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SHORT COMMUNICATION

Microorganisms associated with charophytes under different salinity conditions

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Summary Microorganisms associated with aquatic macrophytes can in various ways interact with a plant and influence its activity and *vice versa*. A low-salinity intrusion into freshwater environment can affect plant-microorganism interactions. In this study, effects of different salinity conditions on the abundance and community composition of associated microorganisms with charophytes in the Curonian Lagoon were assessed. From the results, we found that short term salinity changes affected the abundance of bacteria and fungi associated with charophytes, whereas no response was reflected in the taxa composition of fungi, showing that other factors could be of more importance. The increased fungi abundances and different fungi composition in August in comparison to June was probably related to senescence process of aquatic vegetation. 8 fungi taxa were isolated and identified in association with charophytes, while higher diversity was revealed by DGGE technique.

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Aquatic macrophytes, especially charophytes (*Characeae*), are considered as important biological quality element for fresh and brackish water bodies (Kufel and Kufel, 2002; Mathieson and Nienhuis, 1991 and references therein). In moderately shallow and eutrophic lagoons, charophytes are responsible for stabilization of clear-water states by causing inhibition of phytoplankton (Hilt, 2015). Dense beds of charophytes provide shelter, food and substrate for benthic invertebrates, fish and waterfowls (Dugdale et al., 2006;

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Schmieder et al., 2006). Charophytes are also hosts for various microorganisms such as fungi and bacteria (Berg and Hagemann, 2009; Hempel et al., 2008). Microorganisms associated with aquatic vegetation form biofilm and are rich in antimicrobial compounds, which may protect macrophytes from hostile environment (Berg and Hagemann, 2009). On the other hand, colonies of epiphytic microorganisms, such as cyanobacteria (*Gleotrichia* sp.), might cause decrease of photosynthetic activity, nutrient uptake or very fast decay of macrophytes (Armstrong et al., 2001; Hudon et al., 2009). Although there are studies on the importance of microorganisms associated with macrophytes (Andrews and Harris, 2000; Egan et al., 2014, 2013), little is known about interactions between fungi (Godinho et al., 2013) and macrophytes.

Declining distribution and diversity of macrophytes, including charophytes, have been observed in many regions worldwide including the brackish Baltic Sea (*e.g.* Blindow, 2000; Pitkänen et al., 2013). Eutrophication and its consequences (increased pelagic productivity, water turbidity and reduced light availability) are assumed to be the most important threats to macrophytes (Blindow et al., 2003; Kovtun-Kante et al., 2014). It has been experimentally documented (Blindow and Schütte, 2007) that besides the negative effect of water turbidity the changes in salinity may increase stress for freshwater charophytes in transitional water bodies, such as the Curonian Lagoon. This stress could be enhanced by a human pressure, for instance, by dredging the canal connecting the lagoon and the Baltic Sea (Zemlys et al., 2013). The elevated salinity can affect both sexual reproduction and vegetative propagation (Blindow et al., 2003). Moreover, the changes in salinity may affect charophytes growth rates, incrustation of thalli, *etc.* (Pajusalu et al., 2015; Puche and Rodrigo, 2015; Rodrigo et al., 2015; Urbaniak, 2010 and references therein). Under such unfavorable and changing environmental conditions, stressed plants could be the ideal host for various microorganisms, such as bacteria and fungi, whose effect on a host can be contradictive, *e.g.*, microorganisms can cause disease in their host (Egan et al., 2014, 2013). Eukaryotic fungi have less of an impact than bacteria in aquatic systems unless their habitat is relatively constant over time, either the nutrient source is difficult to degrade or fungi are in specific interactions with other organisms, such as mutualism, commensalism, parasitism and predation (Wurzbacher et al., 2010). Generally, fungi contribute to biofilm processes (Cantrell et al., 2006) and several of endophytic fungi are mutualistic (positively affect hosts while also obtain nutrition for growth and reproduction from tissues of hosts), thus both better resist abiotic stress *via* symbiosis (Rodriguez et al., 2009). However, there is a lack of understanding how freshwater macrophytes (including charophytes) and with them associated bacteria and fungi are affected by more frequent intrusions of saline waters.

Therefore, the aim of this study was to determine salinity effects on the microorganisms associated with charophytes from the Curonian Lagoon. We analyzed the net primary production of charophytes, abundance, and community composition of associated microorganisms from two sites that differ by frequency of brackish water intrusions, and performed *ex situ* experiment manipulating salinity. The composition of fungi associated with charophytes was evaluated using two techniques: nutrient media and PCR-DGGE analysis.

The Curonian Lagoon is situated in the southeastern part of the Baltic Sea (Fig. 1) and it is the largest lagoon (surface area 1584 km^2) in Europe. The lagoon is shallow (mean depth 3.8 m) and hypereutrophic, almost entirely restricted from the Baltic Sea (Bresciani et al., 2012). The Klaipeda Strait provides the only and narrow connection (width of 0.4 km) to the Sea, while in the eastern part of the lagoon the Nemunas River (one of the largest rivers in the Baltic region) is entering the lagoon. Therefore, the Curonian Lagoon is always influenced by mixing masses of brackish and fresh-riverine waters. The inflow of brackish-waters depends on a wind speed and its direction, whereas the largest part of the lagoon consists of fresh water, which is mainly influenced by discharge of the Nemunas River. Intrusions of brackish waters usually occur in the northern part of the Curonian Lagoon, but sometimes they were recorded 40 km away from the Klaipeda Strait (Zemlys et al., 2013).

Two sampling sites (Fig. 1) with different salinity conditions were selected in order to compare microorganisms associated with charophytes. The first site (BW) was situated in the northern part near Kairiai, where annual mean salinity was 2.0–3.0 PSU and salinity $>$ 0.5 PSU was observed 150–250 days per year (Zemlys et al., 2013). The second site (FW) was situated in the central part of the lagoon near Ventė, where annual mean salinity was 0.5–1.0 PSU and salinity $>$0.5 PSU was observed 85–150 days per year.

During two sampling campaigns in June and August of 2014 *in situ* temperature, salinity and dissolved oxygen (DO) were measured using YSI 460 multiple probes. Samples of surface water were collected for analysis of dissolved inorganic phosphorus (DIP), nitrogen oxides ($NO_x = NO_3 + NO_2$), dissolved organic carbon (DOC), alkalinity and total phosphorus (TP). Aliquots for measurements of nutrients, DOC and TP were immediately filtered through Whatman GF/F into 10, 30 and 20 ml glass tubes respectively. For alkalinity analysis unfiltered water samples were used.

Nutrients were analyzed within 12 h by standard methods (Grasshoff et al., 2009) using continuous flow analyzer (San++, Skalar, sensitivity 0.3 μM). DOC was measured by the combustion catalytic oxidation method (at 680°C) with total organic carbon (TOC) analyzer Shimadzu. Water alkalinity was estimated by acid titration (Jenkins and Moore, 1977). TP was spectrophotmetrically analyzed at 850 nm after acid persulphate oxidation (Grasshoff et al., 2009).

The *in situ* rates of net primary production (NPP) of charophytes were measured by the light–dark bottle technique (Blindow et al., 2006). Three replicates of 60 ml Winkler glass bottles were filled with water and immediately fixed with Winkler reagents for initial concentration of dissolved oxygen analysis. In the light and dark bottles (3 replicates each), weighted thalli of charophytes were added for incubation for 4 h in a climate chamber under the conditions close to *in situ* (incident photon flux density was 400 $\mu E\ s^{-1}\ m^{-2}$, temperature 20–22°C). Synchronously, the NPP of pelagic primary producers was measured. However, it was negligible (up to 0.2% of total NPP), therefore total NPP was used in the analysis.

Five charophyte plants were collected for assessment of associated microorganisms.

Two *ex situ* experiments were performed with the most common charophytes (*Chara aspera* and *Chara contraria*) from the Curonian Lagoon: before (June) and after (August)

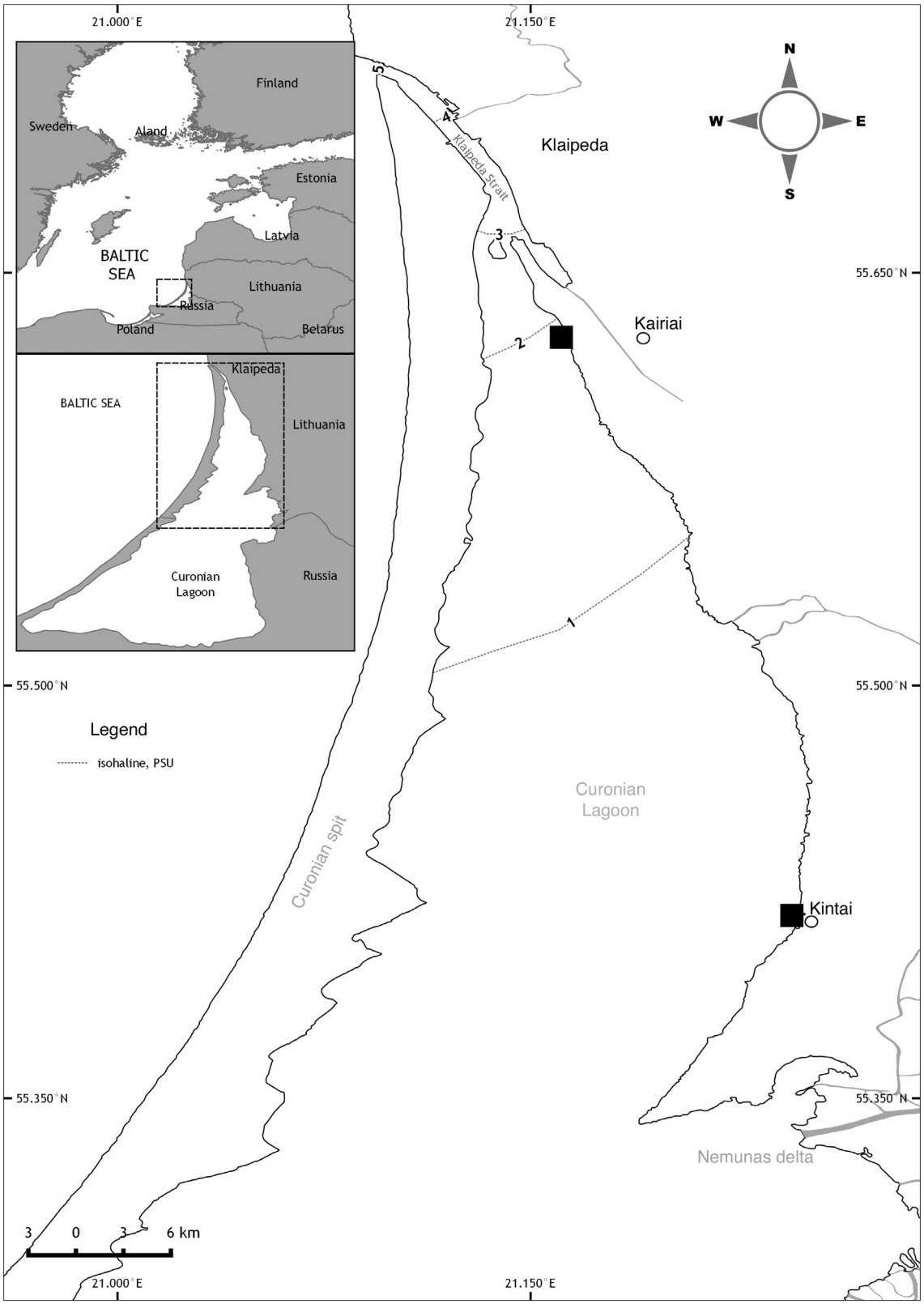

Figure 1 The study area and two sampling sites (indicated by squares), where different water masses dominate: brackish-water (BW) and freshwater (FW). The isohalines of near bottom salinity are taken from the modeled data (Umgiesser et al., 2004) during vegetation period in 2014.

the vegetation peak of charophytes. Each experiment lasted for 2 weeks. Nine intact sediment cores (i.d. 8 cm, height 30 cm) with dominant charophytes inside were collected by hand corer in 1.5 m depth (within 50–150 m from the shore) at each of two stations (Fig. 1). Intact cores were kept in a refrigerator and transferred to the laboratory within 4 h. The cores with charophytes were submerged (with the top open) in 3 incubation tanks with *in situ* water, maintaining its temperature close to natural near bottom conditions in the lagoon. These tanks were manipulated with 3 different treatments of salinity (0 PSU, 3 PSU and 6 PSU). Water was constantly stirred by rotating Teflon-coated magnet, which was fixed to the inner wall of each core for gentle water mixing avoiding sediment resuspension (Kientz et al., 2011). After two weeks, charophytes were taken from each core and pooled into sterile centrifuge tubes, kept in a refrigerator and carried into the lab within 4 h for further analysis of associated microorganisms. Simultaneously, the NPP of charophytes were measured before and after the incubation under different salinity conditions by the light–dark bottle technique as described above for *in situ* NPP measurements. Thalli of charophytes were weighted prior the incubation. Since the weight of charophytes may vary due to species and thalli specific content of calcium or magnesium carbonate (Kufel et al., 2016), each salinity treatment was performed in triplicates.

For associated microorganisms analyses, 5 plants from each experimental core were put into separate sterile centrifuge tubes, weighted, gently washed with sterile water to remove accidentally attached epiphytic microorganisms from the water column. For evaluation of endophytic microorganism presence in *in situ* samples, one set of charophytes were washed with a mixture of ethanol (50%) and sodium hypochlorite (1%) to remove all microorganisms from the surface (Kientz et al., 2011). Samples were homogenized, serially diluted and plated onto Petri dishes with different agar media (3 replicates each). Potato dextrose agar was used for fungi cultivation and Luria broth agar for bacteria. A number of colonies were recalculated into values of colonies forming units (CFU) per gram of fresh charophytes weight. Purified fungal cultures were identified to species or generic taxonomic rank and collected for further molecular analysis. DNA was extracted from freeze-dried parts of charophytes with NucleoSpin kit for soil, according to the instructions of manufacture. The ITS2 region of fungal rDNA was amplified with primer pair ITS3GC and ITS4 (White et al., 1990) and DNA amplified, according to Duarte et al. (2008). Denaturing gradient gel electrophoresis analysis was performed using DGGEK – 2401 denaturing gradient gel electrophoresis system (C.B.S Scientific Company, INC). For fungal DNA, 10 μl samples from the PCR products were loaded on 8% [w/v] polyacrylamide gel in 1× Tris–acetate–EDTA (TAE) with denaturing gradient from 30 to 70%.

The statistical hypotheses about estimates were tested using several parametric and nonparametric statistical criteria depending on data properties (Table 1). All statistical tests were performed in R 3.2.0 (R Core Team, 2015) and using "nparcomp" package (Konietschke et al., 2015).

At freshwater (FW) site, water salinity was <1 PSU during the two sampling periods, whereas at brackish water (BW) site the salinity was almost 2 and 6 times higher in June and August, respectively (Table 2). The water temperature was around 16°C during both sampling campaigns at two sites. At both sites, the concentrations of DO, alkalinity, DIP, and TP were higher in June than in August, whereas the concentrations of DOC were in the opposite. The mean concentrations of water alkalinity and TP concentration were statistically significantly higher at FW site compared to BW site ($t = 5.5$, $df = 10$, $p < 0.001$; $t = 8.3$, $df = 10$, $p < 0.001$, respectively). The concentrations of NO_x during sampling were around

Table 1 Statistical methods used in this study to test the null hypothesis.

Statistical test	Statistical hypothesis
Welch's two sample *t*-test	The concentration of water alkalinity and total phosphorus (TP) do not statistically significantly differ between stations (FW and BW).
	The mean net primary production (NPP) of charophytes does not statistically significantly differ in June and August.
	In situ measured mean NPP (separately in each site) does not significantly differ from the mean NPP in the experiment set-up (corresponding treatments of salinity).
	The mean abundance of fungi from *in situ* samples does not significantly differ from the abundance of bacteria.
Analysis of variance (ANOVA)	The mean NPP of charophytes does not significantly differ between two sites and different salinities.
	The mean abundance of microorganisms (separately bacteria and fungi) does not significantly differ among the stressed conditions in June and August.
Tukey's honest significant difference (HSD) test	The mean abundance of microorganisms (separately bacteria and fungi) does not significantly differ among the contrasts of the two sites in June and August.
	The mean abundance of microorganisms (separately bacteria and fungi) does not significantly differ among the contrasts of the stressed conditions in June and August.
Nonparametric Tukey-type test	The mean NPP of charophytes (separately in each site) does not significantly differ among the salinity treatments.
Spearman rank correlation (r_S)	There is no relationship between the abundance of bacteria and fungi from *in situ*, the experiment set-up and different seasons.

Table 2 The main water column physical and chemical characteristics at two study sites (freshwater – FW and brackish water – BW) in the Curonian Lagoon in June and August, 2014.

Site	Month	Salinity [PSU]	Temperature [°C]	DO [mg l^{-1}]	Alkalinity [mg l^{-1}]	DOC [mmol l^{-1}]	NO_x [μmol l^{-1}]	DIP [μmol l^{-1}]	TP [μmol l^{-1}]
FW	June	0.19	16.3	10.1	2.3 ± 0.7	0.8 ± 0.1	28.3 ± 5.5	0.2 ± 0.02	2.5 ± 0.2
BW	June	1.96	16.3	12.5	1.6 ± 0.1	0.8 ± 0.2	1.8 ± 0.7	0.2 ± 0.01	1.3 ± 0.1
FW	August	0.17	16	8.8	2.6 ± 0.04	0.5 ± 0.05	1.7 ± 0.2	0.3 ± 0.01	3.0 ± 0.2
BW	August	6.44	16	7.9	2.0 ± 0.1	0.3 ± 0.05	2.6 ± 0.3	0.4 ± 0.1	1.6 ± 0.1

2–3 μmol l^{-1}, except in June at FW site, where its concentration reached 28.3 ± 5.5 μmol l^{-1}. The concentrations of DIP ranged from 0.2 to 0.4 μmol l^{-1} at both sites.

The mean net primary production (NPP) of charophytes in June and August were 1.4 ± 0.9 and 2.1 ± 0.6 mg C L^{-1} g ww^{-1} h^{-1}, respectively, which did not significantly differ ($t = -1.57$, $df = 7.79$, $p > 0.05$). However, there was a significant interaction ($F = 7.84$, $df = 1$, $p < 0.05$) between the site and the salinity, therefore NPP was averaged over different salinities and sites for the further analysis (Fig. 2). *In situ* measured means of NPP in FW and BW sites did not significantly differ (respectively, $t = 0.38$, $df = 2$, $p > 0.05$ and $t = 1.13$, $df = 2$, $p > 0.05$) from the means of corresponding treatments of salinity (3 PSU and 0 PSU) in the experiment set-up. Significantly (nonparametric Tukey-type test $p < 0.05$) lower NPP of charophytes at BW site was measured after incubation in the salinity treatments of 0 PSU and 6 PSU than in the treatment 3 PSU, whereas at FW site it was significantly ($p < 0.01$) lower after incubation in the treatments of 3 PSU and 6 PSU. These reduced NPP show a potential stress of charophytes due to the changed salinity conditions in respect to those that commonly occur at BW and FW sites.

In order to test if these stressed conditions also affected bacteria and fungi associated with charophytes, the experiment set-up salinity treatments at sites were classified in the two groups according to the differences in NPP of charophytes: (1) natural conditions – 3 PSU at BW site and 0 PSU at FW site, and (2) stressed conditions – 0 PSU and 6 PSU at BW site, 3 PSU and 6 PSU at FW site.

From *in situ* samples, charophytes were colonized by bacteria in relatively high abundances from 2.2×10^4 CFU g^{-1} fw to 8.0×10^4 CFU g^{-1} fw (Fig. 3a). In June, the mean abundance of bacteria did not significantly differ (Tukey's HSD $p > 0.05$) between the two sites, while in August it was significantly higher only at BW site ($p < 0.05$).

The mean abundance of fungi from *in situ* samples was significantly ($t = 5.25$, $df = 15.97$, $p < 0.01$) lower than the abundance of bacteria and varied from 0.02×10^4 to 1.2×10^4 CFU g^{-1} fw (Fig. 3b). In June, the mean abundance of fungi did not significantly differ (Tukey's HSD $p > 0.05$), while in August the fungal abundance at FW site increased almost 17-fold than in June (Tukey's HSD $p < 0.05$).

Similar patterns of bacterial and fungal abundances were observed in the experimental set-up, respectively. From *in situ* data in June, there was low correlation ($r_S = 0.29$, $N = 9$, $p > 0.05$) between bacteria and fungi abundance, whereas negative moderate correlation ($r_S = -0.61$, $N = 7$, $p > 0.05$) was determined in August. In the experiment set-up, there were negative low correlations in June ($r_S = -0.34$, $N = 18$, $p > 0.05$) and August ($r_S = -0.23$, $N = 18$, $p > 0.05$). All correlations were not statistically significant.

We also tested the effect of stressed conditions by water salinity to the mean abundance of bacteria and fungi associated with charophytes (Fig. 3c and d) from *in situ* and experiment set-up. From *in situ* samples, the mean abundance of bacteria was significantly (Tukey's HSD $p < 0.01$) higher under the stressed conditions in August than in the other contrasts, whereas in the experiment set-up it was significantly higher from the stressed conditions in June. From *in situ* samples, the mean abundance of fungi was

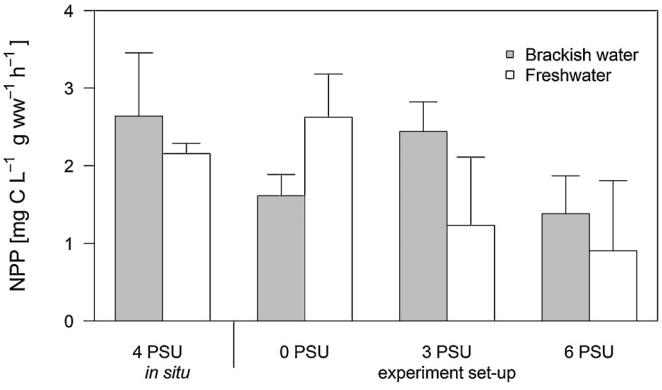

Figure 2 The mean (±standard deviation) net primary production of charophytes in the Curonian Lagoon (*in situ*) and the experiment set-up with different salinities (0, 3 and 6 PSU) at two sites (freshwater and brackish water), 2014. The *in situ* salinity values are averaged field measurements (given in Table 2).

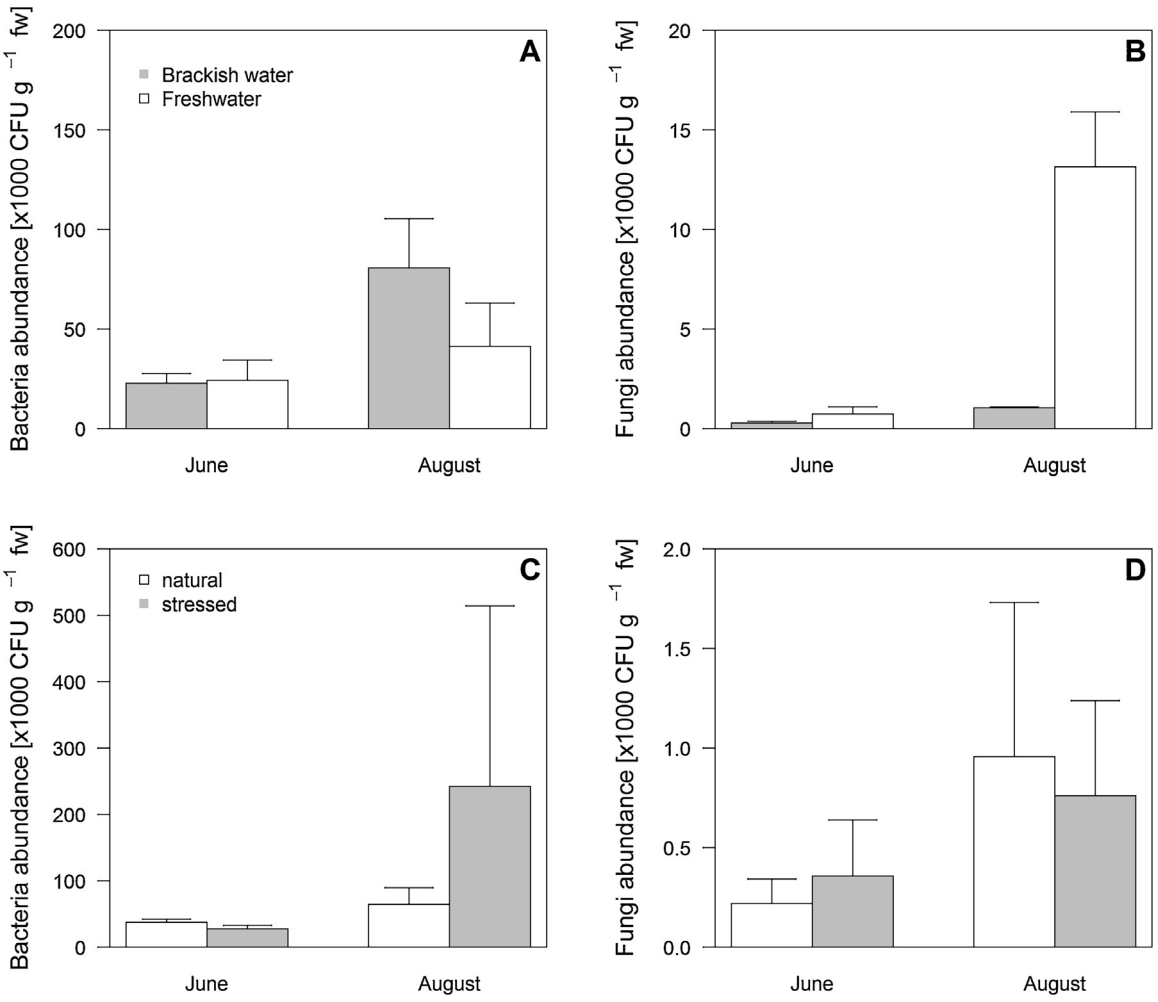

Figure 3 The mean (±standard deviation) abundance of bacteria and fungi associated with charophytes from two study sites (brackish water and freshwater) in the Curonian Lagoon (A and B) and in the experimental set-up under natural and stressed conditions (C and D), in June and August, 2014.

significantly (Tukey's HSD $p < 0.01$) higher under the natural conditions in August than in the other contrasts, whereas in the experiment set-up it was significantly higher from the natural conditions in June. There was no significant ($F = 2.20$, $df = 1$, $p > 0.05$) effect of the stress conditions.

In total, 8 taxa of fungi isolated on nutrient agar were identified to the genus level, whereas several not identified taxa were pooled into mycelia sterile group due to the lack of sporulation or other important features (Table 3). The number of fungi associated with charophytes *in situ* increased from 3 in July to 8 in August. During these periods, the similar pattern was observed from the experimental set-up.

Eighteen fungi operational taxonomical units (OTU) were found throughout the study, where 4 of them occurred frequently (Table 3); bands were considered common if they were found on ≥50% of the dates. Although, the mean number of fungal OTU at BW site was higher compared with FW site, it did not significantly differ ($t = 1.5$, $df = 6.0$, $p > 0.05$).

The most common isolated taxa was *Cladosporium* sp., which was found at various salinity conditions in both months. *Alternaria* sp., *Rhizopus* sp., *Rhodotorula* sp. and *Fusarium* sp. were found only in samples from August (Table 3).

To the best of our knowledge, there are only a few studies about abundance of microorganisms associated with charophytes, and only one of them considers fungi (Berg and Hagemann, 2009; Hempel et al., 2008). Our results showed that the mean abundance of bacteria associated with charophytes was significantly higher than the one of fungi. The determined abundance of bacteria in the Curonian Lagoon was similar to the results obtained by Berg and Hagemann (2009), where *Chara hispida* plants from a lake habitat were colonized by bacteria in 0.3×10^5–4×10^5 CFU g^{-1} fw abundance. However, the mean abundance of fungi was 1×10^2 CFU g^{-1} fw, which was two times lower than in our study. Bacteria dominate over fungi on living and healthy plant due to different colonization and nutrition strategy: most bacteria are more efficient than fungi in the use of simple polysaccharides and peptides (Romani et al., 2012 and references therein) and are interacting as epiphytes (Egan et al., 2013), while fungi usually live as endophytes (Porras-Alfaro and Bayman, 2011; Rodriguez et al., 2009) and only to some degree act as epiphytes in the phyllosphere of a plant.

In our study, the *in situ* abundance of bacteria and fungi increased at both sites during August (Fig. 3a). This pattern can be explained to some extent by findings in the studies of

Table 3 Presence (+) of fungi associated with charophytes after isolation on nutrient agar in June and August from two sites (freshwater and brackish water) and at different salinity treatments: from 0 to 6.44 PSU (* – *in situ* measurements). The number of fungal taxa assessed by cultivation on nutrient media and taxonomic units by denaturing gradient gel electrophoresis (DGGE). DGGE analysis data from August are missing due to the failed analysis.

Fungal taxa (abbreviation)	June								August							
	Brackish water				Freshwater				Brackish water				Freshwater			
	0	1.96*	3	6	0	0.19*	3	6	0	3	6	6.44*	0	0.17*	3	6
Alternaria sp.											+	+	+			
Aspergillus sp.		+				+		+					+	+	+	+
Cladosporium sp. (CLA)		+	+	+	+	+		+			+	+		+		
Mucor sp.		+							+	+	+	+		+	+	
Penicillium sp.		+				+		+					+	+	+	+
Rhizopus sp.												+			+	+
Rhodotorula sp.												+		+		
Fusarium sp.												+	+	+		+
Mycelia sterile	+		+	+		+		+	+	+	+	+	+	+	+	+
Yeasts			+	+					+			+		+		
Total fungal taxa	1	4	3	3	1	4	1	3	3	2	4	8	5	8	5	5
Fungal operational taxonomic units (DGGE)	4	8	10	11	5	6	8	1								

bacterias (Hempel et al., 2008; Romani et al., 2012), where older shoots usually enhance the growth of bacteria due to increased leak of organic compounds and inorganic nutrients. In the Curonian Lagoon, almost 12 fold difference in fungi abundance between FW and BW sites in August (Fig. 3b) indicates earlier senescence of charophytes at FW site. This could be explained by the different hydrological conditions at those two sites: a higher amount of organic material and more turbid waters from river inflow at FW site than at BW site (Krevs et al., 2007). Shallow freshwater aquatic ecosystems having abundant and diverse amount of organic matter (from both autochthonous and allochthonous origins) are a suitable habitat for microbial communities (Wetzel and Søndergaard, 1998; Wurzbacher et al., 2010). Abundant saprophytic fungi spores or hyphae from the water column and sediment can attach to a plant with lower photosynthetic activity and develop as epiphytes, meanwhile most of the endophyte fungi begin to act as saprobes and rapidly colonize decaying material (Porras-Alfaro and Bayman, 2011; Zuccaro et al., 2008). Bacteria are also abundant in such environment (Wetzel and Søndergaard, 1998). However, in our study, the observed significantly lower abundance of bacteria at FW site compared with BW in August could be affected by fungal activities. It is known that microorganisms sharing the common host and substrate can interact in a synergistic or antagonistic manner (Mille-Lindblom et al., 2006; Zuccaro et al., 2008). Fungi are more efficient than bacteria in the use of complex polysaccharides and peptides and can inhibit the growth of bacteria by producing antibiotic substances at the same time (Romani et al., 2012 and references therein).

Our results from the *in situ* and the experimental set-up clearly showed the optimal salinities for the net primary production (NPP) of charophytes (see Fig. 2). The *in situ* rates of NPP from FW and BW sites were closest to NPP from the treatments that corresponded salinity appropriate for the sites (0 and 3 PSU, respectively). The similar pattern was found in the southern Sweden (Blindow et al., 2003), where the highest rate of photosynthesis of *C. aspera* collected from freshwater lake was at 0 PSU, whereas plants collected from brackish water of the Baltic Sea (*ca.* 8 PSU) had their optima at 5–10 PSU. On the other hand, the significant decrease of NPP in our experiment was determined in the treatments that did not correspond *in situ* salinities, and therefore indicated stress of charophytes due to changes in salinity. According to Blindow et al. (2003), plants do not gradually adapt to increasing salinities and probable salinity shock can be feasible and affect physiological processes such as germination and photosynthesis.

Based on the data from our experimental set-up, we found that bacteria are partly affected by short term salinity changes (3 PSU at FW and 6 PSU at BW sites), which resulted in the increase of bacteria abundance. This can be due to increased leak of organic compounds and inorganic nutrients (Hempel et al., 2008; Romani et al., 2012) from stressed plants. Meanwhile, higher organic and lower phenolic content in a host plant seems to explain (Hempel et al., 2008) that the higher mean abundance of bacteria are observed at BW site than at FW site in August. Fungi abundance in the experimental set-up was significantly suppressed by short term changes in salinity only at FW site in August where the highest fungal activity was observed in *in situ* conditions. This could be caused by direct inhibition effect of saline water on fungi activity in the decomposition processes (Connolly et al., 2014) or indirect effect as salinity can trigger plant responsive mechanism against environmental stress (Holzinger and Pichrtová, 2016). Plant starts to produce secondary metabolites that protect the cell against environmental stress and possess antifungal properties (Ghazala et al., 2004; Juan et al., 2014), which can suppress epiphytic fungi.

In this study, the fungal diversity indicated by culturing method was lower in comparison with DGGE method. Microscopic or culture based methods usually underestimate the

diversity of fungi in comparison with molecular methods (Duarte et al., 2012) due to a small size of identified organisms, the absence of distinguishing phenotypic characters and that most of the microorganisms cannot be cultured (Duarte et al., 2012). On the other hand, molecular methods can show a presence of not active (dead) microorganisms (Duarte et al., 2012). The combination of traditional (fungal strain cultivation on agar media) and molecular techniques is the best option for identification of fungal community associated with macrophytes.

From our isolated fungi, *Alternaria* sp. and *Cladosporium* sp. are considered as one of the most common fungi taxa of the aquatic algae (Loque et al., 2009; Zuccaro et al., 2003). Both taxa appear to lack host specificity, having been isolated as fungal endophytes from numerous locations and hosts (Flewelling et al., 2015 and references therein). Fungi taxa observed from the samples in August (*Fusarium*, *Penicillium*, *Aspergillus*, *Cladosporium*, *Mucor* and *Rhizopus*) are dominating genera of terrigenous micromycetes, which propagules are abundant in the water column and bottom sediments (Voronin, 2014). Most of the fungi belonging to those genera are saprophytes and their spores or hyphae from the water column and sediment can attach to a plant and take part in decomposition processes (Wurzbacher et al., 2010). In August, their presence in association with charophytes and increased abundance could indicate senescence processes of charophytes.

There were a similar number of culturable fungi taxa at both study sites. Slightly higher diversity at BW site was observed in June based on DGGE analysis. It is known that diversity of fungi usually decreases with increasing salt concentration (Kis-Papo et al., 2001). Although, fungi can exhibit broad salt tolerance, higher fungal diversity is observed in brackish than in marine or freshwater environment (El-Sharouny et al., 2009; Mohamed and Martiny, 2011; Ristanović and Miller, 1969). Osmotic pressure by salinity can affect the production of conidia and growth of filamentous fungi (Mert and Ekmekçi, 1987). Nevertheless, other abiotic factors can influence fungal diversity, such as temperature, substrate (nutrient availability), pH, hydrostatic pressure and oxygen (Bärlocher and Boddy, 2016).

Thus, it is evident that more understanding is needed in ecological interactions between microorganisms and their hosts under dynamic environmental conditions (such as estuaries) in order to predict ecosystem changes according to ecological scenarios (*e.g.* climate change). The discovered diversity of fungi, especially of endophytic origin, could be an interesting study object for applied scientists to assess capabilities for production of bioactive compounds (Schulz et al., 2002). It is known that *Alternaria* sp. possess antimicrobial and anticancer activity, *Cladosporium* sp. — antimicrobial and insecticidal activity (Flewelling et al., 2015; Schulz et al., 2008), *Penicillium* and *Aspergillus* — antialgal, antimicrobial, anticancer, antioxidant, and insecticidal bioactivity (Flewelling et al., 2015).

In this study, we have shown that with charophytes associated bacteria dominate over fungi. In the end of the vegetation period, considerably lower fungi abundance at the site frequently affected by brackish water than at the freshwater site indicates that charophyte beds are in better status in the brackish water environment, whereas at the freshwater site saprophytic fungi are most likely to take part in senescence process of vegetation. The short salinity changes partly influenced the abundance of associated bacteria and fungi; therefore, other environmental factors than salinity could be important, including the reaction of hosts (*e.g.*, production of secondary metabolites). The revealed diversity of fungal taxa associated with charophytes may serve for future applied research on the importance of these fungi for biologically active metabolites.

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