The importance of the polyphasic approach in a comparative study of *Nodularia* (*Nostocales*, *Cyanobacteria*)

Význam komplexního přístupu při srovnávacím studiu sinic rodu *Nodularia* (*Nostocales*, *Cyanobacteria*)

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This paper focuses on the morphology, taxonomy and ecology of the widespread cyanobacteria of the genus *Nodularia* Mertens ex Bornet & Flahault. In this study the benthic strain of *N. sphaerocarpa,* isolated from a sand-pit near Olomouc (Czech Republic), is compared with brackish and seawater strains. Changes in morphology and growth parameters (biomass and chlorophyll *a*) recorded in varying salinity gradients were studied and a 16S rRNA sequencing and AFLP analysis conducted. Morphological and ecophysiological characteristics found were in congruence with molecular data. Three major subgroups of the benthic *Nodularia* (*N. sphaerocarpa*, *N. moravica* and *N. harveyana*) were found using the polyphasic approach. The results of both the molecular and morphological study clearly separated *N. moravica* and *N. sphaerocarpa*, as freshwater species prefering a low salinity and the *N. harveyana* strains originating from a marine environment preferring a high salinity.

K e y w o r d s: AFLP, cyanobacteria, ecology, morphology, *Nodularia*, salinity, 16S rRNA

Introduction

The genus *Nodularia* Mertens ex Bornet & Flahault is a widespread group of ecologically and morphologically complicated species, which usually occur in brackish coastal waters and freshwater alkaline water bodies. This genus rarely occurs in the Czech Republic being represented exclusively by bentic species (Kaštovský et al. 2010). Previous identification was primarily based on the width of the filament. Based on Geitler's species concept (Geitler 1932), two main groups of species are recognized; the *N. harveyana* group (filaments narrower than 8 μm) and the *N. spumigena* group (filaments wider than 8 μm). Before the 1980s a great number of species, forms and varieties were described. After this date several taxonomic revisions were made and many taxa were combined (e.g. Nordin & Stein 1980, Komárek et al. 1993). The number of species varies depending on the authors. Komárek et al. (1993) and Hindák et al. (2003) classified *Nodularia* species, using the presence of gas vesicles as the main diacritical feature, with filament width secondary. Following this treatment *Nodularia* species were divided into a benthic group without gas vesicles (*N. harveyana*, *N. moravica*, *N. sphaerocarpa*, *N. turicensis*, *N. willei*) and a planktonic water bloom forming group with gas vesicles (*N. baltica*, *N. crassa*, *N. litorea*, *N. spumigena*). However, the occurrence of gas vesicles is not a stable feature and gas vesicles can disappear under unfavourable conditions (Poulíčková et al. 2004).

The taxonomically important character should be the ability to form gas vesicles and not their actual presence (Komárek et al. 1993).

Current identification of *Nodularia* species is based on the morphology of vegetative cells, heterocytes, akinetes, ecology and molecular biology (Laamanen et al. 2001, Lyra et al. 2005). Cell size can be variable and can overlap among different species (for detail see Komárek et al. 1993, their Fig. 2), however, in combination with the other features all *Nodularia* species are identifiable. The taxonomic position of *N. sphaerocarpa* is still unclear. Numerous authors consider this species to be a variety of *N. harveyana* (e.g. Geitler 1932, Elenkin 1938, Starmach 1966, Kondrateva 1968, Bourrelly 1970, Nordin & Stein 1980), whereas others classify it as a separate species (Komárek et al. 1993, Hindák et al. 2003).

The aims of this study are: (i) to evaluate the relationship of morphology, ecophysiology and molecular variability in the taxonomic classification within the genus *Nodularia* and (ii) to evaluate the taxonomic position of *N. moravica* and *N. sphaerocarpa*.

Material and methods

A benthic population of *N. sphaerocarpa* was obtained from a eutrophic sandpit near Olomouc (Czech Republic; 49°34'3.775"N; 17°14'58.131"E) in 2007 (pH 8.03, conductivity 1040 µS/cm, salinity 0.7‰) using the sampling methods published in Špačková et al. (2009). Samples of bottom sandy sediments were incubated under standard laboratory conditions (temperature t = 15±1 °C, illumination 20 µmol·m^{-2}·s^{-1}, photoperiod L/D 16/8 hrs) and studied using a Zeiss AxioImager light microscope (AxioCam HRc 13MPx, objectives Planapochromat 100/1.4, Oil, DIC, EC Plan-Nefluar 40/1.3, Oil, DIC). A strain of *N. sphaerocarpa* was isolated in liquid Bristol-Bold (BB) medium (Bold 1949). Several strains including those of *N. harveyana* (strain CCAP 1452/1, origin: marine; SAG 44.85, origin: salt marsh), *N. moravica* (strain Hindák 2000/15; Institute of Botany, Slovak Academy of Sciences, origin: freshwater, benthic), *N. sphaerocarpa* (strain SAG 50.79, origin: thermal water) and *Nodularia* sp. (strain CCAP 1452/6, origin: marine) were used for comparing with the above strain of *N. sphaerocarpa* (strain Dvořák 2009, origin: freshwater, benthic; Table 1).

Experimental growth in salinity gradient

The liquid medium BB was adjusted with sodium chloride to a final salinity gradient as follows: 10, 20, 30, 40, 50, 60 and 70‰. Serological plates (12 wells) were filled with 3.5 ml of culture media and 100 µl of *Nodularia* inoculum (3000 cells per ml) added to each well, with three replicates of each salinity. The plates were kept at the same culture conditions as incubated natural material of *N. sphaerocarpa*. Cultures were maintained for 30 days and regularly checked using an inverted microscope, the Zeiss Axiovert 40C. During the following 30 days, the number of vegetative cells, heterocytes, akinetes and vegetative cells between heterocytes were counted in a Bürker chamber. Morphological parameters (cell length and width) were measured for one hundred filaments per sample. The growth of cultures was evaluated using the chlorophyll-a concentration method of Vernon (1960).

Table 1. – List and characterization of the *Nodularia* strains used in this study.

Strain name and designation	Locality/Habitat	Reference	GeneBank access no.
N. harveyana, CCAP 1452/1	no data/marine	this study	HQ394172
N. harveyana, SAG 44.85	Lincolnshire, near Gibraltar/salt marsh	this study	HQ394175
N. moravica, Hindák 2000/15	Podivín, Czech Republic/freshwater, benthic, sand-pit lake	this study	HQ394173
N. sphaerocarpa, Dvořák 2009	Olomouc, Czech Republic/freshwater, benthic,	this study	HQ394177
N. sphaerocarpa, SAG 50.79	Dax, France/thermal water	this study	HQ394174
Nodularia sp., CCAP 1452/6	Dunstaffnage Bay, Oban, UK /marine, intertidal sediment	this study	HQ394176
N. harveyana, BECID29	Gulf of Finland, Baltic Sea/littoral zone, rock surface	Lyra et al. 2005	AJ781146
N. harveyana, Hübel 1983/300	Hiddensee, Baltic Sea/benthic microbial mat	Lyra et al. 2005	AJ781142
N. harveyana, Bo53	Boiensdorf/shallow coastal water	Lyra et al. 2005	AJ781143
N. harveyana, BECID27	Gulf of Finland, Baltic Sea /littoral zone, plant surface	Lyra et al. 2005	AJ781145
N. sphaerocarpa, BECID35	Gulf of Bothnia, Baltic Sea/littoral zone, mat-like colony	Lyra et al. 2005	AJ781149
N. sphaerocarpa, BECID36	Gulf of Finland, Baltic Sea/littoral pool, rock surface	Lyra et al. 2005	AJ781147
N. sphaerocarpa, Fä19	Fährdorf, Isle of Poel/shallow coastal water	Lyra et al. 2005	AJ781144
N. sphaerocarpa, Hübel 296	no data	Lyra et al. 2005	AJ781141
N. sphaerocarpa, PCC 73104	Spotted lake, Canada/alkaline soil	Lyra et al. 2005	AJ781139
N. sphaerocarpa, Up16a	Gulf of Finland, Baltic Sea/plankton	Lyra et al. 2005	AJ781140
N. sphaerocarpa, UTEX B 2092	Osoyoos, Canada/alkaline soil	Lyra et al. 2005	AJ781151
N. spumigena, AV1	Gulf of Finland, Baltic Sea/plankton	Lyra et al. 2005	AJ781136
N. spumigena, F81	Gulf of Finland, Baltic Sea/plankton	Lyra et al. 2005	AJ781137
N. spumigena, PCC 9350	Gulf of Finland, Baltic Sea/plankton	Lyra et al. 2005	AJ781131
N. spumigena, UTEX B 2093	San de Fuca, Whidbey Island, WA, USA/pond	Lyra et al. 2005	AJ781148

Statistical analyses

Morphological variability was analysed using one-way ANOVA (NCSS software; Hintze 2006). Polynomial correlation was made on significance level of $\alpha = 0.05$. Hierarchical clustering was based on Ward's minimum variance method (NCSS software).

DNA extraction, amplification and sequencing

DNA extraction was performed using the protocol of Doyle & Doyle (1990). The integrity and quality of DNA was checked on 1.8% agarose gel. Concentrations of DNA samples were assessed by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, USA). The PCRs with 16S rRNA primers CYA106F, CYA359F, CYA781R(a) and CYA781R(b) (Nübel et al. 1997) were performed using a FastStart PCR Master Kit (Roche) following the PCR protocol of Nübel et al. (1997). The PCR products were checked by agarose electrophoresis, purified using GeneElute PCR Clean up Kit (Sigma-Aldrich Co., USA) or cloned into the pGEM®-T vector (Promega Corporation, Madison, USA) and sequenced.

Table 2. – List of primer sets used in the reactions with the total number of scored and polymorphic bands.

Amplification Primer Sets Sequences	Number of bands	Number of polymorphic bands
EcoRI primer E-CAG / MseI primer M-CAAC	77	77
EcoRI primer E-CAG / MseI primer M-CAAT	86	85
EcoRI primer E-CAG / MseI primer M-CGAT	65	65
EcoRI primer E-ACC / MseI primer M-CAAC	74	72
EcoRI primer E-ACC / MseI primer M-CAAT	50	50
EcoRI primer E-ACC / MseI primer M-CGAT	77	76
EcoRI primer E-ATC / MseI primer M-CGAT	72	72
EcoRI primer E-ACA / MseI primer M-CGAT	42	42
lEcoRI primer E-ACT / MseI primer M-CGAT	67	67
EcoRI primer E-ACG / MseI primer M-CAAC	48	48
EcoRI primer E-ACG / MseI primer M-CTT	53	53

Total number of bands: 711
Polymorphic bands: 99.43%

AFLP analysis

The original procedure published by Vos et al. (1995) with the modification proposed by Kitner et al. (2008) was used for AFLP analysis of six *Nodularia* strains. In total eleven selective primer combinations were chosen to generate the AFLP profiles (Table 2). Products of amplification were separated on a 6%, 0.4 mm thick denaturing polyacrylamide gel (0.5× TBE buffer) using the T-REX (Thermo Scientifis Owl Separation Systems, Rochester, NY, USA) sequencing gel electrophoresis apparatus. Subsequent silver staining was used for visualizing the AFLP patterns.

Phylogenetic analyses

The 16S rRNA sequences of selected *Nodularia* strains (see Table 1 for access numbers) obtained above and from EMBL Nucleotide Sequence Database were aligned with ClustalW2 (EMBL Sequence Analysing tool; available from http://www.ebi.ac.uk/Tools/clustalw2/). The phylogenic tree was obtained using Bayesian MCMC (Markov chain Monte Carlo) analysis, which also enables a molecular clock analysis (Drummond & Rambaut 2007). The 95% confidence interval for the divergence of *Nodularia* isolates was inferred by an analysis with BEAST 1.4.2. (available from http://beast.bio.ed.ac.uk). The analysis was run for 10M generations and the burn-in was set to 100K generations. Then program TreeAnnotator summarized a tree sample from BEAST annotating it with posterior probabilities, HPD node heights and rates. This tree was viewed in the program FigTree (http://tree.bio.ed.ac.uk/software/figtree). Minimum evolution and maximum likelihood bootstrap analysis was carried out using PAUP* (Swofford 2001). Visualized AFLP gels were scored for presence (1) or absence (0) of bands. The binary matrix was constructed from primary data and subjected to FreeTree for cluster analysis (Pavlíček et al. 1999; method UPGMA, Jaccard similarity coefficient). The resulting cluster was visualized in TreeView (Page 1996). To validate how consistently the AFLP data support given isolate bipartitions, bootstrap analysis was carried out using 1000 replicates (Felsenstein 1985).

Results

Investigation of natural population of Nodularia sphaerocarpa

The morphology of *N. sphaerocarpa* accords with the description in Komárek et al. (1993) and differences between the species studied are compared in Table 3. *Nodularia sphaerocarpa* was initially observed in December 2007 when it formed an important part of the benthic cyanobacterial assemblages (25%) on the surface of sandy bottom sediments. Filaments were usually straight, bent or occasionally wavy, attenuated or not, 5–8 µm wide (Figs 1–5). Sheaths were colourless and diffluent. Cells without gas vesicles were short, barrel-shaped, 2–5 × 5–7 µm. Heterocytes were elliptical, rectangularly-rounded or barrel-shaped, 5–9 × 6–10 µm. Akinetes were spherical or elliptical, 6–10 µm in diameter, with wart-like incrustations on their surface (Figs 6–9) and often in chains (more than four cells). Occasionally, short hormogonia (up to 20 cells), which had germinated from akinetes, were found.

Table 3. – Comparison of the morphology of the *Nodularia* species included in this study.

	N. sphaerocarpa	*N. moravica*	*N. harveyana*	*N. spumigena*
Vegetative cells	2–5 × 5–7	2–6 × 7–15	1.5–3.5 × 4–5	2–5.6 × 6.8–12
Heterocytes	5–9 × 6–10	4–11 × 8.5–11	3–6 × 4–7	2–5 × 9–13.7
Akinetes	6–10 in diam.	6–10 × 8–12	4–8 × 5–7	5.7–15 × 8–12
Gas vesicles	not present	not present	not present	present
Habitat	periphytic, benthic, alkaline waters	alkaline littoral	periphytic, benthic, saline or mineral water	planktonic, marine
Reference	this study	Hindák et al. 2003	Komárek et al. 1993	Komárek et al. 1993

Effect of salinity on morphology of Nodularia strains

Five strains of *Nodularia,* from culture collections, were compared with the isolate (Table 1, Figs 10–43). Growth and filament shape were variable and depended on salinity. *Nodularia sphaerocarpa* (both strains), *N. harveyana* (both strains) and *Nodularia* sp. were mostly long and straight (Figs 1–30) or slightly bent. Short filaments and hormogonia of *N. sphaerocarpa* (strain Dvořák 2009) were recorded in standard BB medium (Fig. 10).

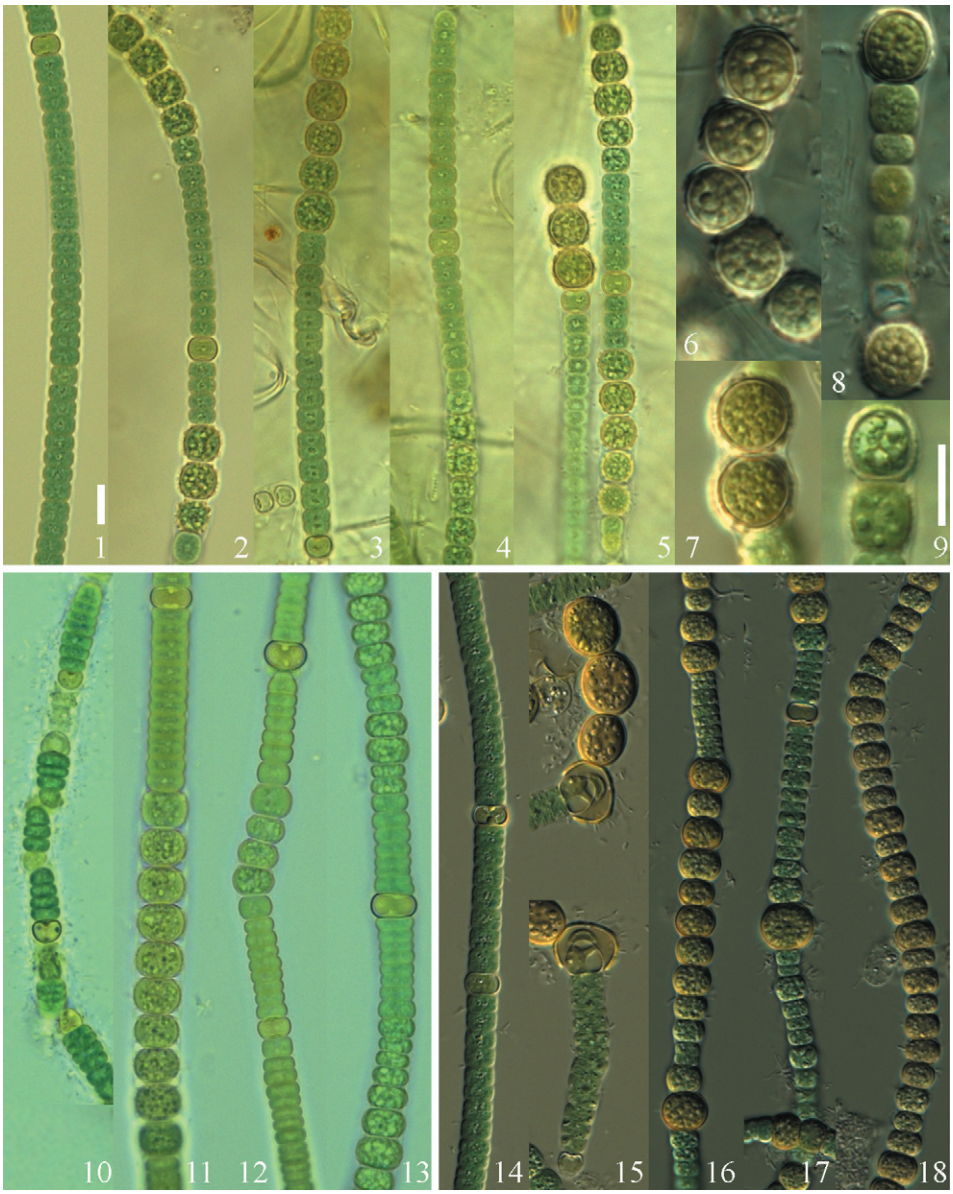
Growth of *N. sphaerocarpa* (strain Dvořák 2009) was best when the salinity was 10–20‰ (Table 4) and inhibited when it was 40‰, whereas the second strain (SAG 50.79) grew well only in basic BB medium and at a salinity of 10‰ (1920000 cells per ml). *Nodularia moravica* (Hindák 2000/15) grew well in media with salinities of up to 40‰ (with the highest abundance of 4,138,000 cells per ml at 10‰ salinity). Strain *N. harveyana* (CCAP 1452/1) grew in basic BB medium with salinities up to 50‰, but the growth was similar over the entire range. Strains of *N. harveyana* (SAG 44.85) and *Nodularia* sp. (CCAP 1452/6) grew in basal BB medium with salinities up to a 40‰ and *N. harveyana* and *Nodularia* sp. grew best at salinities of 30‰ and 10‰, respectively (Table 4). Growth parameters (abundance and chlorophyll-a) were significantly correlated with gradient of salinity, except for strain *N. harveyana* CCAP 1452/1 (Table 5).

Table 4. – Culture growth parameters on salinity gradient: abundances (thousands·ml^{-1}) of vegetative cells, heterocytes, akinetes and the chlorophyll *a* concentration (μg·l^{-1}) of *Nodularia* species when kept at particular salinities; n.d. – no data (no akinetes appeared or strain was not growing); salinity gradient: BBM – Bristol-Bold medium without addition of NaCl (= 0‰) and salinity range (10–50‰).

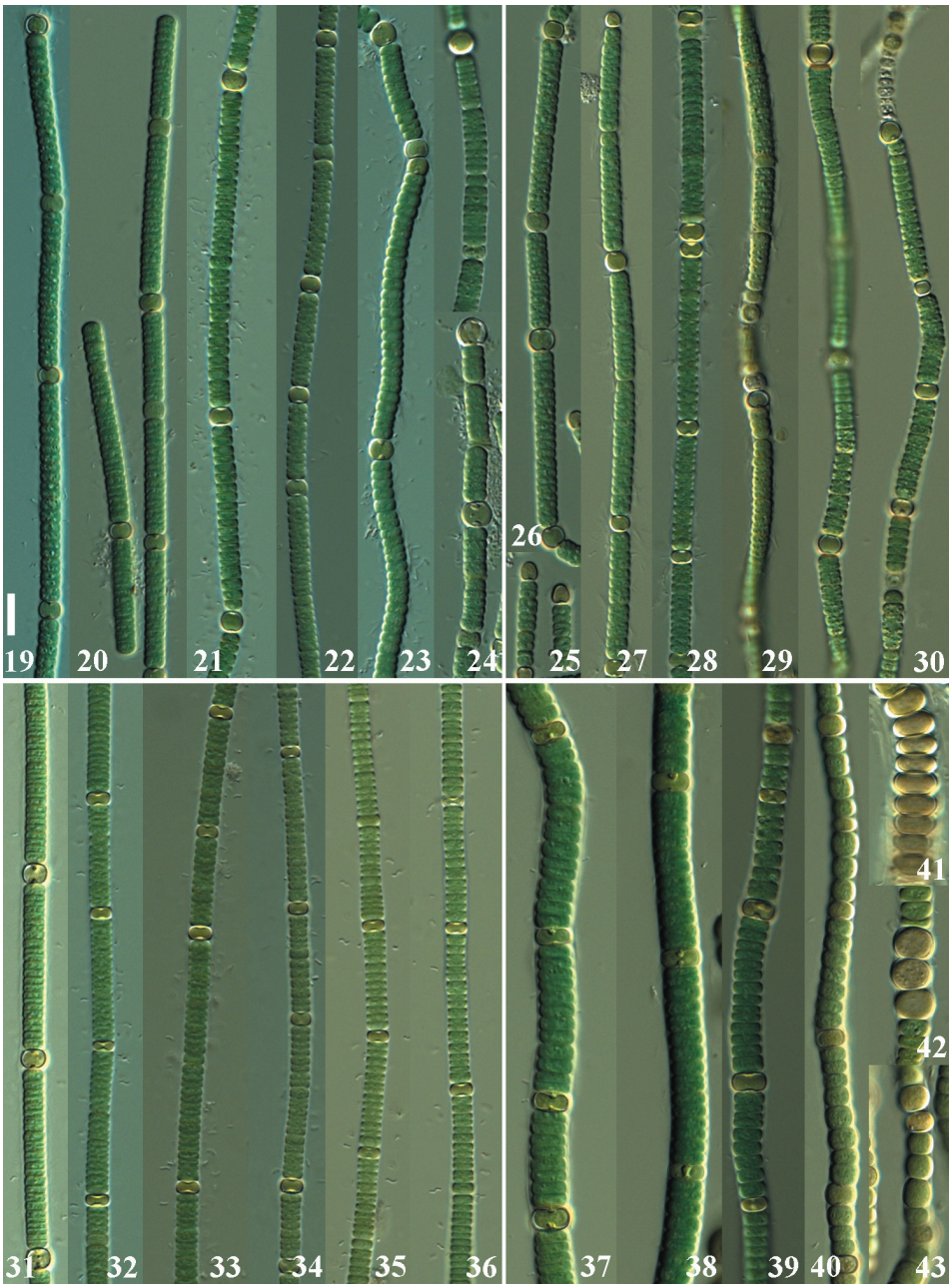
Species (strain)	Abundance	Salinity					
		BBM	10‰	20‰	30‰	40‰	50‰
N. sphaerocarpa	cells	2904	13737	18205	2558	n.d.	
(strain Dvořák 2009)	heterocytes	149	290	349	49	n.d.	
	akinetes	4	27	37	4	n.d.	
	total cells	3058	14053	18592	2611	n.d.	
	chlorophyl *a*	11.89	31.39	22.63	5.72	n.d.	
N. sphaerocarpa	cells	5893	1920	n.d.	n.d.	n.d.	
(SAG 50.79)	heterocytes	209	43	n.d.	n.d.	n.d.	
	akinetes	n.d.	130	n.d.	n.d.	n.d.	
	total cells	6102	2093	n.d.	n.d.	n.d.	
	chlorophyl *a*	37.43	8.89	n.d.	n.d.	n.d.	
N. moravica	cells	3222	4138	3940	3247	320	
(strain Hindák 2000/15)	heterocytes	471	347	273	210	27	
	akinetes	n.d.	n.d.	n.d.	n.d.	63	
	total cells	3693	4484	4213	3457	410	
	chlorophyl *a*	110.62	76.58	54.10	13.08	3.43	
N. harveyana	cells	19653	13871	13113	12573	19431	10071
(CCAP 1452/1)	heterocytes	1327	809	847	740	870	542
	total cells	20980	14680	13960	13313	20306	10613
	chlorophyl *a*	25.02	40.90	40.37	31.66	52.96	16.00
N. harveyana	cells	3849	2960	6422	9600	3871	
(SAG 44.85)	heterocytes	658	596	556	756	191	
	total cells	4507	3556	6978	10369	4076	
	chlorophyl *a*	19.56	19.94	17.91	15.24	5.97	
Nodularia sp.	cells	15607	18853	8044	10449	4787	
(CCAP 1452/6)	heterocytes	747	900	484	716	223	
	total cells	16353	19753	8529	11164	5010	
	chlorophyl *a*	49.07	32.54	24.54	16.90	10.31	

Table 5. – Coefficient of determination (r^2) of the correlation between the growth parameters and salinity. Salinity ranges as in Table 4; the strain *N. harveyana* CCAP 1452/1 growth of which was not significantly correlated with salinity is not shown. * significance level 0.05.

Growth parameters	*N. sphaerocarpa* (strain Dvořák 2009)	N. moravica (strain Hindák 2000/15)	*N. sphaerocarpa* (SAG 50.79)	*N. harveyana* (SAG 44.85)	*Nodularia sp.* (CCAP 1452/6)
Vegetative cells	0.949*	0.987*	0394	0.214	0.711*
Heterocytes	0.930*	0.972*	0.549	0.694*	0.649*
Total cells	0.949*	0.979*	0.369	0.241	0.710*
Chlorophyll–a	0.948*	0.984*	0.980*	0.440	0.993*



Figs 1–18. – Morphological variability of *Nodularia sphaerocarpa*. **1–9** seminatural population of *N. sphaerocarpa* from a sand pit at Olomouc: **1–5** filaments; **6–9** akinetes; **10–18** changes in the filaments recorded at different salinities. **10–13** strain Dvořák 2009: **10** standard BB medium; **11** salinity 10‰; **12** salinity 20‰; **13** salinity 30‰; **14–18** strain SAG 50.79: **14–15** standard BB medium; **15** hormogonia production; **16–18** salinity 10‰. Scale bars 10 µm (1–5, 10–18; 6–9)



Figs 19–43. – Morphological variability of *Nodularia harveyana* and *Nodularia moravica*. **19–24** strain CCAP 1452/1: **19** standard BB medium; **20** salinity 10‰; **21** salinity 20‰; **22** salinity 30‰; **23** salinity 40‰; **24** salinity 50‰; **25–30** strain SAG 44.85: **25–26** standard BB medium; **27** salinity 10‰; **28** salinity 20‰; **29** salinity 30‰; **30** salinity 40‰; **31–36** strain CCAP 1452/6: **31** standard BB medium; **32** salinity 10 ‰; **33** salinity 20‰; **34** salinity 30‰; **35–36** salinity 40‰; **37–43** strain Hindák 2000/15: **37** standard BB medium; **38** salinity 10‰; **39** salinity 20‰; **40** akinete formation in standard BB medium; **41–43** salinity 10 and 30‰. Scale bar 10 µm.

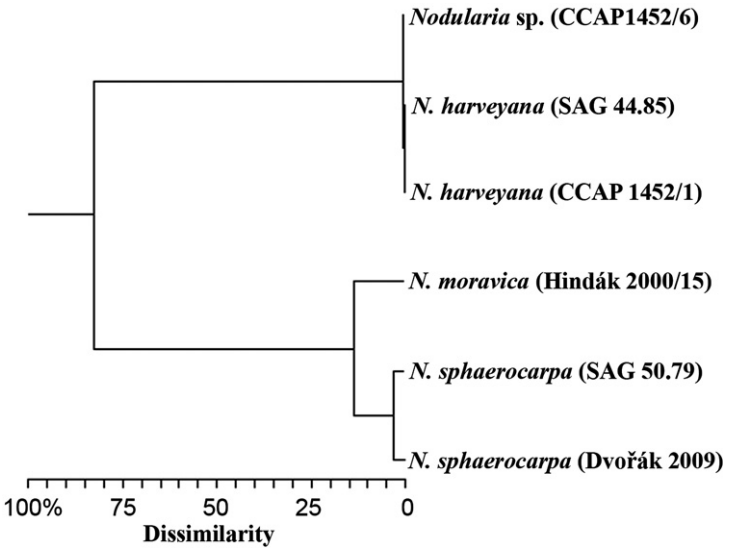


Fig. 44. – Hierarchical clustering based on morphology of vegetative cells and heterocytes (Ward's minimum variance method).

Vegetative cells were always wider than long. Barrel-shaped cells occurred occasionally. Cell morphology was significantly influenced by the salinity gradient (Table 6). Hierarchical clustering analysis based on cell morphology clearly separated the marine group of *N. harveyana* and *Nodularia* sp. from the freshwater group of *N. sphaerocarpa* and *N. moravica*. *Nodularia moravica* was also slightly separated from *N. sphaerocarpa* (Fig. 44). There was no significant influence of salinity on cell size within the *N. harveyana* group. A wide variation in cell dimensions was observed in *N. moravica*, but without any general trend relative to salinity. Cells, akinetes and heterocytes seemed to be wider at the higher salinities in *N. sphaerocarpa* (Table 6). Cells and heterocytes were the widest in a medium adjusted to a salinity of 30‰. Cell dimensions of the strain (SAG 50.79) of *N. sphaerocarpa* were similar to those of strain Dvořák 2009. There were no significant differences in cell dimensions in the standard medium with a salinity of 10‰ (except for that of the heterocytes). Cells of *N. moravica* were wider than long, occasionally barrel-shaped or elliptical. Cell and heterocyte width in the *N. harveyana* group were not as variable as in *N. sphaerocarpa* and *N. moravica*. Cells and heterocytes were always wider than long and the minimum and maximum values were similar (for details see Table 6).

Within the first 10 days of the experiment vegetative cells and heterocytes were observed in cultures of all strains. No akinetes were formed by *N. harveyana* and *Nodularia* sp. After 20 days akinetes occurred in the cultures of *N. sphaerocarpa* and *N. moravica*, particularly at salinities between 10–30‰ and sporadically in the basic BB medium. Akinetes of *N. sphaerocarpa* (strain Dvořák 2009) were not of the same shape as in the natural population and were usually wider than long and lacked wart-like incrustations on their surfaces (Figs 11–13). Low salinities stimulated *N. sphaerocarpa* to produce short hormogonia from filaments by means of necridic cells (Fig. 10). Akinetes of *N. sphaerocarpa* (SAG 50.79) occurred in long chains (0–10‰ salinity) and those in stan-

Table 6. – Changes in morphology of *Nodularia* strains kept at different salinities. Salinity gradient as in Table 4; n.d.– no data. Effects of salinity on the length (μm) and width (μm) of each cell type were tested using one-way Anova; * significance level 0.05–0.01; ** significance level < 0.01, n = 100 cells for each salinity.

		Vegetative cells		Heterocytes		Akinetes		Cells HTC-HTC
		Length	Width	Length	Width	Length	Width	
Nodularia sphaerocarpa	BBM	2.51±0.64	6.90±0.58	4.96±0.77	7.54±0.81	5.67±0.71	9.43±1.15	31±7
(strain Dvořák 2009)	10‰	2.40±0.70	8.02±0.73	4.98±0.90	9.06±1.00	5.03±1.31	10.00±0.81	95±15
	20‰	2.51±0.62	7.57±0.67	5.29±0.90	9.28±0.96	6.09±0.94	9.81±1.29	69±20
	30‰	2.24±0.46	9.19±1.02	6.08±1.29	11.06±1.44	5.73±0.64	10.89±1.53	70±31
	F	3.83**	141.71**	25.15**	160.04**			
	r^2	0.50	0.74	0.69	0.93			
Nodularia sphaerocarpa	BBM	3.28±0.92	6.97±0.47	6.43±1.16	8.75±0.88	9.77±1.76	10.56±1.31	17±7
(SAG 50.79)	10‰	2.93±0.76	7.13±0.94	5.62±1.80	7.92±0.95	7.96±1.42	10.40±0.98	23±4
	F	2.64	0.70	3.32	7.78**			
Nodularia moravica	BBM	3.13±0.85	9.81±0.65	5.48±1.03	9.67±0.60	n.d.	n.d.	13±4
(strain Hindák 2000/15)	10‰	3.00±0.89	8.90±0.60	4.52±0.68	9.16±0.64	n.d.	n.d..	13±5
	20‰	3.58±0.92	7.71±0.59	5.29±0.78	8.26±0.68	8.00±0.00	12.00±0.00	9±3
	30‰	3.06±0.89	7.00±0.68	5.55±0.76	8.03±0.60	n.d..	n.d..	11±4
	40‰	3.81±0.75	8.69±1.25	6.06±1.18	10.00±0.63	7.32±2.04	9.84±1.60	14±8
	F	3.88**	69.05**	10.31**	47.78**			
	r^2	0.39	0.36	0.38	0.01			
Nodularia harveyana	0‰	2.29±0.59	5.42±0.51	5.16±0.58	5.93±0.44	n.d.	n.d.	18±6
(CCAP 1452/1)	10‰	2.29±0.46	5.29±0.46	4.58±0.81	5.61±0.50	n.d.	n.d.	17±5
	20‰	2.29±0.46	5.16±0.27	4.90±0.83	5.55±0.57	n.d.	n.d.	15±4
	30‰	2.58±0.50	4.90±0.60	4.74±0.58	5.45±0.57	n.d.	n.d.	13±5
	40‰	2.09±0.65	5.00±0.51	4.77±0.92	5.58±0.62	n.d.	n.d.	17±6
	50‰	2.45±0.63	5.37±0.49	5.09±0.88	6.27±0.65	n.d.	n.d.	15±7
	F	2.73*	6.89**	2.38*	9.22**			
	r^2	0.02	0.13	0.01	0.07			
Nodularia harveyana	0‰	2.44±0.62	5.25±0.57	5.03±0.53	5.53±0.88	n.d.	n.d.	12±6
(SAG 44.85)	10‰	2.06±0.56	5.19±0.40	4.94±0.80	5.62±0.61	n.d.	n.d.	17±9
	20‰	2.03±1.18	5.19±0.57	4.72±0.77	5.81±0.64	n.d.	n.d.	15±5
	30‰	2.16±0.37	5.16±0.37	4.60±0.71	5.88±0.55	n.d.	n.d.	14±7
	40‰	2.16±0.57	5.31±0.47	4.69±0.78	5.91±0.47	n.d.	n.d.	11±4
	F	1.61	0.63	2.03	2.06			
	r^2	0.20	0.06	0.79	0.93			
Nodularia sp.	0‰	2.06±0.57	4.87±0.50	5.13±0.62	6.10±0.60	n.d.	n.d.	26±9
(CCAP 1452/6)	10‰	2.06±0.68	5.26±0.68	3.94±0.81	5.94±0.73	n.d.	n.d.	22±8
	20‰	2.00±0.63	4.98±0.48	4.19±0.75	5.58±0.50	n.d.	n.d.	24±9
	30‰	1.68±0.60	5.39±0.56	3.58±0.81	6.03±0.60	n.d.	n.d.	26±12
	40‰	1.94±0.57	5.16±0.45	3.71±0.94	6.00±0.77	n.d.	n.d.	25±12
	F	2.13	4.68**	18.73**	3.04*			
	r^2	0.39	0.29	0.68	0.01			
Pooled data	F	17.26**	233.43**	20.13**	200.98**			

dard medium were more spherical than in the 10‰ medium (Figs 16–18). Germination was observed in the standard BB medium (Fig. 15). Akinetes of *N. moravica* occurred throughout the whole salinity gradient and were the widest of all of the cultivated strains (Table 6). Their shape changed from flatly elliptical to round (Figs 41–43). Unlike in

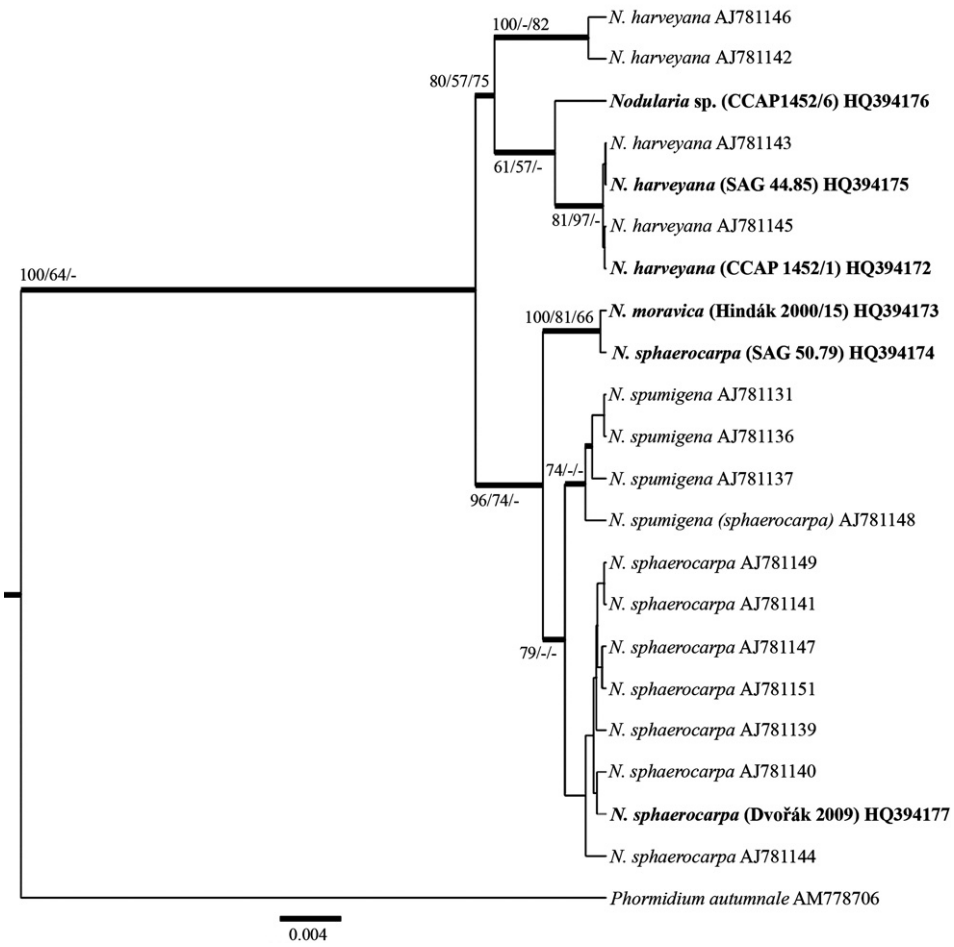


Fig. 45. – Phylogram of single tree generated from Bayesian MCMC (Markov chain Monte Carlo) analysis based on 16S rRNA sequences (length = 421bp) and with *Phormidium autumnale* as the outgroup. Labelled branches indicate Bayesian posterior probabilities ≥ 95%. Bootstrap support values (≥ 50%) are shown above the branches (posterior probability from Bayesian analysis/minimum evolution/maximum likelihood). Strains studied are in bold.

N. sphaerocarpa, the content of the akinetes was less granulated, rather fine granulated to nearly homogenous and pale brown or green-brown. Akinetes of *Nodularia moravica* germinated to produce hormogonia in media with salinities of between 10 and 20‰.

Analysis of 16S rRNA gene sequences

The 16S rRNA sequences from the *Nodularia* strains studied were compared with those available from the GenBank and one *Phormidium* isolate (outgroup). Following Lyra et al. (2005), only well-defined sequences were used. This analysis clearly divided the genus *Nodularia* into two major groups: *N. harveyana* and a group represented by *N. moravica*,

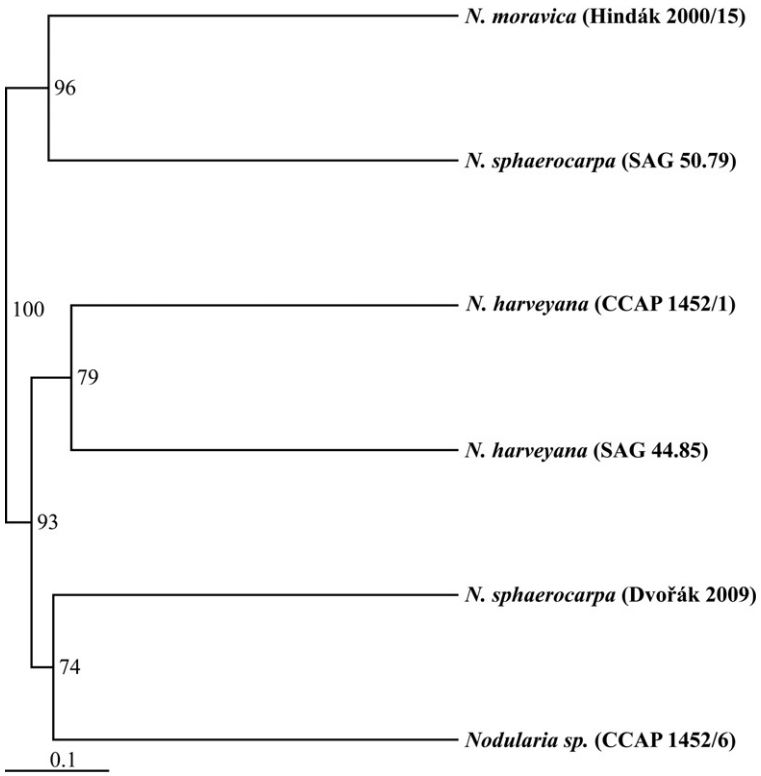


Fig. 46. – UPGMA analysis dendrogram (Jaccard's similarity coefficient) based on 711 AFLP fragments. Significant values of bootstrap analysis are shown on branches.

N. spumigena and *N. sphaerocarpa* (Fig. 45). The *N. harveyana* clade included the strains CCAP 1452/1 and SAG 44.85 (*N. harveyana*) and strain CCAP 1452/6 (*Nodularia* sp.). There was not a high degree of similarity within the *N. harveyana* clade.

The second group is represented by *N. moravica*, *N. spumigena* and *N. sphaerocarpa* and its inner separation is supported by high bootstrap values (Fig. 45). The most dissimilar group inside this clade is *N. moravica,* which is separate from the *N. sphaerocarpa* and *N. spumigena* groups.

AFLP analysis

In total six *Nodularia* spp. strains were analysed using eleven AFLP primer combinations, which generated 711 unambiguously scored fragments, of which 99.4% were polymorphic. The statistical analysis (Fig. 46) supported the results of the 16S rRNA sequencing (Fig. 45). The only difference was the co-segregation of *Nodularia* sp. (CCAP 1452/6) strain together with *N. sphaerocarpa.* Nevertheless, the co-segregation of *N. moravica* and *N. sphaerocarpa* support the theory that *N. moravica* has diverged significantly from other *Nodularia* species. Moreover, *N. harveyana* is considered to be a monophyletic species clearly diagnosed both by molecular and morphological characters.

Discussion

Cyanobacteria play a key role in the functioning of many ecosystems and because they produce toxins they are potentially harmful organisms. Despite their importance, however, many aspects of their biodiversity and ecology are poorly understood. Routine species identification, mostly using morphology-based classifications, may not provide sufficient taxonomic resolution as cyanobacteria with similar or identical morphology can differ significanly in their physiology. In recent years, the analysis of 16S rRNA gene sequences has demonstrated that the morphological classification of cyanobacteria in some cases corresponds to phylogenetically coherent taxa (Garcia-Pichel et al. 1996), whereas in other cases the traditional classification greatly underestimates extant diversity (Ferris et al. 1996). For example, in bacteriology, the tolerance and requirements for high salt concentrations and high temperatures are recognized as important phenotypic properties that are correlated with phylogeny (Imhoff et al. 1998).

The genus *Nodularia* consists of apoheterocytic nostocalean cyanobacteria, for which filament and cell morphology can be used for intraspecific taxonomy (e.g. Geitler 1932, Kondrateva 1968, Nordin & Stein 1980, Komárek et al. 1993). Altogether 28 species, varietes and forms were revised by Nordin & Stein (1980) and only *N. harveyana* and *N. spumigena* were considered as valid species. They hypothesized that the other morphological species may only be a result of adaptation to growth conditions, e.g. salinity and pH values, which are the most important factors influencing the distribution of *Nodularia*. Thus these authors do not consider cell and heterocyte dimensions and morphology in general to be reliable taxonomic characters.

However, hierarchical cluster analysis based on morphological data of five *Nodularia* strains clearly separated the species *N. sphaerocarpa*, *N. harveyana* and *N. moravica*. While *N. harveyana* differs in trichome width, shape and number of chained akinetes (2–4), *N. sphaerocarpa* forms spherical akinetes and has chains of high numbers of akinetes (Komárek et al. 1993). These features were probably overlooked by previous authors (Geitler 1932, Elenkin 1938, Starmach 1966, Kondrateva 1968, Bourrelly 1970, Nordin & Stein 1980). Similarly, *N. moravica* differs from both of the species mentioned above (Hindák et al. 2003) in terms of its morphology (vegetative cells, heterocytes and akinetes) and ecology.

The classification based on morphology accords with molecular data. *Nodularia harveyana* is clearly separated from other *Nodularia* species. Although the validity of *N. sphaerocarpa* is confirmed by molecular studies (Bolch et al. 1999, Laamanen et al. 2001, Lyra et al. 2005) it is heterogeneous. *Nodularia sphaerocarpa* (strain Dvořák 2009) belongs to the main cluster of *N. sphaerocarpa*, however, *N. sphaerocarpa* (SAG 50.79) is separated from this group and has a high similarity with *N. moravica*. An identical strain of *N. sphaerocarpa* from a thermal spring (strain PCC 7804; Dax, France) studied by Bolch et al. (1999) has the same dissimilarities in the nucleotide sequences of its cpcBA-IGS as *N. sphaerocarpa*. However, the position of strain PCC 7804 is closer to *N. sphaerocarpa* than the other species included in his study.

Nodularia moravica was described by Hindák et al. (2003) and currently there are no molecular studies on this species. This author provided us with the type strain of *N. moravica* (Hindák 2000/15) for DNA analysis. The molecular, morphological and ecophysiological data support the claim of Hindák et al. (2003) that *N. moravica* differs significantly from

the other *Nodularia* species. The taxonomic position and/or determination of the thermophilic *N. sphaerocarpa* (SAG 50.79) strain remains unresolved and it is proposed to keep this strain as *Nodularia* sp.

A similar situation exists for *N. harveyana*, whose separation from other species was supported by morphology and 16S rRNA sequences. Strain *Nodularia* sp. CCAP 1452/6 belongs to the *N. harveyana* cluster, although it was not supported by AFLP profiles, due to the fact that the few strains tested could not be compared with database data. Based on the 16S rRNA sequencing results it is proposed to identify *Nodularia* sp. CCAP 1452/6 as *N. harveyana*.

Nordin & Stein (1980) studied *Nodularia* isolates kept in various salinity and pH gradients and concluded that although this genus can tolerate low salinities and pH values, it does not have the features of a "true freshwater" species. Warr et al. (1984) record a negative influence of increasing salinity on the growth of *N. harveyana*. The results presented here support the idea that halotolerance in *Nodularia* is species-specific. Unlike species isolated from marine environments (*N. harveyana*) those from freshwater (*N. sphaerocarpa*, *N. moravica*) grew better at low salinities. The highest range of salinities tolerated by species in the study was recorded for *N. harveyana* and *Nodularia* sp. and the narrowest for *N. sphaerocarpa* isolated from thermal water. However, its extremely narrow range compared to that for *N. sphaerocarpa* (strain Dvořák 2009) may be because of the age of the culture and the length of time it was maintained under laboratory conditions.

The dependence of the growth rate of genetically variable strains on salinity and species-specific halotolerance is also reported for *Spirulina* spp. by Nübel et al. (2000). Their data do not support the traditional opinion that a few closely related species of cyanobacteria with *Spirulina* morphology have a broad ecological euryvalence and ubiquitous distribution (Anagnostidis & Golubić 1966). Three of the isolates originated from hypersaline water and were similar in their high halotolerance and broad euryhalinity. Phylogenetic analysis placed them in a monophyletic cluster apart from all the other species (Nübel et al. 2000).

The ecophysiological characteristics of *Nodularia* strains studied support the hypothesis, that ecologically distinct organisms thriving in different habitats have different physiological capabilities and different evolutionary histories, which are reflected in their genetic divergence.

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Souhrn

Studie se zabývá morfologií, taxonomií a ekofyziologií sinic rodu *Nodularia,* který zahrnuje sladkovodní, brakické i mořské zástupce. Experimentální práce spočívala v hodnocení morfologické variability a růstových parametrů v gradientu salinity. Ekofyziologické a morfologické charakteristiky velmi dobře korespondují s výsledky molekulárních metod (16S rRNA, AFLP) a mohou vysvětlit rozdělení bentických druhů rodu *Nodularia* na dva sladkovodní druhy (*N. sphaerocarpa a N. moravica*) a jeden mořský (*N. harveyana*).

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