and their ecological niche in limnic ecosystems of Poland. Our objectives are as follows: (i) to analyze the molecular and morphological relationships between the freshwater and saline subspecies of *U. flexuosa*, (ii) to assess incongruence of habitats of the inland *U. flexuosa* complex inferred from ecological data.

Materials and methods

Study area and sampling

The study included inland *Ulva flexuosa* Wulfen populations from 21 aquatic ecosystems located in Poland (central Europe) (Fig. 1). Thalli of *Ulva flexuosa* subsp. *pilifera* and subsp. *paradoxa* were found in rivers, ponds, lakes, streams and single stands located in an oxbow lake, mine and peat depression, water canal, and dam reservoir (Table 1).

Thallus samples were collected from the centers of macroalgae mats formed by *Ulva flexuosa*. Small parts of the thalli (1 g) were preserved for molecular analysis in silica gel (Sigma-Aldrich, Seelze, Germany). Voucher specimens are deposited in the Herbarium of the Faculty of Biology, Adam Mickiewicz University in Poznań.

Samples of water and *Ulva* thalli were collected simultaneously at each site from May to September during the years 2004-2012. Water was sampled by hand from directly under the macroalgae mats using 1.0 l sterile plastic bottles (Roth, Karlsruhe, Germany). Long-sleeved veterinary-grade gloves were used for sampling to avoid any pollution. On the lakes and ponds, water samples were collected from a rowboat to approach an *Ulva* mat cautiously from the side of the pelagic zone to avoid potential deposit displacement (stagnant water). Each sample was filtered through a coarse plastic sieve to separate the vascular plants from water. Next, the water samples were placed in 0.5 l sterile plastic containers (Roth, Karlsruhe, Germany), and cooled at 4°C. In the laboratory, the samples were filtered through a nitrocellulose microbiological filter with a pore size of 0.45 microns.

Microscopic observation

Laboratory analyzes included measurement of the thalli (presence of proliferations) and examination of the cell morphology (the number of pyrenoids, shape and arrangement of cells) using a light microscope Zeiss Axioskop 2 MOT.

Physical and chemical analysis of water

The physicochemical parameters of the water samples from all sites containing inland *Ulva flexuosa* were analyzed. Field measurements included total dissolved solids (TDS) and conductivity (EC), all measured using a field probe Professional Plus Series with sensor No. 605103 (YSI, Yellow Springs, OH, USA). The concentrations of chloride (Cl^-) and sodium chloride (NaCl) were determined using standard methods with a HACH DR 2800 spectrophotometer (APHA 1998). Chloride and sodium chloride were measured in filtered water samples immediately after collection (< 4 h).

Molecular analysis

Total DNA from the silica-gel-preserved thalli was extracted using the CTAB method (Doyle & Dickson 1987), modified for 50 mg of starting material, with an initial rehydration step as described by Hayden et al. (2003). Polymerase chain reaction (PCR) amplifications of the ITS1-5.8S-ITS2 (part of the rRNA gene area) and the chloroplast *rbc*L gene were performed (Shimada et al. 2003, Mareš 2009, Mareš et al. 2011). The genome area containing ITS1, ITS2 and the 5.8S ribosomal subunit was amplified with primers ITS1 and ITS4 (Table 2). The reaction sequence consisted of an initial denaturation step (5 min at 95°C), followed by 30 s at 95°C, 1 min at 50°C and 1 min at 72°C for 35 cycles; and a final 5 min extension at 72°C. The *rbc*L gene was amplified

Figure 1

Distribution of sampling locations of inland *Ulva* in Poland (black circles: stands with *Ulva flexuosa* subsp. *pilifera*; gray circles: stands with *Ulva flexuosa* subsp. *paradoxa*)

Table 1

Taxon sampling, voucher information and GenBank accession numbers (ITS regions and *rbc*L gene) for inland subspecies of *Ulva flexuosa* Wulfen examined within the study

Location	Ecosystem	Voucher	Year	GenBank Accession No.
Ulva flexuosa subsp. *pilifera* (Kűtz.) M.J. Wynne				
Poland, Poznań, N 52° 22' 22.55"/E 16° 51' 35.93"	mine pit	U0027	2004	KP941128, KP941148
Poland, Stęszew, N 52° 17' 28.1"/E 16° 41' 03.8"	river	U0025	2006	KP941135, KP941154
Poland, Szamocin, N 53° 04' 1.4"/E 17° 03' 11.8"	river	U0021	2008	KP941134, KP941155
Poland, Koło, N 52° 12' 19.30"/E 18° 30' 48.23"	river	U0023	2008	KP941125, KP941145
Poland, Poznań , N 52° 22' 1.27"/E 16° 51' 51.79"	pond	U0028	2008	KP941133, KP941153
Poland, Środa Wielkopolska, N 52 ° 12' 57.9"/E 17° 16' 47.79"	peat depression	U0007	2009	KP941121, KP941142
Poland, Lubowo, N 52° 30' 40.18"/E 17° 22' 30.46"	lake	U0011.80.90	2009	KP941129, KP941149
Poland, Poznań, N 52° 21' 37"/E 17° 02' 40"	stream	U0012.13.15	2009	KP941137, KP941157
Poland, Kleszczewo, N 52° 20' 20"/E 17° 02' 44"	stream	U0014	2009	KP941123, KP941143
Poland, Wągrowiec, N 52° 48' 7.41"/E 17° 12' 31.43"	river	U0024.63	2010	KP941130, KP941150
Poland, Kórnik, N 52° 12' 02.1"/E 16° 59' 40.5"	oxbow lake	U0026	2009	KP941139, KP941159
Poland, Poznań, N 52° 24' 10.5"/E 16° 57' 45.2"	water reservoir	U0032	2009	KP941136, KP941156
Poland, Konin, N 52° 18' 05.6 ,'/E 18° 16' 34.9"	lake	U0083.84	2010	KP941132, KP941152
Poland, Konin, N 52° 17' 17.9 ,'/E 18° 12' 45.2"	lake	U0085.86	2010	KP941127, KP941147
Poland, Ślesin, N 52° 20' 21.2"/E 18° 21' 28.7"	lake	U0087	2010	KP941122, KP941120
Poland, Poznań, N 52° 28' 03.8"/E 16° 55' 58.2"	pond	U0097	2012	KP941138, KP941158
Poland, Nowe Skalmierzyce, N 51° 39' 32.9"/E 17° 56' 31.5"	river	U0115	2012	KP941131, KP941151
Poland, Konin, N 52° 19' 19.4"/E 18° 16' 16'	pond	U0116	2012	KP941135, KP941144
Poland, Jacewo, N 52° 48' 1.78"/E 18° 17' 14.23"	pond	U0122	2012	KP941126, KP941146
Ulva flexuosa subsp. *paradoxa* (C. Agardh) M.J. Wynne				
Poland, Lechowo, N 52° 47' 16.75"/E 18° 6' 34.85"	canal	U0121	2012	KP941141, KP941160
Poland, Pakość, N 52° 48' 08.1"/E 18° 06' 24.0"	pond	U0098	2012	KP941140, KP941161

Table 2

Primers used for DNA amplification and sequencing

Primer	Sequence	Target	Direction
[1]ITS1	5' TCCGTAGGTGAACCTGCGG 3'	ITS	Forward
[1]ITS4	5' TCCTCCGCTTATTGATATGC 3'	ITS	Reverse
[1]RH1	5' ATGTCACCACAAACAGAAACTAAAGC 3'	*rbc*L	Forward
[1]1385r	5' AATTCAAATTTAATTTCTTTCC 3'	*rbc*L	Reverse
[2]rbc571	5' TGTTTACGAGGTGGTCTTGA 3'	*rbc*L	Forward
[2]rbc590	5' TCAAGACCACCTCGTAAACA 3'	*rbc*L	Reverse

[1]amplification and sequencing; [2]only sequencing

using primers RH1 and 1385r (Table 2). The reaction profile included initial denaturation at 95°C for 5 min; followed by 30 s at 95°C, 1 min at 50°C and 1.5 min at 72°C for 35 cycles; and finished by extension for 5 min at 72°C. DNA sequencing was performed as described by Leskinen & Pamilo (1997), Kawai et al. (2007) using a HITACHI 3130 xL Genetic Analyzer (Applied Biosystems, CA, USA) automated sequencer. The primers used for amplification and sequencing are listed in Table 2. All of the raw data from the DNA sequencing were corrected manually, both ends were trimmed, and the regions of the highest quality were assembled into final sequences using the BioEdit software (Hall 2007). In further analyzes of the dataset, only specimens with available sequences of both genes were used. Three *Ulvaceae* taxa: *Ulvaria obscura* var. *blyttii*, *Umbraulva olivascens* and *Percursaria percursa* were included as outgroups for further phylogenetic analysis (Hayden & Waaland 2002, Hayden et al. 2003, Ichihara et al. 2009a, b). Sequences of reference taxa were obtained from the National Center for Biotechnology Information (NCBI) database (Table 3).

Table 3

Summary of the analyzed sequences

Taxon	Source	GenBank accession No.	
		ITS	*rbc*L
Outside taxa from *Percursaria, Ulvaria* and *Umbraulva* genus			
Percursaria percursa	Hayden & Waaland 2002, Hayden et al. 2003	AY260570	AF499674
Ulvaria obscura var. *blyttii*	Hayden & Waaland 2002, Hayden et al. 2003	AY260571	AF499673
Umbraulva olivascens	Hayden et al. 2003	AY260564	AY255876
Internal taxa from *Ulva* genus			
Ulva australis	Kraft et al. 2010	EU933985	EU933957
Ulva californica	Hayden et al. 2003	AY260560	AY255866
Ulva californica	Hayden & Waaland 2004	AY422515	AY422558
Ulva clathrata	Blomster et al. 1999, Hayden & Waaland 2004	AF127170	AY422563
Ulva compressa	Blomster et al. 1998, Hayden et al. 2003	AF035350	AY255859
Ulva fasciata	Hayden & Waaland 2004	AY422524	AY422565
Ulva flexuosa subsp. *flexuosa*	Mareš et al. 2011	HM447564	HM447574
Ulva flexuosa subsp. *paradoxa*	Tan et al. 1999	AJ234306	HM447575
Ulva flexuosa subsp. *paradoxa*	Mareš et al. 2011	HM447561	HM447565
Ulva flexuosa subsp. *pilifera*	Mareš et al. 2011	HM447579	HM447566
Ulva flexuosa subsp. *pilifera*	Mareš et al. 2011	HM447584	HM447568
Ulva flexuosa subsp. *pilifera*	Mareš et al. 2011	HM447583	HM447567
Ulva flexuosa subsp. *pilifera*	Mareš et al. 2011	HM481175	HM447569
Ulva flexuosa subsp. *pilifera*	Mareš et al. 2011	HM447580	HM447576
Ulva flexuosa subsp. *pilifera*	Mareš et al. 2011	HM447582	HM447577
Ulva flexuosa subsp. *pilifera*	Mareš et al. 2011	HM447581	HM447578
Ulva flexuosa subsp. *pilifera*	Mareš et al. 2011	HM481176	HM447570
Ulva flexuosa subsp. *pilifera*	Mareš et al. 2011	HM481171	HM447572
Ulva flexuosa subsp. *pilifera*	Mareš et al. 2011	HM481173	HM447573
Ulva intestinalis	Kraft et al. unpublished	EU933966	EU933939
Ulva intestinalis	Hayden & Waaland 2004	AY422508	AY422552
Ulva lactuca	Hayden & Waaland 2004	AY422499	AY422543
Ulva lactuca	Tan et al. 1999, Hayden & Waaland 2002	AJ234310	AF499669
Ulva limnetica	Ichihara et al. 2009, Ichihara et al. 2013	AB425969	AB425968
Ulva linza	Hayden et al. 2003	AY260557	AY255861
Ulva pertusa	Hayden & Waaland 2004	AY422504	AY422549
Ulva procera	Hayden & Waaland 2004	AY422521	AY422562
Ulva procera	Hayden et al. 2003	AY260558	AY255863
Ulva prolifera	Tan et al. 1999, Hayden et al. 2003	AJ234304	AY255864
Ulva prolifera	Hayden et al. 2003	AY260559	AY255865
Ulva pseudocurvata	Tan et al. 1999, Hayden et al. 2003	AJ234312	AY255869
Ulva rigida	Hayden & Waaland 2004	AY422522	AY422564
Ulva scandinavica	Tan et al. 1999, Hayden et al. 2003	AJ234317	AY255870
Ulva stenophylla	Hayden et al. 2003	AY260569	AY255874
Ulva taeniata	Hayden & Waaland 2004	AY422525	AY422566
Ulva tanneri	Hayden & Waaland 2004	AY422519	AF499672

Phylogenetic analysis

Initial alignments of two data sets were prepared using the MUSCLE software (Edgar 2004). The first set contained ITS sequences enlarged by a group of reference and outgroup sequences (totally 60 sequences). The second set contained *rbc*L sequences similarly enlarged by a group of reference and outgroup sequences (totally 60 sequences). The alignments were trimmed at the ends and corrected manually. Based on the final multiple sequence alignments, the phylogenetic trees were built using the neighbor-joining distance method with the MEGA4 software (Tamura et al. 2007) and the Kimura 2-parameter substitution model (Kimura 1980) (data not presented). The stability of individual nodes was calculated using the bootstrap test (for 1 000 replicates). In most cases, the analyzes were not sufficient to infer a tree with predefined subfamilies grouped into monophyletic branches. For this reason, a concatenated set of data was constructed. It combined ITS and *rbc*L with reference taxa and outgroup sequences together with additional characteristic morphometric features (e.g. the number of pyrenoids, the cell arrangement in the thallus and the cell shape) and environmental features (such as the origin of the ecosystem, the chloride supply and the occurrence of co-dominants). The full list of the features used is provided in Table 4.

The partition homogeneity test in PAUP 4.0 (Swofford 2002) was used to assess whether the data could be concatenated into a single set.

The final tree was calculated using the Bayesian approach with the MrBayes 3.2.1 software (Ronquist & Huelsenbeck 2003). This approach combines the relative reliability of ML (Maximum Likelihood) with fast scanning of the parameter landscape using the Markov chain Monte Carlo (MCMC) approach implemented in the MrBayes3 program. Preliminary runs with MrBayes using a mixture of model priors demonstrated conclusively that GTR (generalized time-reversible) matrices provided the best fit to the sequence data. Therefore, the GRT model was used to provide substitution priors for the sequence partition of the data. A Metropolis-

Table 4

Set of morphometric and environmental features used to calculate Bayesian tree with concatenated data

	Coding of features
Morphometric features	
Branching	**0** – absent; **1** – present
Pyrenoids	**1** – number of pyrenoids < 3; **2** – number of pyrenoids > 3
Thalli surface architecture	**1** – curly bubbled with a strongly corrugated and often highly furcated thallus; **2** – intestinally undulating thallus with a smooth surface
Cells arrangement in thallus	**1** – cells in regular rows; **2** – cells randomly distributed
Life form	**1** – thalli creeping along the bottom; **2** – thalli slowly floating on the water surface
Surface of macroalgae mats	**1** – surface < $1m^2$; **2** – surface > $1m^2$
Branche's apex cells	**1** – rounded and broad; **2** – long and thin
Cell shape	**1** – polygonal and rounded; **2** – polygonal and not rounded
Proliferation	**0** – absent; **1** – present
Proliferation apex cells	**1** – rounded, wide and thin-walled; **2** – elongated, narrow and thin-walled
Environmental features	
Origin/genesis system	**1** – system of anthropogenic origin; **2** – system of semi-natural origin; **3** – system of natural origin
Chlorides supply	**0** – no chlorides supply; **1** – supply of natural origin; **2** – supply of anthropogenic origin
Water flow	**1** – stagnant water; **2** – flowing water
Co-dominants	**1** – filamentous algae co-dominance; **2** – vascular plants co-dominance
Type of ecosystem	**1** – lake; **2** – river; **3** – stream; **4** – pond; **5** – peaty cavity; **6** – retention reservoir; **7** – oxbow lake; **8** – ditch; **9** – excavation reservoir; **10** – rain water reservoir; **11** – sea
Lemnids' occurrence	**1** – > 50% of lemnids in the assemblage; **2** – < 50% of lemnids in the assemblage
Chloride	**1** – Cl^- in water at a concentration of < 500 mg l^{-1}; **2** – Cl^- in water at a concentration of >500 mg l^{-1}
NaCl	**1** – NaCl in water at a concentration of < 500 mg l^{-1}; **2** – NaCl in water at a concentration of > 500 mg l^{-1}
Conductivity	**1** – conductivity < 1500 µS/cm^2; **2** – conductivity > 1500 µS/cm^2
Total dissolved soils	**1** – TDS < 1000 mg l^{-1}; **2** – TDS > 1000 mg l^{-1}
$CaCO_3$ crystals on thalli surface	**0** – absent; **1** – present
No data	**?**

coupled Markov chain Monte Carlo analysis was performed with 2 to 3 million generations, two runs and eight chains (four per run). The Markov chain was sampled every 100 generations. Convergence of the runs was confirmed by the value of the average standard split deviation factor falling below the recommended value of 0.01. The final trees were obtained after removing the first 25% of the samples. All of the phylogenetic trees were edited in MEGA4 and TreeView (Page 1996, Tamura et al. 2011).

Results

Anatomical, morphological and habitat characteristics were included in the analysis of the ITS and *rbc*L sequences for the inland taxa of *Ulva*. Ten morphometric and 11 environmental features were assigned to the samples and reference taxa for *U. flexuosa* subsp. *pilifera* (Kützing) M.J. Wynne and *U. flexuosa* subsp. *paradoxa* (C. Agardh) M.J. Wynne. The morphometric-environmental features' series were also assigned to species representing the external group in the tree. Because of the lack of sufficient data, additional features were not attributed to the remaining marine *Ulva* species.

The tree-connected ITS and *rbc*L sequences and morphometric-environmental features basically did not change the topology of the obtained tree with respect to the tree obtained as a result of the analysis of the nucleotide sequences only. Incorporation of additional characteristics resulted in standardizing the division of the groups representing the inland taxa. This effect is very clear for the group of *U. flexuosa* subsp. *pilifera* (Fig. 2).

Completely separate sequences of *U. limnetica* were included in the analysis because of its high ecological similarity to the inland populations of *U. flexuosa* (particularly to *U. flexuosa* subsp. *pilifera*). On the basis of the tree combining the ITS and *rbc*L sequences, *Ulva limnetica* formed an indistinct group together with weakly related species of other representatives of marine *Ulva* such as *U. compressa*, *U. intestinalis* and *U. pseudocurvata* (Fig. 2). Freshwater *Ulva limnetica* is a species that differs considerably from the remaining marine *Ulva* species in its clearly faster pace of sequence evolution in comparison to other *Ulva* species.

Ulva rigida, *U. scandinavica*, *U. clathrata*, *U. fasciata* and *U. teaniata* were combined in separate

Figure 2

Bayesian tree based on a concatenated data combining ITS, rbcL and sequences of outgroups together with characteristic morphological and environmental features. Bootstrap support is given for branches.

clade. This complex of species is an example of a considerable resemblance of closely related marine species of *Ulva*.

The samples of *U. flexuosa* subsp. *pilifera* formed a very standardized complex characterized by a number of common features. Incorporation of the external features in the analysis did not result in individual taxa of *U. flexuosa* subsp. *paradoxa* moving to the *U. flexuosa* subsp. *pilifera* complex. Features that clearly distinguished the inland populations existed. On account of general morphometric features, *U. flexuosa* subsp. *pilifera* is a taxon with more than 3 pyrenoids per cell, thalli with curly, bubbled, strongly corrugated and often highly furcated or intestinally undulating smooth surfaces, randomly distributed cells and polygonal and rounded cell shapes (Fig. 3 and 5, Table 5).

Moreover, taking environmental characteristics into consideration, *U. flexuosa* subsp. *pilifera* was found in the entire spectrum of aquatic ecosystems of natural and anthropogenic origin. The concentrations of chlorides in the habitats of *U. flexuosa* subsp. *pilifera* did not exceed 500 mg l^{-1}, the electrolytic conductivity was < 1 500 µS cm^{-1}, and the TDS was < 1 000 mg l^{-1} (Table 6).

The second studied inland taxon, *U. flexuosa* subsp. *paradoxa* is characterized, in terms of its general morphometric features, as having fewer than 3 pyrenoids per cell, thalli that are curly, bubbled, strongly corrugated and often highly furcated, cells arranged in their thalli in regular rows; and cells that are mainly polygonal rather than rounded and whose proliferation is always present (Fig. 4 and 5, Table 5). The analyzed populations of *U. flexuosa* subsp. *paradoxa* came exclusively from ecosystems of anthropogenic origin with constantly high chloride levels (concentrations > 500 mg l^{-1}). The electrolytic conductivity of habitats of this taxon was > 1 500 µS cm^{-1}, and TDS was > 1 000 mg l^{-1} (Table 6).

Table 5

Selected morphological, anatomical and habitat characteristics useful for identification of *Ulva flexuosa* subspecies from inland ecosystems

Feature	Subspecies	
	U. flexuosa subsp. *pilifera* (Kűtz.) M.J. Wynne	*U. flexuosa* subsp. *paradoxa* (C. Agardh) M.J. Wynne
Habitat	fresh waters or slightly brackish, abundant in lakes, fish ponds, oxbow lake, streams, rivers, and canals	saline water of salt marshes, peat pits and anthropogenic systems (e.g. canals and reservoirs along the roads and highways; ponds and ditches near graduation towers and salt plants)
Abiotic factors of water (range)	Cl^- (mg l^{-1}): 23.2-265.0 NaCl (mg l^{-1}): 23.5-437.25 Conductivity (µS cm^{-1}): 465.0-1307.0 TDS (mg l^{-1}): 230.0-760.0	Cl^- (mg l^{-1}): 6187.5-8317.3 NaCl (mg l^{-1}):10 209.38-11 965.50 Conductivity (µS cm^{-1}): 3477.5-5813.5 TDS (mg l^{-1}): 3659.5-5122.0
Development	attached or free floating, in many causes in masses (macroalgal mats)	attached or free floating, rare in macroalgal mats
Morphology	tube-like, and intestine-like thalli	tube-like, and intestine-like thalli
Surface of thalli	intestinally undulating thallus with a smooth surface or curly bubbled with a strongly corrugated and often highly furcated thallus	strongly corrugated and often highly furcated thallus
Color of thalli	green or green-yellow	green or green-yellow
Branching of thalli	abundant or absent	numerous and highly developed
Proliferation (uniseriate branches)	numerous or absent, apical cells of proliferation short and obtusely-rounded	very numerous, apical cells of proliferation elongated and tapered or unrounded (blunt)
Shape of cells	polygonal often rounded quadrilateral with sharp edges	rectangular, square, polygonal with rounded or sharp edges
Size of cells (µm)	3.5 – 28.44 × 3.1 – 27.47	4.8 – 24.8 × 4.9 – 33.5
Cell arrangement in thalli	irregular or in small groups and short rows	in long rows, sometimes irregular
Number of pyrenoids	(1) 2 – 4	1 – 3 (4)
Diameter of pyrenoids (µm)	0.75 – 5.0	1.3 – 2.4

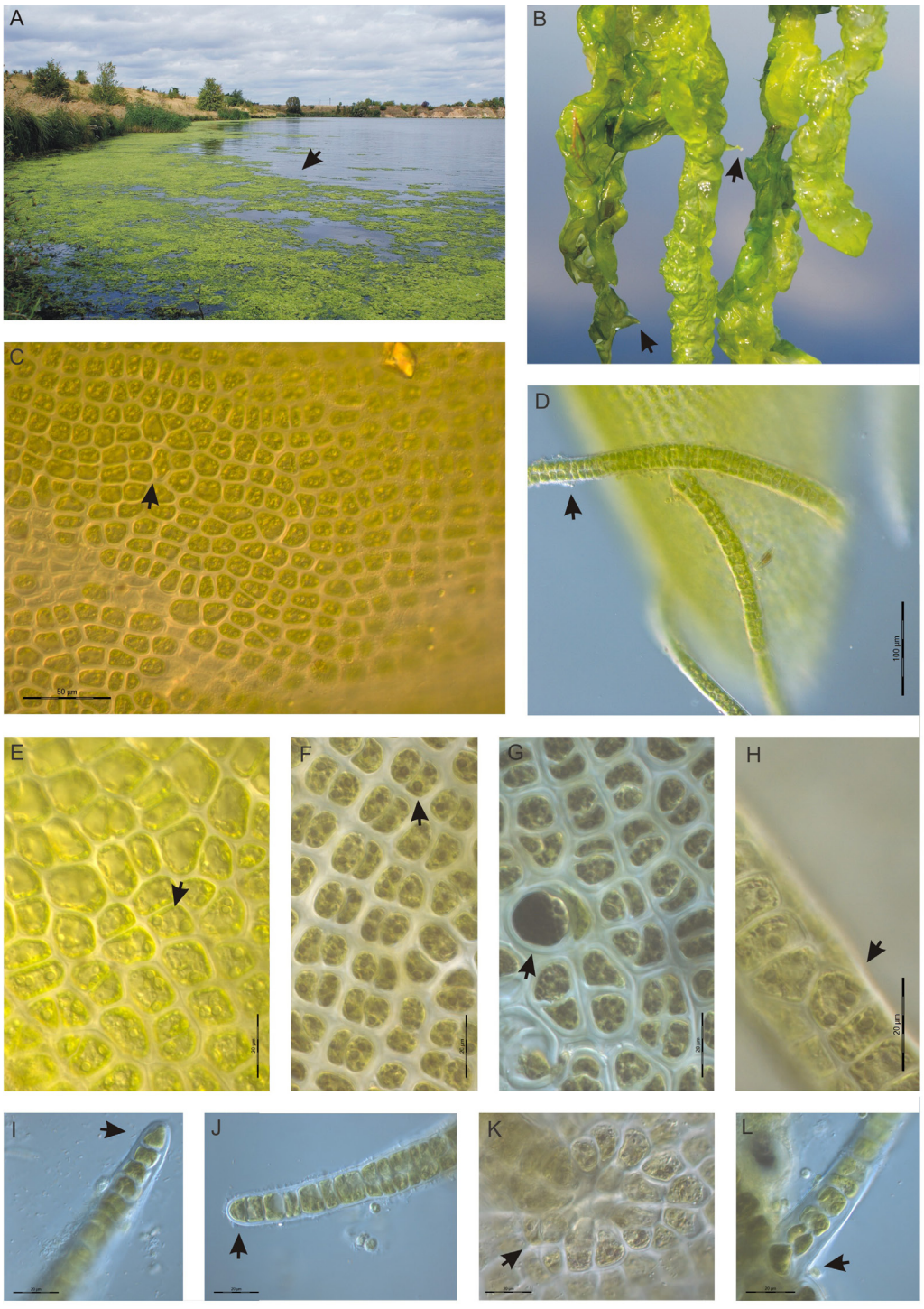

Figure 3

U. flexuosa subsp. *pilifera* from inland waters. A – green tide of *Ulva* (arrow) in the littoral zone of a pond in Konin City (Wielkopolska Region, Poland). B – a tube-like thallus with many primary macroscopic branches, C – very common disordered cell arrangement (most frequently in apical and central part of thalli), D – multiseriate branches of thalli (with 3 – 4 rows of cells), E – young cells with cup-shaped chromatophores, F – cells with two or more pyrenoids (max. 4) are seen in each plastid, G – formation of proliferations from a swollen cell in basal and central parts of the thallus, H – branches with two rows of cells, I and J – obtusely rounded apical cell of uniseriate branches, K – basal part of a young branch (view from top), L – basal part of a young uniseriate branch (view from side). Photo A and B by S. Mielczarek, photos C – L by A. Rybak

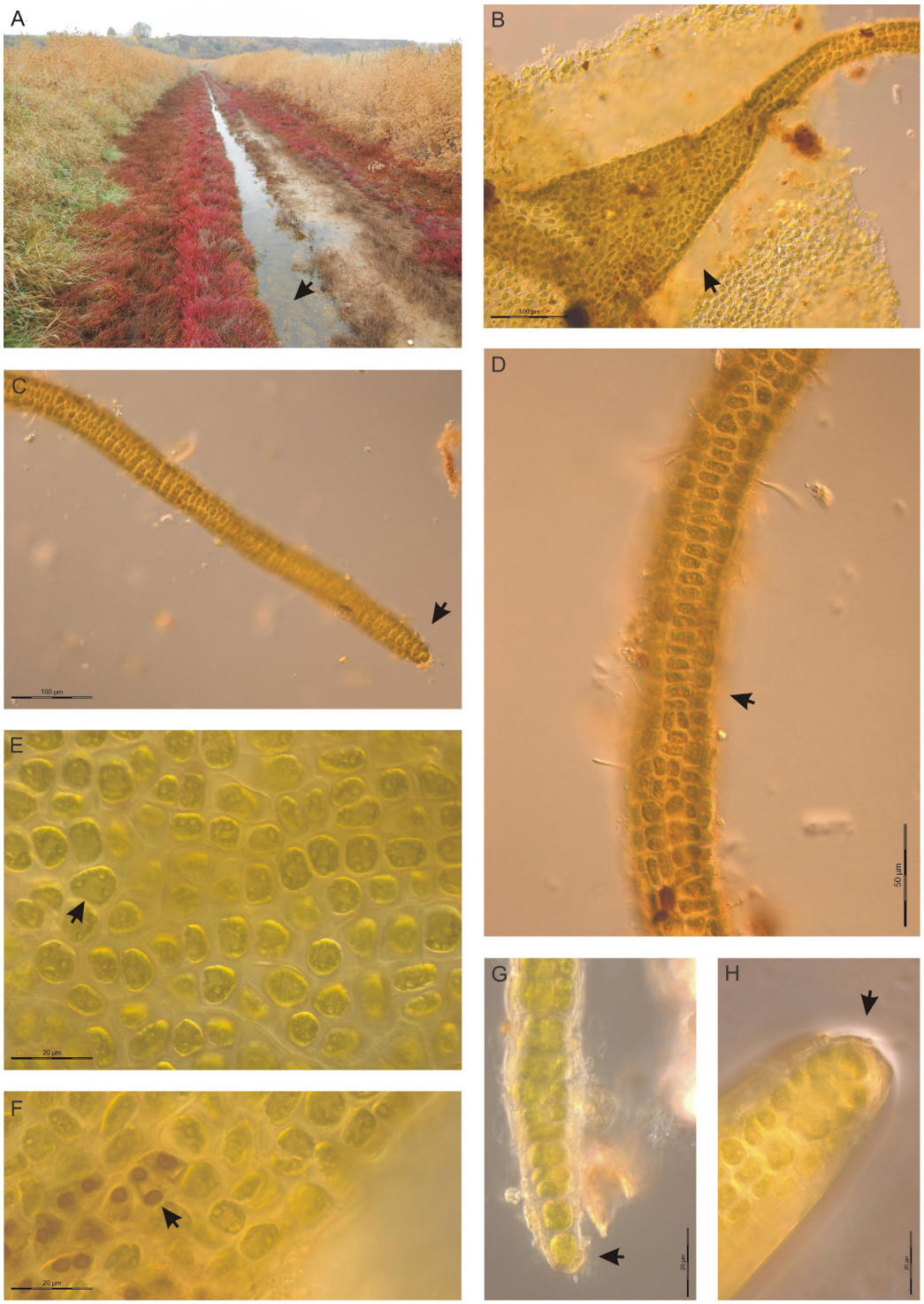

Figure 4

U. flexuosa subsp. *paradoxa* from inland waters. A – artificial canal with *Ulva* on the bottom (arrow) near Lechowo village (Kuyavian-Pomeranian Region, Poland), B – basal parts of a branch, C – blunt-tipped young branch with multiple apical cells, D – branch with several rows of cells, E – disordered cell arrangement in the basal part of thalli; cells with cup-shaped chromatophores; each plastid has 1 – 3 pyrenoids, F – pyrenoids stained with Lugol's solution, G – juvenile branch with a uniseriate apex, H – gently tipped branch with multiple apical cells. Photos A – H by A. Rybak

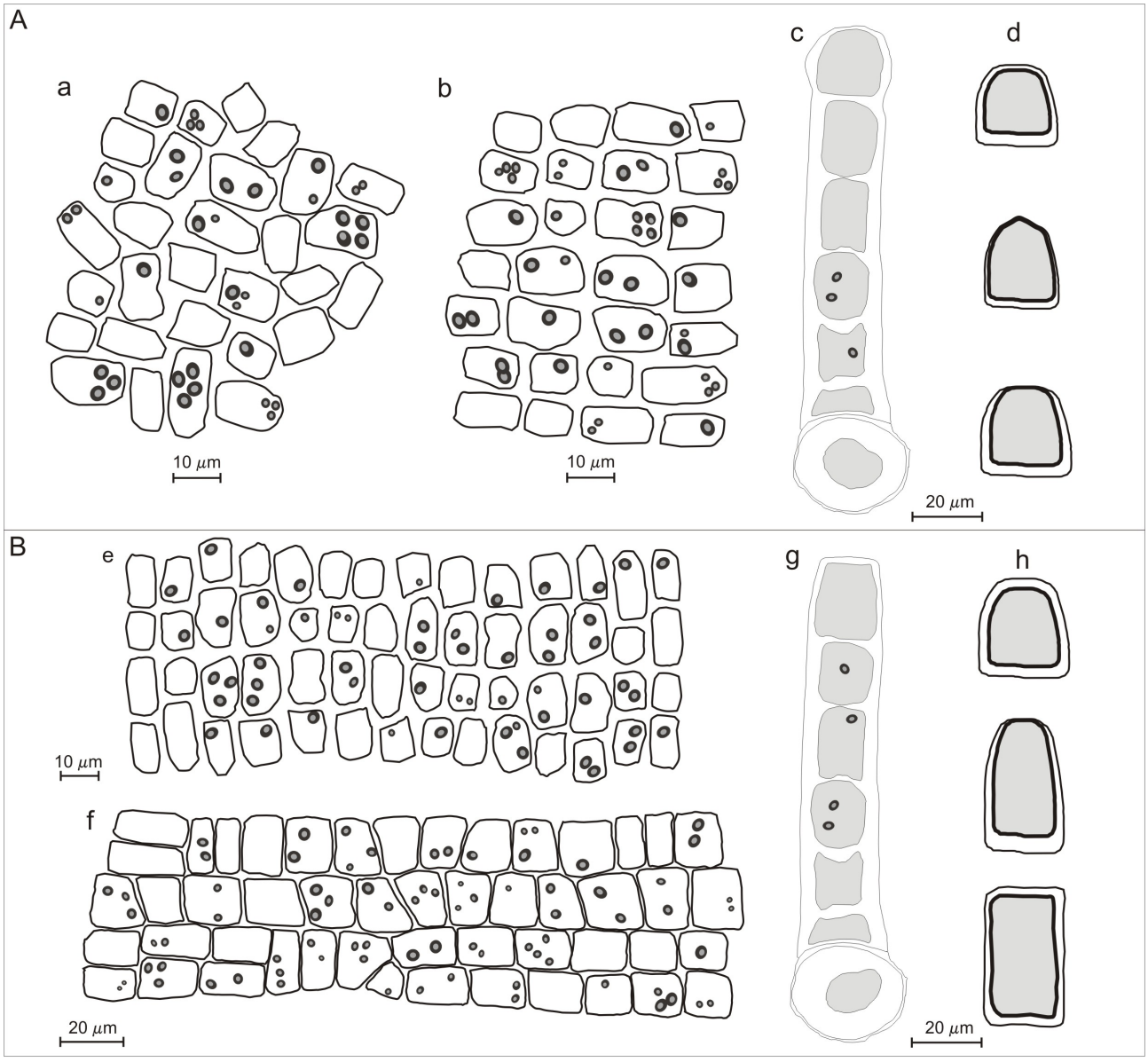

Figure 5

Shape of cells and apical regions of uniseriate branches of inland *Ulva flexuosa* species. A – *U. flexuosa* subsp. *pilifera*, B – *U. flexuosa* subsp. *paradoxa*. a – disordered cell arrangement, b – cells arranged in short regular rows, c –uniseriate apex of a juvenile branch with an obtusely rounded apical cell, d – different shapes of apical cells, e – cells arranged in short regular rows, f – cells arranged in long rows, g – elongated apical cells with a blunt apex, h – different shapes of apical cells

Discussion

Morphological plasticity of *Ulva* species is linked to environmental factors such as salinity, concentration of nutrients, temperature of water and depth of habitats (Reed & Russell 1978, Leskinen & Pamilo 1997, Messyasz & Rybak 2009, Messyasz et al. 2013, Rybak et al. 2013). Reliable identification of taxa from the *Ulva* genus requires detailed analysis of their morphological differentiation (morphometry of the thalli, thallus cells, and apical proliferations), as well as the use of technologies applied in molecular biology. Given their abundance and easy application, two or more molecular markers (nrDNA ITS and chloroplast *rbc*L) (Blomster et al. 1998, 1999, 2000; Mareš et al. 2011; Ichihara et al. 2009a, b; Ichihara

Table 6

Average values of physicochemical factors of water at the examined sites

Location and Specimen Voucher	Cond. (µS cm^{-1})	TDS (mg l^{-1})	Cl^{-} (mg l^{-1})	NaCl (mg l^{-1})
Ulva flexuosa subsp. *pilifera* (Kűtz.) M.J. Wynne				
mine pit; U0027; (n = 1)	621.00	297.00	124.00	23.50
Samica Stęszewska River; U0025; (n = 1)	564.00	230.00	56.50	64.50
Noteć River; U0021; (n = 1)	782.00	351.00	235.00	387.75
Kiełbaska River; U0023; (n = 1)	685.00	350.00	156.00	257.40
Rozlany Pond; U0028; (n = 1)	752.00	458.00	107.00	176.55
peat depression; U0007; (n = 1)	1042.00	635.00	265.00	437.25
Lednica Lake; U0011.80.90; (n = 1)	821.00	429.00	98.00	161.70
Świątnica Stream; U0012.13.15; (n = 22)	1307.68	528.00	132.83	217.14
Michałówka Stream; U0014; (n = 25)	1063.90	617.39	102.63	169.33
Nielba River; U0024.63; (n = 22)	781.45	531.09	64.80	106.91
oxbow lake; U0026; (n = 1)	694.00	345.00	215.00	354.75
Malta Reservoir; U0032; (n = 34)	650.89	426.26	63.11	104.11
Pątnowskie Lake; U0083.84; (n = 2)	571.00	373.75	27.00	44.55
Gosławskie Lake; U0085.86; (n = 2)	518.1	349.05	26.00	42.90
Licheńskie Lake; U0087; (n = 1)	604.00	377.00	23.25	38.36
Moraski Pond; U0097; (n = 1)	824.00	536.00	80.00	132.00
Ołobok River; U0115; (n = 1)	465.00	336.50	142.50	235.10
pond; U0116; (n = 1)	968.00	676.00	77.00	127.05
pond; U0122; (n = 1)	1003.00	760.00	264.10	435.70
Ulva flexuosa subsp. *paradoxa* (C. Agardh) M.J. Wynne				
pond; U0098; (n = 4)	3477.50	3659.50	8317.30	11965.50
canal; U0121; (n = 2)	5813.50	5122.00	6187.50	10209.38

Cond. – Conductivity; TDS – Total Dissolved Solids. The number of samples are given in brackets

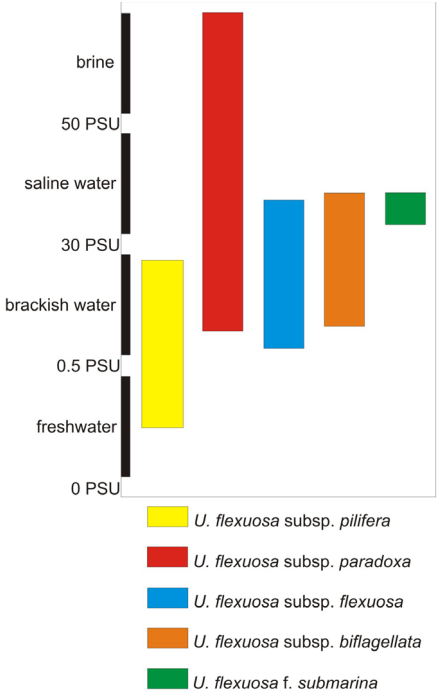

Figure 6

Comparison of salinity and different types of aquatic ecosystems where *Ulva flexuosa* taxa occurred (by Guiry & Guiry 2014)

et al. 2013) are most often used for identification of *Ulva* species. In many cases, the application of traditional technologies for species identification has proven insufficient (Hayden and Waaland 2002) due to modifications in the taxonomy of the *Ulva-Enteromorpha* genus in recent years, which resulted from the revision of the species that have been classified to date as belonging to the *Enteromorpha* genus. The enormous phenotype plasticity among the *Ulva* species depends on environmental conditions and the seasonal nature of the *Ulva* development and has frequently contributed to misidentification of *Ulva* species.

In all probability, varying biological and abiotic conditions were behind most reports of *Ulva* occurrence in inland ecosystems that referred to several species and subspecies and many forms (mostly *Enteromorpha intestinalis* (syn.: *Ulva intestinalis*), *E. compressa* (syn.: *Ulva compressa*), *E. salina* (syn.: *Ulva flexuosa*), and *E. prolifera* (syn.: *Ulva prolifera*). Revision of freshwater *Ulva* thallus samples collected in natural environments, combined with herbarium analysis (including holotypes of

U. flexuosa subsp. *flexuosa* and *U. flexuosa* subsp. *pilifera*) using traditional and molecular taxonomic methods, leads to the conclusion that it is likely that only one species of the *Ulva* genus, i.e. *U. flexuosa* (Mareš et al. 2011), occurs in European inland waters. Marine species like *Ulva intestinalis* and *U. compressa* or other species from *Ulva* genera were not confirmed to occur in the inland water ecosystems of central Europe.

Thalli of *Ulva flexuosa* subsp. *pilifera* were found mainly in rivers and lakes. However, *U. flexuosa* subsp. *paradoxa* was found at two sites in the vicinity of a fertilizer factory (an artificial pond and a canal supplied with salted slurry). Despite several years of searching for *U. flexuosa* subsp. *paradoxa* in anthropogenic areas and natural settlements (inland saltings and mineral spring fens) where high concentrations of chlorides have been recorded, we did not manage to find any other sites of this taxon.

In coastal ecosystems, the increased fertility of the habitats is the main cause of *Ulva* blooms (Littler and Littler 1980). In freshwater ecosystems, *Ulva* often occurs abundantly in the form of dense floating mats (Messyasz & Rybak 2009; Messyasz & Rybak 2011; Rybak et al. 2012a, b). To date, environmental research on freshwater *Ulva* at multiple independent sites has been lacking, and hence, the reasons for abundant occurrence of *Ulva* in water bodies such as lakes, rivers, and ponds have not been explicitly clarified. Although high levels of ammonia nitrogen and mineral salt discharges that penetrate into aquatic ecosystems from e.g. roadways, have been empirically found to cause mass development of freshwater *Ulva*, the data still seem to be insufficient to allow us to understand freshwater *Ulva* ecology (Messyasz & Rybak 2011). Hence, the view has persisted that the increasing eutrophication and salinization of aquatic ecosystems, combined with aquatic ecosystem contamination with heavy metals, results in freshwater *Ulva* expansion in European waters (Wohlgemuth et al. 1984, Messyasz & Rybak 2009, Messyasz & Rybak 2011, Mareš et al. 2011). Nonetheless, freshwater *U. flexuosa* has also been found in ecosystems where biogenic and mineral salt discharges were very low. In spite of the said charges, permanent *Ulva* blooming in such ecosystems has been observed, and *Ulva* has become a very distinctive local dominant in the hydrocenosis (Rybak & Messyasz 2009, 2011). According to Mareš et al. (2011), detailed research will be necessary to determine the concentrations of the dissolved ions that are responsible for the formation of freshwater *Ulva* blooms. Furthermore, information on the freshwater *Ulva* life cycle and reproductive methods will be required. These studies are all the more essential in light of the increasingly common occurrence of freshwater *Ulva* blooms reported from lakes and rivers (Rybak & Messyasz 2011). The abundant occurrence of freshwater *Ulva* has not been previously classified as having a negative impact on humans, but such negative impacts may exist at the level of aqueous biocenosis, because freshwater *Ulva* mats may locally change the physical and chemical conditions of water, due to e.g. the dark patches they create, and also modify a habitat's thermal circulation. Intensive biogenic consumption may also drain macro and microelements from the habitat (Messyasz et al. 2013). Dramatic ecological consequences of *Ulva* mat ("green tide") formation, primarily for benthos, have been observed in marine ecosystems. A deficiency of oxygen under the mat stimulates hydrogen sulfide production inside the deposits and affects flora and fauna in sea littoral zones (Sundbäck et al. 1990, Bonsdorff et al. 1997). In addition, the natural level of vascular plant vegetation and benthic fauna may suffer, depending on the surface mat cover rate (Norkko & Bonsdorff 1996). Romano et al. (2003) reported that the rate of structural redevelopment of zoobenthos groups and zoobenthos predators (fish and birds in this case) would become very violent if stimulated by the mats. It was also noted that some bird species, such as *Calidris alpina*, avoid areas covered by abundant *Ulva* thalli in the summertime. In the winter, *Numenius arquata*, *Tringa tetanus*, and *Pluvialis squatarola* avoid regions affected by abundant *U. intestinalis* thalli occurrence (Nicholls et al. 1981). The above-mentioned reports suggest that macroscopic alga mats significantly reduce the occurrence of zoobenthos and hence the food base for many macroscopic marine fauna species. Such environmental effects may also be observed for inland *Ulva* populations. Information on the causes of the (often massive) occurrence of taxa from the *Ulva* genus in fresh waters is still lacking, and the environmental requirements of freshwater *Ulva* are not determined either.

Due to the small number of *U. flexuosa*

subsp. *paradoxa* sites identified in inland aquatic ecosystems in Europe, we need an *ex situ* culture in various salinity settings to define the tolerance of the taxon to this environmental parameter. Initial findings by the authors (data not presented in this article) indicate that *U. flexuosa* subsp. *paradoxa* cultures in the Wang medium (Wang 1990), with no addition of chlorides, will results in thalli necrosis in as little as 48 hours from the moment they are placed in the medium. Similar laboratory research was carried out by Ichichara et al. (2013), who studied the tolerance of freshwater *Ulva limnetica* to increasing salinity. *U. limnetica* is the only species of the *Ulva* genus in the world that can only exist in fresh water and has not been found in coastal regions to date (Ichichara et al. 2009a, b). The cited research included other marine species of *Ulva*, such as *U. linza, U. pertusa* or *U. prolifera*, which were found to be unable to tolerate fresh water (PSU < 5) and to die within 3-4 days from the beginning of incubation. In the case of *U. limnetica*, the researchers noted 100% thallus vitality at salinity levels of 0, 5, and 30 PSU for the entire 7-day period of the study. Hence, *Ulva limnetica* is the only known *Ulva* species which has managed to adapt itself to freshwater habitats without having lost all of its ability to survive in sea waters (Ichihara et al. 2013). Previous experiments by Ichihara et al. (2011) demonstrated the excessive expression of *ULL* (*Ulva*-lectin-like) genes in *U. limnetica*. The said genes in this species are most likely strongly engaged in the adaptation to or tolerance to freshwater conditions. This species is the only known representative of the *Ulva* genus in the world with a tubular thalli (Enteromorpha-like *Ulva*) found exclusively in inland waters.

Given that the occurrence of *Ulva flexuosa* subsp. *paradoxa* is associated with saline waters of either natural or anthropogenic origin, the populations of this taxon occurring in inland ecosystems should not be called "freshwater" populations. The authors suggest using the term "inland taxon" for *U. flexuosa* subsp. *paradoxa* in future studies. On the other hand, in the case of *Ulva flexuosa* subsp. *pilifera*, the term "freshwater taxon" is obviously relevant because the populations of this taxon develop most often in waters with low chloride levels. In accordance with the Venice system, limnetic waters can have salinity levels up to 5 PSU (Venice System 1958, Oertli 1964). Inland *U. flexuosa* subsp. *pilifera* populations have not been found to date in waters with salinity levels higher than this limit value for fresh waters (Fig. 6).

Conclusions

At the present stage of identification of phylogenetic relations of the inland *Ulva*, and on the basis of the findings of previous studies by Mareš et al. (2011), we support the idea that the two *Ulva* subspecies found in European inland waters are close evolutionary entities. These taxa may be interpreted as subspecies or even young species that separated as a result of habitat isolation and adaptation to very different conditions. The occurrence of full isolation of young species, however, should be further verified by experimental hybridization of *U. flexuosa* subsp. *pilifera* and *U. flexuosa* subsp. *paradoxa*, as suggested by Mareš (2009). Such studies will preclude the occurrence of another type of physiological isolation if hybrids capable of further reproduction are obtained. This type of verification may be complemented by the use of markers that are more sensitive to the differences between the taxa, such as micro-satellite sequences and DNA – fingerprinting techniques (ISSR and AFLP) which have been successfully used in phylogenetic studies of closely related species (Kagami et al. 2008, Kostamo et al. 2008), hydrides (Gupta et al. 2015) and Baltic populations of *Ulva intestinalis* and *U. compressa* (Alström-Rapaport & Leskinen 2002, Alström-Rapaport et al. 2010).

Our results thus not only clarify the identity of the inland *Ulva* living in Central Europe, but also have significant methodological implications for future taxonomic studies on other Enteromorpha-like *Ulva* from freshwater ecosystems.

Acknowledgments

The author thanks Professor Emeritus Lubomira Burchardt for her considerable contribution to this study. Andrzej Rybak received a PhD fellowship from the Adam Mickiewicz University Foundation. We also thank the anonymous reviewers of this paper for the valuable, critical and helpful comments on the manuscript.

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