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Bacterial community on submersed plants in running water

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ABSTRACT

The Ceyhan River basin consists of a large number of rivers, lakes and dams and since the basin area is important for agricultural and industrial activities, it receives an extensive discharge of pollutants. There is a possibility that toxic (e.g. some cyanobacteria) and pathogenic, such as Salmonella species, bacteria present in the bacterial community. Therefore these bacteria could be responsible for water quality and the health diseases of the aquatic organisms. In this study the epiphytic bacterial densities on different submersed species, collected from Ceyhan River Basin, in different seasons have been compared. The impacts of physico-chemical variables of the surrounding water on epiphytic bacteria population has also been tested. Average densities of bacteria on the leaves of all tested plants were higher in autumn and lower in spring. In all seasons, the density of bacteria on the leaves of *Myriophyllum spicatum* was higher than that of the other two species, *Ceratophyllum demersum* and *Groenlandia densa*. Bacterial density on the leaves of all tested plants significantly differed between the season. Epiphytic bacterial density correlated significantly with pH, conductivity and NO_3^-. On the other hand, epiphytic bacterial density was strongly correlated with temperature, TN, NH_4^+, TP, SRP and Chl *a*, while no significant correlation could be determined between bacterial population and DO.

Keywords: Myriophyllum spicatum; Ceratophyllum demersum; Groenlandia densa; Environmental factors; Epiphytic bacteria; Running water

1. Introduction

In aquatic environments, bacterial development is generally associated with surfaces (e.g. in biofilms), lakes or marine snow [1]. Biofilms not only occur on abiotic surfaces, but also on aquatic plants [2]. The number of epiphytic bacteria on the surface of macrophytes has been estimated to be between 105 and 106 cells/cm^2 [3–5]. Epiphytic bacteria colonisation on the leaf-surface is the principal food source for many invertebrates such as snails, mayfly larvae and Chrinomidae larvae [6]. Macrophytes, in turn, can use the growth substances produced by epiphytic bacteria [6]. It has been suggested that epiphytic bacteria are metabolically more active than planktonic and benthic species [7,8]. It has been also reported that [9,10] epiphytic bacteria found on aquatic plants are in high abundance and are generally larger than free-living bacteria [11]. There is also a possibility that toxic (e.g. some cyanobacteria) and pathogenic (such as Salmonella species) bacteria are present in the bacterial community. Therefore, these bacteria could be responsible for water quality and diseases in aquatic organisms. Therefore, in recent years, the focus has been on the study of the bacterial colonisation of macrophyte surfaces [12–15].

Submerged macrophytes are key factors in freshwater systems. They are not only the main primary producers, in addition to algae, but also provide shelter and spawning areas for many organisms in the system. Aquatic macrophytes secrete dissolved organic materials such as Dissolved Organic Carbon (DOC) [16], which could be the main reason for epiphytic bacterial colonisation on macrophytes. On the other hand, leaf age, leaf position, and leaf surface (abaxial or adaxial), etc. are affected by the density of epiphytic bacteria on macrophytes.

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Ceratophyllum demersum L. is frequent, sometimes abundant, in ponds, canals, dykes and streams with high ionic contents, including brackish and mesotrophic to eutrophic water [17]. Since it lacks roots, it is shade-tolerant and can persist in water under low light conditions [18]. It also has a strong preference for eutrophic waters [19].

Groenlandia densa (L.) Fourr. grows in shallow, clear, base-rich water in rivers, streams, canals, ditches and ponds [18]. It is a characteristic species of streams flowing from calcareous springs and is rarely found in lakes and reservoirs [18]. It can grow in mesotrophic to eutrophic water with high alkalinity.

Myriophyllum spicatum L. also usually grows in water with high alkalinity and in mesotrophic to eutrophic water [18]. Although normally found in eutrophic water, it also occurs in highly calcareous but nutrient-poor limestone lochs [18].

In this context, the aim of this study was to compare epiphytic bacterial densities on different submersed species in different seasons and to test the impacts of physiochemical variables of the surrounding water on the epiphytic bacteria population.

2. Material and methods

2.1. Study area

The Ceyhan Basin is located in the provinces of Adana, Osmaniye, Kahramanmaraş and Adıyaman, in the south-eastern Anatolian Region in Turkey (Fig. 1). The river basin covers 20 670 km^2 [20] and consists of a large number of rivers, lakes and dams. The area is an important breeding site for migratory birds [21]. Since the basin area is important for agricultural and industrial activities, it receives an extensive discharge of pollutants.

2.2. Sampling and counting

Submersed macrophyte species, *C. demersum*, *M. spicatum* and *G. densa*, were collected during three field trips (autumn, spring, summer) in 2014 and 2015 from three different sites of running water in the Ceyhan River Basin. As the present study covered the Ceyhan River Basin, three different sampling points were chosen to represent the entire river basin. During the survival studies, which included 33 stations, three of the species analysed in this study were found together at only three sampling points. Therefore, these three sites were selected as sampling stations for the present study. According to the physiochemical analyses, the waters of the Ceyhan Basin are hypertrophic and eutrophic, but the sampling stations remained meso- to eutrophic during the study period. In total, nine (three plants from each site) plants of each species were examined. The leaves were divided into three fractions: tip (apex) leaves (from leaf-node 1), middle leaves (from leaf-node 5) and lower leaves (from leaf-node 10). Leaves from each fraction of the plants were removed with forceps and stained with phenolic aniline blue (PAB) [22], and the bacteria on the abaxial surface were counted using light microscopy. The density of the bacterial population was evaluated by counting the number of bacteria in a minimum of 10 quadrats on each leaf. A Whipple Square eyepiece graticule and a magnification of 1,000 were used. Mean values were used for the results.

Temperature, conductivity, pH and dissolved oxygen were determined in situ using commercial metres. For chemical analysis, water samples were taken from each sampling location and analysed according to APHA [23].

All statistical analyses were performed with Minitab12, and the Principal Components Analysis (PCA) was performed using SPSS 22.0 to assess the influences of physiochemical variables on the density of epiphytic bacterial populations on macrophytes.

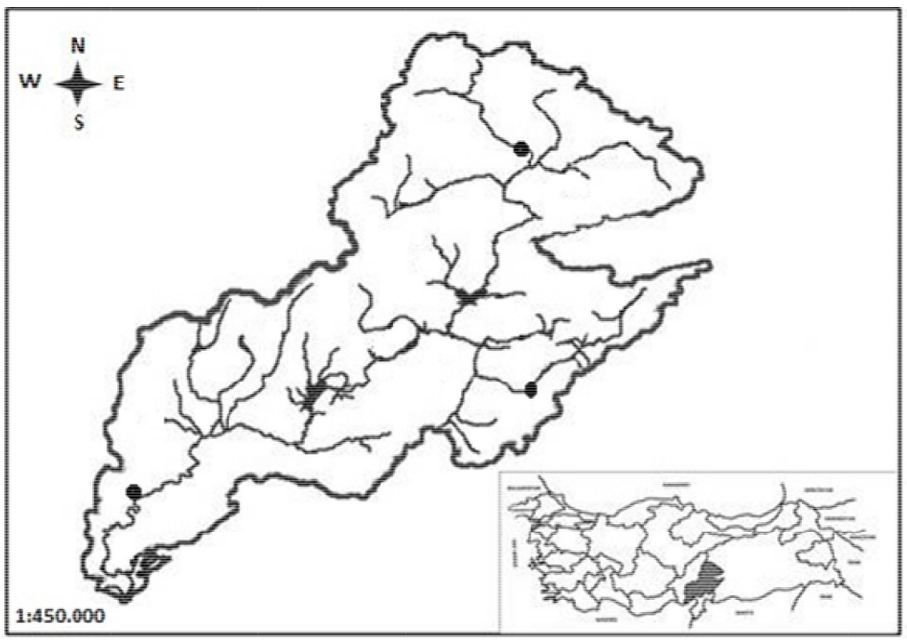

Fig. 1. Map of study area and location of sampling points.

2.3. Isolation of bacterial strains

Epiphytic bacteria were transferred to the surface of the sterile agar media using the following method [24]: from each tested plant, three leaves (from leaf-node 1, 5 and 10) were gently washed in 100 ml of buffer solution, containing 6.75 g KH_2PO_4 and 8.75 g K_2HPO_4 per litre, with a pH of 7. Leaves were gently shaken manually for 3 min in flask containing buffer. The washing solution was filtered through a 25-µm Millipore filter to eliminate debris and ruins. The bacteria were collected from the filter and re-suspended in 5 ml of buffer. Aliquots (1:2 ml) of serial dilutions from this suspension were placed on a sterile agar surface. The infected plates were incubated at a temperature of 20°C for 6 d. After checking the cleanliness of the bacterial cultures in the slide coloured with the Gram method, the strains were stored at a temperature of +4°C.

2.4. Identification of epiphytic bacteria

Bacteria isolated from the leaves of the tested plants were identified to the genera and group level, according to the relevant literature [25–28].

3. Results

Average densities of bacteria on the leaves of all tested plants were higher in the autumn and lower in the spring (Fig. 2). In all seasons, the density of bacteria on the leaves of *M. spicatum* was higher than that of the other two species, *C. demersum* and *G. densa*. Epiphytic bacteria population on the leaves of *G. densa* was less than that of both *M. spicatum* and *C. demersum* in all seasons.

Bacterial density on the leaves of all tested plants significantly differed between seasons ($p < 0.01$ for each species based on ANOVA). Significant differences were also determined between the plants in the spring, summer and autumn ($p = 0.035$, $p = 0.009$, $p < 0.01$, respectively).

Temperature, pH and oxygen values ranged from 14.4 to 23.8°C, 7.91 to 8.13 and 7.00 to 8.1 mg. L^{-1}, respectively. Chlorophyll *a* levels ranged from 0.17 to 3.17 mg. L^{-1}. However, maximum levels were determined in spring 2015 for phosphate and in summer 2015 for nitrogen (Table 1). The results concerning the identification of bacteria inhabiting the tested plant leaves in this study are given Table 2.

The influences of the measured physiochemical variables on the density of epiphytic bacteria on macrophytes was assessed using PCA. The two main factors extracted by PCA explained 69% of the total variance at the rivers (Fig. 3). The first axis was strongly associated with bacteria and soluble reactive phosphorus (SRP), while the second axis was reasonably related to dissolved oxygen (DO).

Epiphytic bacterial density correlated significantly with pH, conductivity and NO_3 ($p < 0.05$ in all cases). On the other hand, epiphytic bacterial density was strongly

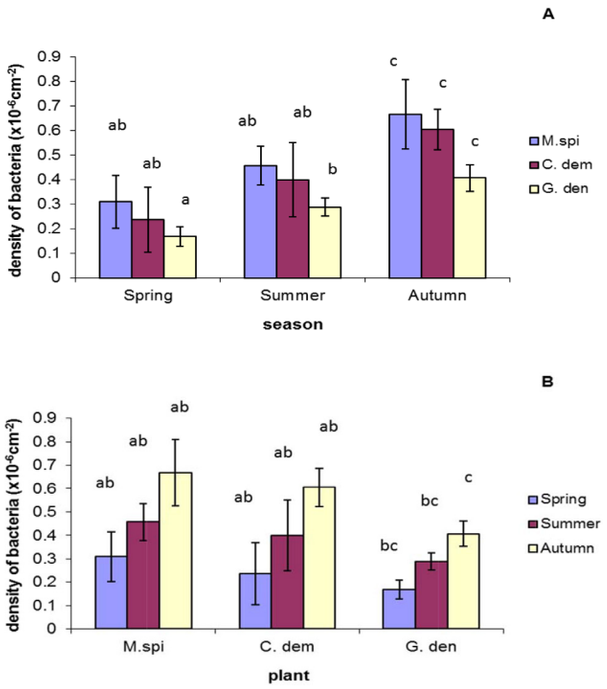

Fig. 2. Average density of bacteria on the leaves of macrophytes according to season (A) and according to species (B), error bars are shown as ± SD and statistical differences are shown with letters.

Table 1
Mean values and ± SD of physico-chemical variables, and chlorophyll *a* concentration determined at the sampling points of Ceyhan basin during the sampling period

Variables	Spring	Summer	Autumn
Temperature(°C)	14.4 ± 4.36	23.8 ± 2.06	15.6 ± 3.26
Oxygen (mg·L^{-1})	7.64 ± 2.69	8.51 ± 0.97	7.0 ± 0.43
pH	7.93 ± 0.13	7.91 ± 0.29	8.13 ± 0.25
Conductivity (µS·cm^{-1})	427.6 ± 75.63	396.3 ± 62.40	523.6 ± 39.04
TN (mg·L^{-1})	2.86 ± 1.88	3.44 ± 1.37	1.48 ± 0.88
N-NO_3 (mg·L^{-1})	1.58 ± 0.91	1.17 ± 0.37	1.29 ± 0.68
N-NH_4 (mg·L^{-1})	0.33 ± 0.14	0.13 ± 0.01	0.11 ± 0.009
TP (mg·L^{-1})	0.47 ± 0.06	0.06 ± 0.04	0.04 ± 0.012
SRP (mg·L^{-1})	0.20 ± 0.004	0.01 ± 0	0.016 ± 0.004
Chlorophyll *a* (mg·L^{-1})	0.17 ± 0.01	3.17 ± 0.03	0.96 ± 0.12

Table 2
Generic composition of epiphytic bacteria isolating surface of the tested macrophytes leaves (average-bacteria in percent)

Bacteria	Spring	Summer	Autumn
Pseudomonas	5	7	9
Flavobacterium-Cytophago	41	43	38
Enterobacteriaceae	29	24	17
Arthrobacter-Corynebacterium	11	9	10
Bacillus	4	2	6
Aeromonos-Vibrio	3	6	8
Achromobacter	7	9	12

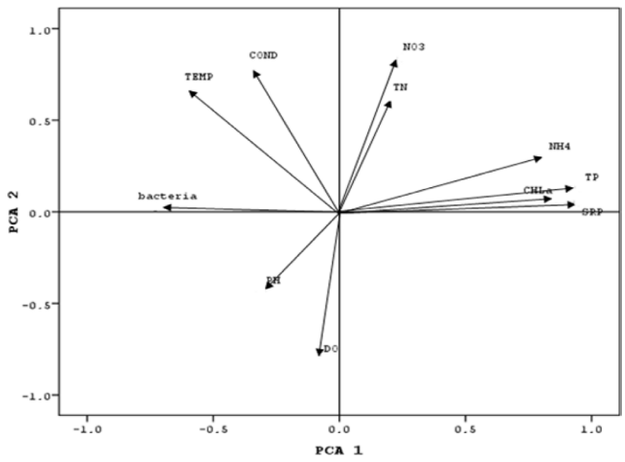

Fig. 3. Principal component analysis (PCA) of physico-chemical variables and epiphytic bacteria. Abbreviations: CHL *a*, chlorophyll *a*; DO, dissolved oxygen; TN, total nitrogen; NH_4-N, ammonia nitrogen; NO_3-N, nitrate nitrogen; TP, total phosphate; SRP, soluble reactive phosphorus; TEMP, temperature.

correlated with temperature, TN, NH^+_4, TP, SRP and Chl *a* ($p < 0.01$), while no significant correlation could be determined between bacterial population and DO.

4. Discussion

In the present study, bacterial densities on submersed macrophytes ranged between 0.168 and 0.666×10^{-6}. In general, average densities of bacteria on the leaves of all tested plants are higher in the autumn and lower in the spring. Based on their life cycle stage, macrophytes may release essential amounts of macro nutrients [29] and high concentrations of micro nutrients [30]. In particular, old plant parts may excrete organic compounds and inorganic nutrients at the same time [31]. Therefore, because the plants are older in the autumn, bacterial colonisation should be higher than in the spring when the plants are young.

In all seasons, the densities of epiphytic bacteria on the leaves of *M. spicatum* were higher than those of both *C. demersum* and *G. densa*. The leaves of *M. spicatum* have several segments, which increases the surface area for bacterial colonisation; the opposite is true for *C. demersum*, with only a few segments, or *G. densa*, with no segments. Hempel et al. [14] also found higher bacterial cell numbers on *M. spicatum* than on *Potamogeton perfoliatus*; the authors concluded that this might be the result of the higher surface to volume ratio and the whorl-like structure of *M. spicatum* leaves, which encourages the colonisation of epiphytic organisms.

Flavobacterium-Cytophago and *Enterobacteriaceae* were the most abundant group in all seasons. The *Flavobacterium-Cytophago* group was the dominant group in all seasons (38 to 43% of the total). Bacterial populations can represent both advantages and disadvantages for submersed macrophytes.

Epiphytic bacteria provide organic compounds and carbon dioxide to the macrophytes, thereby enhancing nutrient recycling [32]. On the other hand, bacterial populations on the submersed macrophyte leaves could increase shading effects. Maximillien et al. [33] and Hubel et al. [34] suggested that bacterial colonisation on marine red algae is inhibited by the secondary metabolites released by the algae.

Similar to macroalgae, freshwater macrophytes such as *M. spicatum* [35,36], *Chara globularis* [37,38] and *P. pefroliatus* [39] produce secondary compounds such as polyphenol and cyclic sulphur, which show allelopathic activity against algae and cyanobacteria [35,36]. Flavonoids, glycosides, phenols, aponins and tannins have been analyzed from *C. demersum* extracts [40], which are also responsible for antimicrobial activity. On the other hand, the macrophytes analyzed in this study were sampled from running water, which was an open water environment; the concentration of allelo chemicals might have a minimal effect against bacterial growth on submersed plants.

According to the PCA results, average densities of bacterial populations were significantly correlated with the physicochemical variables of water, such as temperature, TN, NH^+_4, TP, SRP and Chl *a* ($p < 0.001$), and were less significantly correlated with pH, conductivity and NO^-_3 ($p < 0.05$). Previous studies suggested that oligotrophic lakes with high inputs of nutrients supported bacterial growth compared to clear water oligotrophic lakes which are less affected by nutrient inputs [41,42]. Furthermore, studies have demonstrated that the addition of macrophyte-derived nutrients experimentally to both the Ogeechee and Hudson River increased bacterial assemblages [43,44].

It is known that bacteria and other microorganisms in aquatic systems cooperate in the recycling of organic matter and nutrients such as NO^-_3 and NH^+_4 in the surrounding water [41]. This microbial cycle is thought to enrich the ecosystem ability due to rapid recycling and decreased sinking rates. Therefore, the loss of nutrients was in organisms remaining in the water column [41]. Denitrification, the reduction of nitrate to gaseous nitrogen is an important process for nitrate removal from aquatic systems [45]; bacteria have an active role in this pro-process. Conditions become anoxic and sufficient amounts of nitrate and organic carbon are required [46]. In aquatic ecosystems, denitrification mainly takes place in the sediment [47], but has also been seen in bacterial biofilms on the macrophyte surface [47]. Therefore, macrophytes may affect denitrification by providing a surface area for epiphytic bacteria and release DOC into the environment. Similarly, Huss and Wehr [48] suggested that nutrient conditions affect the impacts of submersed macrophytes on bacterial populations, e.g. *Vallisneria americana* has a positive impact on bacterial populations under high NH^+_4 conditions, but a neutral or negative impact under limited NH^+_4 conditions. Their results specifically suggest that bacteria in NH^+_4-poor environments remained N-limited, but become DOC- or P-limited in NH^+_4 rich environments. Therefore, under NH^+_4-rich conditions the release of DOC or P by macrophytes has a positive effect on bacterial growth; on the other hand, under NH^+_4 poor conditions, the release of DOC or P by macrophytes has little effect on bacteria. These findings are supported by the PCA results of the present study, where a strong correlation was determined between the bacterial population and NH^+_4.

5. Conclusion

It has been suggested that macrophytes may release essential amounts of macro nutrients [29] and high concentrations of micro nutrients [30]. Submersed macrophytes had strong but complex effects on bacterial numbers and these effects were influenced by water chemistry through the availability of limiting nutrients such as N, P or DOC. Therefore TN, NH^{+}_{4}, TP and SRP exhibited significant effects on bacterial populations in this study.

Different submersed macrophyte species have different effects on epiphytic bacterial colonisation. This study showed that the morphological structure of submersed macrophytes plays an important role in the colonisation by epiphytic bacteria on leaves. On the other hand, future studies including detailed experiments are needed to fully understand the mechanisms of epiphytic bacteria colonisation on submersed macrophytes.

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