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ORIGINAL ARTICLE

Bacterial diversity and hydrography of Etoliko, an anoxic semi-enclosed coastal basin in Western Greece

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Abstract Etoliko, an anoxic semi-enclosed basin, is part of a complex wetland in Western Greece extremely rich in biodiversity. It covers an area of 1,700 ha with an atypical orientation that has been formed tectonically. In order to identify the main factors influencing the bacterial profile at the Etoliko basin, 48 samples were collected, representing seasonal variation at four sampling stations. Physico-chemical analysis of the samples indicates the presence of three layers in the Etoliko basin: (1) low-density surface layer, (2) a layer with a steep density gradient, and (3) dense water below a depth of 20 m. A permanent halocline, whose thickness is varying seasonally, has been identified in the Etoliko basin water column, while the spatiotemporal salinity distribution was highly affected by the basin's interaction with the nearby Messolonghi lagoon. The anoxic zone extends from 20 m below the surface to the bottom of the Etoliko basin in summer, while the bottom layer was hypoxic during winter. Bacterial populations were analyzed by Automated Ribosomal Intergenic Spacer Analysis (ARISA). Bacterial richness and diversity were calculated and compared across samples. Hierarchical analysis showed that ARISA clustered the surface water samples according to seasonal variation, while sediment and near-to-bottom water samples appear to be stable and to cluster together. Non-metric multidimensional scaling (MDS) indicates that bacterial composition depends on dissolved oxygen and salinity. Increase in salinity of the ecosystem leads to a significant reduction of the microbial diversity.

Keywords Bacterial diversity · ARISA · Etoliko · Anoxia · Salinity

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Introduction

During the last decade, changes in the oceans' dissolved oxygen content have become a focal point of scientific research. Low oxygen areas have spread in the coastal oceans during the last 50 years and have been reported for waters around America, Africa, Europe, Australia, Japan, and China (Nixon 1990; Diaz and Rosenberg 1995; Wu 1999). The relative contribution of the physical, chemical, and biological processes that lead to oxygen depletion varies for different water bodies.

In coastal water bodies, morphology, nutrients/organic load, and salt/fresh water budget control anoxic conditions. Morphology is the dominant factor responsible for permanent stratification of fjords, semi-enclosed basins, and continental deep depressions, while shallow and narrow sills are responsible for bottom water stagnation and anoxia. The Black Sea (Neretin et al. 2001; Glazer et al. 2006; Hiscock and Millero 2006; Konovalov et al. 2006), the Framvaren Fjord (Millero 1991; Dyrsenn 1999; Yao and Millero 1995; Mandernack et al. 2003), and the Cariaco Basin (Astor et al. 2003) provide characteristic examples of morphology-induced anoxia; the Adriatic Sea (Justić et al. 1987, 1993) and the Danish coasts

(Josefson and Hansen 2004) are nutrient-induced anoxic environments. The prevalence of anoxic/hypoxic conditions in a coastal environment modulates their chemistry (Yemenicioglu et al. 2006; Percy et al. 2008) and their biology (Josefson and Widbom 1988; Nilsson and Rosenberg 1997; Powilleit and Kube 1999).

The upper oxic layers of meromictic ecosystems are usually characterized by aerobic, fresh or brackish water, whereas the deeper layers are anoxic and saltier. These different physicochemical properties lead to the development of distinct microbial communities within relative short vertical distances. The oxic layer favors communities typically found in rivers and lakes (Zwart et al. 2002), while the suboxic and anoxic layers favor the development of chemolithotrophs and photolithotrophs. The suboxic and anoxic layers of semi-enclosed basins host a variety of microbial metabolic pathways such as anoxygenic photosynthesis, manganese oxidation, nitrification, denitrification, anaerobic ammonium oxidation (anammox), sulfide and thiosulfide-oxidation, and methane oxidation (Madrid et al. 2001; Kuypers et al. 2003; Vetriani et al. 2003; Manske et al. 2005; Lam et al. 2007; Clement et al. 2009; Fuchsman et al. 2011). In the case of the Black Sea, the microbial communities of the suboxic and anoxic zones exhibit a remarkable continuity (Vetriani et al. 2003; Fuchsman et al. 2011). On the other hand, the bacterial community composition in oxic zones is mainly correlated with phytoplankton and is seasonal, which might reflect interactions between these communities (Kent et al. 2007; Giovannoni and Vergin 2012).

Etoliko is a tectonically formed semi-enclosed coastal basin in Western Greece (Fig. 1). Its surface extends over approx. 1,700 ha, with a mean depth of ~12 m and a maximum depth of about 27.5 m. It is connected with the Messolonghi lagoon through two shallow and narrow openings with a total cross-section area of about 200 m^2 under the bridges of Etoliko Island (Fig. 1) and a mean depth of 1.2 m. The Etoliko basin is permanently stratified with an isolated aphotic deep layer being anoxic/hypoxic and sulfuric (Dassenakis et al. 1994; Leonardos and Trilles 2003; Papadas et al. 2009; Gianni et al. 2011, 2012). Anoxia is controlled predominantly by the morphologies of the Etoliko basin and the Messolonghi lagoon and by the presence of a narrow and shallow sill. The salt/fresh water budget and nutrient load play supplementary roles. The Etoliko basin behaves like a typical anoxic basin, as many other semi-enclosed coastal basins and fjords (e.g. Black Sea and Framvaren Fjord) (Gianni et al. 2012).

A number of studies have been carried out in recent years, examining the physicochemical parameters of the Etoliko basin (Papadas et al. 2009; Gianni et al. 2012); however, the microbial diversity has so far been ignored. The characterization of the microbial community structure is, however, essential to fully understand the ecosystems.

The goal of this study was to characterize the bacterial diversity of the Etoliko basin using an ARISA fingerprinting approach and also to decipher the main factors shaping the bacterial profile of the Etoliko basin. In addition, physicochemical parameters were examined and human intervention affecting the seawater quantity entering the Etoliko basin was also assessed.

Materials and methods

Sampling

Samples were collected on a seasonal basis at four stations, A2, A4, A8, and A9 (Fig. 1) from July 2007 to April 2008. Due to the oxygenation of the hypolimnion observed during February 2008, we decided to analyze this sample instead of the January 2008 sample. Surface and near-to-bottom water samples were collected from each sampling station, while for the deeper stations, A8 and A9, water from the halocline layer was also collected. Sampling was done using a Niskin bottle (free flow 5-l Hydro-Bios sampler). All samples were kept in sterilized screw-capped bottles. Sediment samples from each sampling station were obtained with an Eckman grabber.

Vertical profiles of temperature (°C), conductivity (mS/cm), and dissolved oxygen (DO in mg/l) were measured in situ using a Multi-Parameter Troll 9500 (In-Situ). Salinity (‰) and density (σ-t) were calculated from temperature, conductivity, and pressure data.

Analytical methods

DNA extraction, ARISA amplification

For the isolation of genomic DNA, 4 l from each water sample were first filtered through a sterile 3-μm glass fiber filter (Whatman, Florham Park, NJ, USA) and then through a sterile 0.2-μm membrane filter (Whatman). Total DNA was extracted as previously described (Katsaveli et al. 2012) from the 0.2-μm membrane filter. DNA was quantified with a Qubit fluorometer (Invintrogen, USA), according to the manufacturer's instructions. The sediment samples (150 g each) were suspended in 2 l of sterile near-to-bottom water with mechanical shaking. The homogenate was then treated in the same way as the water samples.

PCR for ARISA was performed as previously described by Cardinale et al. (2004). In brief, the ITSF (5′-GTCGTAAC AAGGTAGCCGTA-3′) and ITSReub (5′-GCCAAGGCAT CCACC-3′) primers set was used with 10–300 ng of environmental DNA in an amplification reaction mixture containing 1× PCR buffer, 1.5 U of Taq DNA polymerase (Takara Mirus Bio, WI, USA), 0.2 mM of each deoxynucleoside triphosphate and 0.25 μM primers in a final volume of 20 μl. The mixture was held at 94 °C for 3 min, followed by 30 cycles of

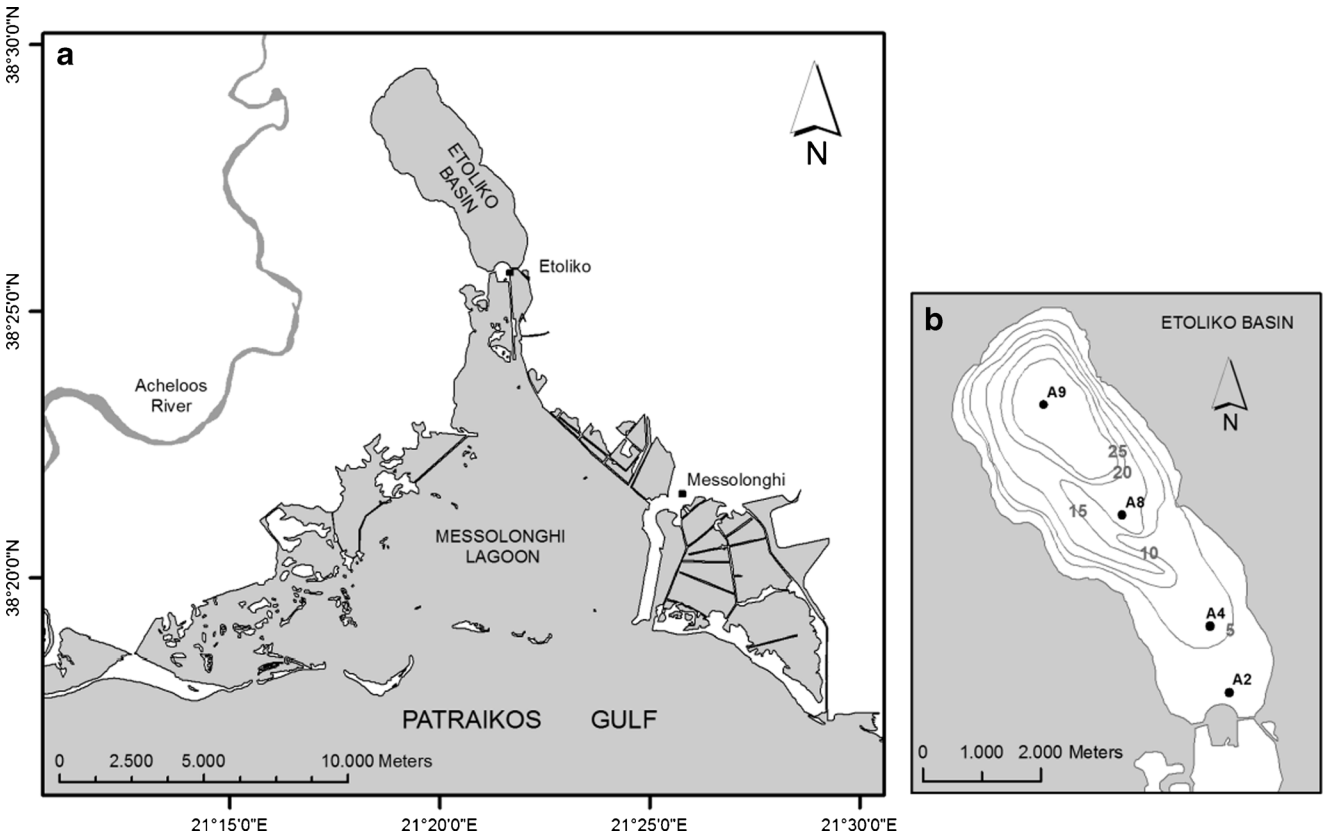


Fig. 1 **a** The extended study area. **b** Sampling stations in the Etoliko basin

94 °C for 45 s, 55 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 7 min.

For the ARISA analysis of the samples, an amount of 0.5–1 μl of the PCR products, along with 0.5–0.8 μl of an internal size standard (1,000 or 2,500 ROX; Applied Biosystems) was added to 13 μl of deionized formamide, and the mixture was denatured at 95 °C for 5 min, followed by 2 min on ice. All samples were processed in triplicate. The sample fragments were then analyzed using an ABI 310 genetic analyzer (Applied Biosystems). All ARISA data were analyzed using the GeneScan 4.0 software program (Applied Biosystems), and the data were transferred to a spreadsheet for further analysis, including the calculation of ecological indices. The Shannon–Weaver index (*H*) reflects the amount of disorder in the species distribution of the observed community. Evenness is an index providing a sense of how evenly the different categories contribute to the Shannon–Weaver index, while the Margalef index was used as a simple measure of species richness (Legendre and Legendre 2000).

Statistical analysis

The matrix of aligned fragments and fluorescent values was imported to the PRIMER6 software package (v.6.1.12) (Clarke and Gorley 2006). Data were: (1) normalized using presence/absence pretreatment, (2) analyzed using the Bray–Curtis similarity method, and (3) clustered in the group average mode with the default parameters (5 % significance, mean number of permutations 1,000, number of simulations 999) for generating a dendrogram based on percent similarity. The null hypothesis that there were no differences between the seasonal bacterial communities has been tested using the non-parametric statistical test PERMANOVA (Anderson 2001). The test for significant relationships between different concentrations of oxygen and the bacterial profile was examined using a distance-based multivariable analysis (DISTLM) and a distance-based redundancy analysis (dbRDA). Analysis was performed using the PERMANOVA+ plugin utilized through PRIMER 6 (Anderson 2005; Anderson et al. 2008).

Results and discussion

Physicochemical analysis

Throughout the sampling period, the Etoliko basin appeared spatially homogenous with no significant differences in the measured physicochemical characteristics at the respective depths.

The water column at the Etoliko basin was permanently stratified and throughout the sampling period, three layers were

distinguished: (1) a surface low-density layer, (2) a layer with steep density gradient, and (3) dense water below the depth of 20 m (Fig. 2a-A1). The depth of the surface and pycnocline layers, as well as the stratification intensity, depended on seasonal salinity and temperatures.

The vertical temperature distribution indicated a limited surface layer (0–4 m) with a mean temperature of about 30.5 °C during summer. In the same period, the seasonal thermocline was extended down to a depth of 12 m, controlling the vertical stratification of the basin. In autumn, the surface temperature decreased (~19 °C) and the thermocline layer was gradually destroyed. Thus, during wintertime, the surface layer extended down to 12 m depth and was characterized by temperature values of about 9.5 °C. The bottom layer temperature was constant at about 13.5 °C throughout the sampling period (Fig. 2a-A2).

A permanent halocline was found in the Etoliko basin water column throughout the sampling period (Fig. 2a-A3). Its thickness, as well as the surface layer depth, varied seasonally. During summer, the surface layer was limited (up to 4 m depth) and was characterized by mean salinity values of 22.5‰. During the winter months, it extended down to a depth of 12 m and the salinity decreased to 21.5‰.

The surface layer was saturated and often super-saturated with dissolved oxygen throughout the sampling period. This layer was 6–8 m deep during summer and spring, while its depth increased to 12 m in the winter months. From this depth downwards, the oxycline followed with its lower limit also being season-dependent. During summer, the anoxic layer extended from a depth of 20 m down to the bottom of the basin. Interestingly, its thickness gradually decreased during the autumn months. In February 2008, the bottom layer of the Etoliko basin became hypoxic. About 2 mg/l dissolved oxygen was recorded in a depth of 20 m, while the oxygen concentration gradually decreased with depth. Values of about 1 mg/l characterized the lowest sampling depths. In April 2008, 1 mg/l of DO was measured at 25 m, and values slightly lower than 0.5 mg/l were recorded for the water sediment interface (Fig. 2a-A4).

The spatiotemporal salinity distribution provides evidence for the interaction between the Messolonghi lagoon and the Etoliko basin. In July 2007, a high salinity water layer extended from 4 to 9 m depth of the Etoliko basin water column (Fig. 2b, top row). This abnormal vertical salinity distribution was noticed in the entire lagoon and was ascribed to salt water inflow from the Messolonghi lagoon. This saltier water flowed as a bottom current near the connection channel between the two lagoons and when it reached the same density as the surrounding water interleaved with that of the Etoliko basin (Fig. 2b, top row). Particularly interesting was the salinity distribution in the basin during October 2007. Increased values characterized the entire basin, with a surface salinity as high as ~24.5‰ and bottom values increased by about 0.5‰ (Fig. 2a-A2, b, 2nd row). This was probably due to the high salinity water inflow from Messolonghi lagoon which took place during the summer and the early autumn months and influenced the salinity of the entire coastal basin in October 2007.

In recent years, water exchange between Messolonghi and Etoliko lagoons was changed through reconstruction of the connecting channel between the two basins. The technical interferences in the Etoliko/Messolongi system were completed in May 2006, and the channel's total cross-section has increased by about 30 %, facilitating the inflow of denser water from the saltier Messolonghi lagoon, thus affecting Etoliko lagoon hydrography. According to Gianni et al. (2011), the limited deepening of the sill created a mild increase of the water flow into the Etoliko lagoon. This inflow of the oxygenated saltier water from the Messolonghi lagoon has resulted in a weak mixing of the water column. Such a small-scale mixing introduced oxygen into the halocline waters during the winter period without destroying the stratification. Regarding the physicochemical status of Etoliko basin, this study is reporting conditions similar to those recorded in the period 2006–2007 (Gianni et al. 2011, 2012). The exception is the recorded increase of salinity in the entire water column of Etoliko lagoon during October 2007.

ARISA analysis

Bacterial community profiles were generated using ARISA. Individual ARISA amplicons and their seasonal relative abundances at each of the four sampling stations were examined. Unique Operational Taxonomic Units (OTUs) were indicated as unique amplicons on the ARISA profile. The majority of the OTUs were 50–1,000 bp.

Ecological diversity indices were calculated from the normalized ARISA peak matrix (Table 1). A high Shannon–Weaver index ranging between 3.2 and 5.08 indicated a very high bacterial diversity, confirmed by an also high Margalef index. High equitability indicated substantial evenness in all samples. Interestingly, the Shannon–Weaver indices of the autumn samples (3.735±0.29) had lower values than those of the other seasons (summer: 4.54±0.27; winter: 4.49±0.25; and spring 4.63±0.32), which can be attributed to the high salinity surface water that entered the Etoliko basin (Fig. $2B_2$). This is also apparent in the number of taxa (autumn: 106.66±22.37; winter: 243.42±57.45; spring: 268.83±44.51; summer: 243±50.95), with a steep decline when compared with the other seasons (Table 1). Summer and spring samples exhibited the highest bacterial diversity. Compared with other anoxic environments, like the Black Sea and the Cariaco Basin, the bacterial diversity of the Etoliko basin is elevated (Bosshard et al. 2000; Humayoun et al. 2003; Madrid et al. 2001; Vetriani et al. 2003). Similar diversity indices have been used for comparable ecosystems like the Clipperton atoll, which exhibits a high Shannon–Weaver index of 3.0–4.1 (Galand et al. 2012).

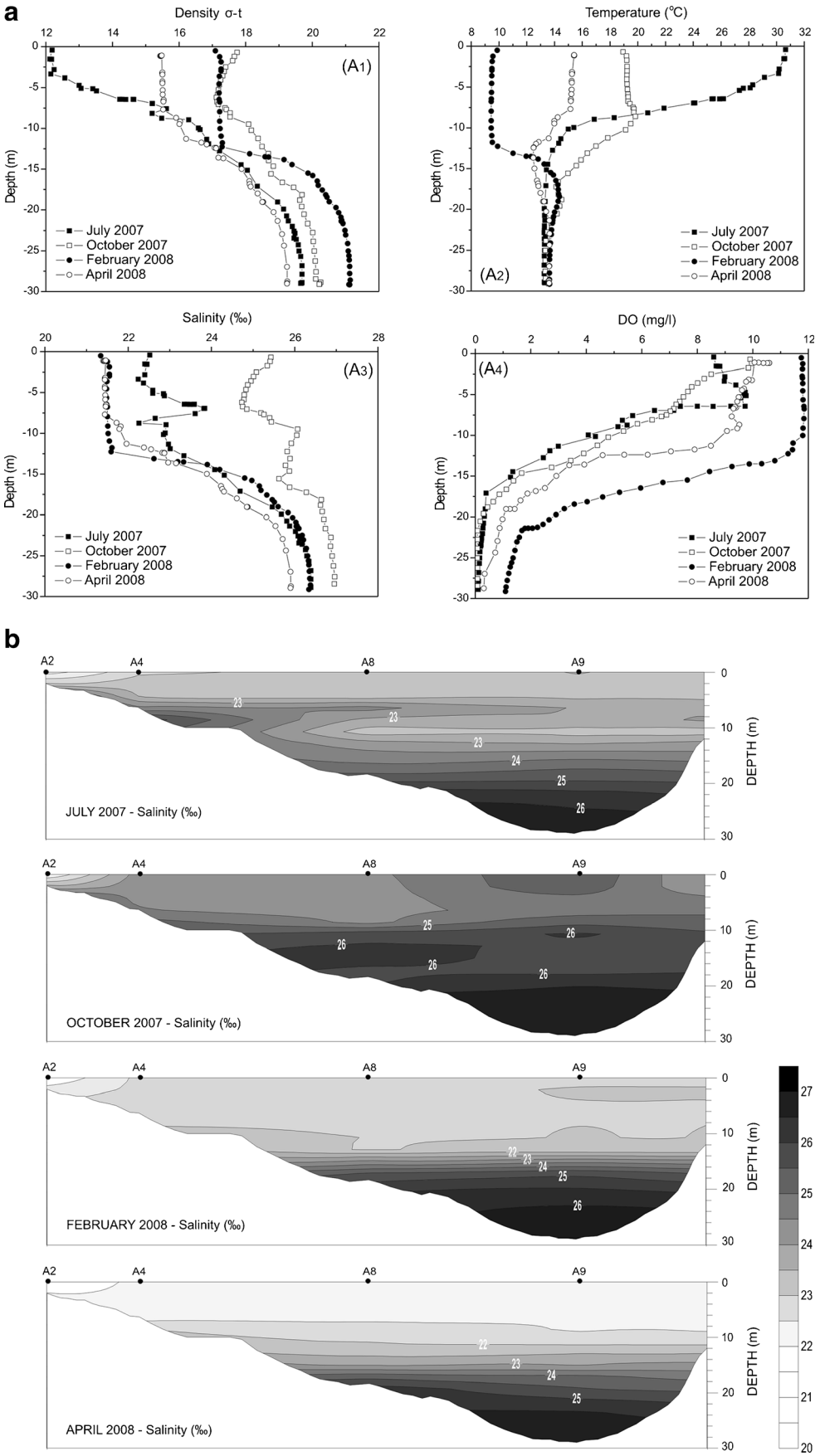
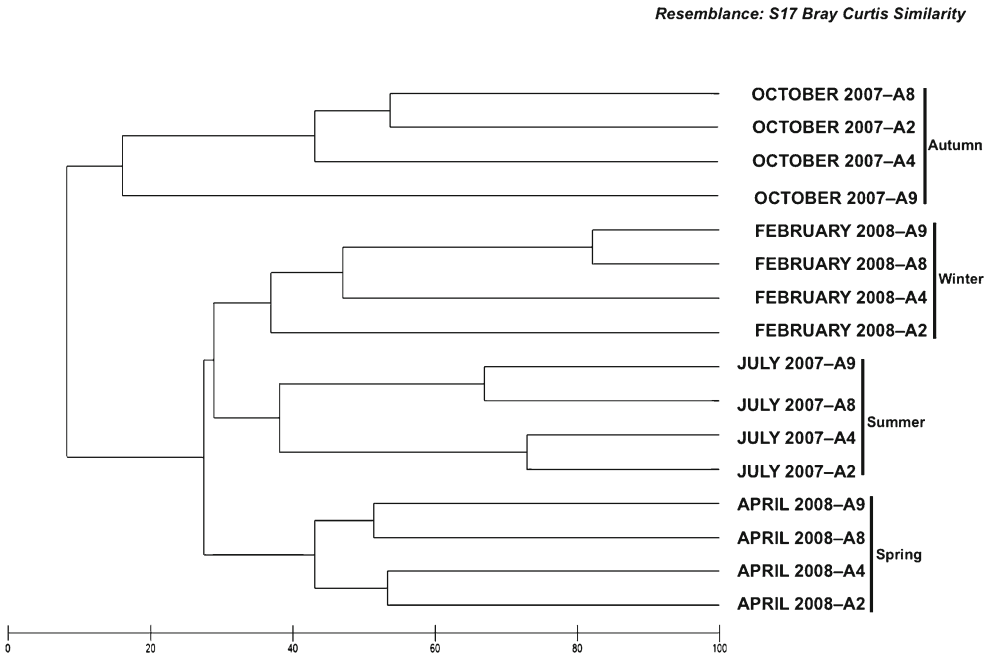


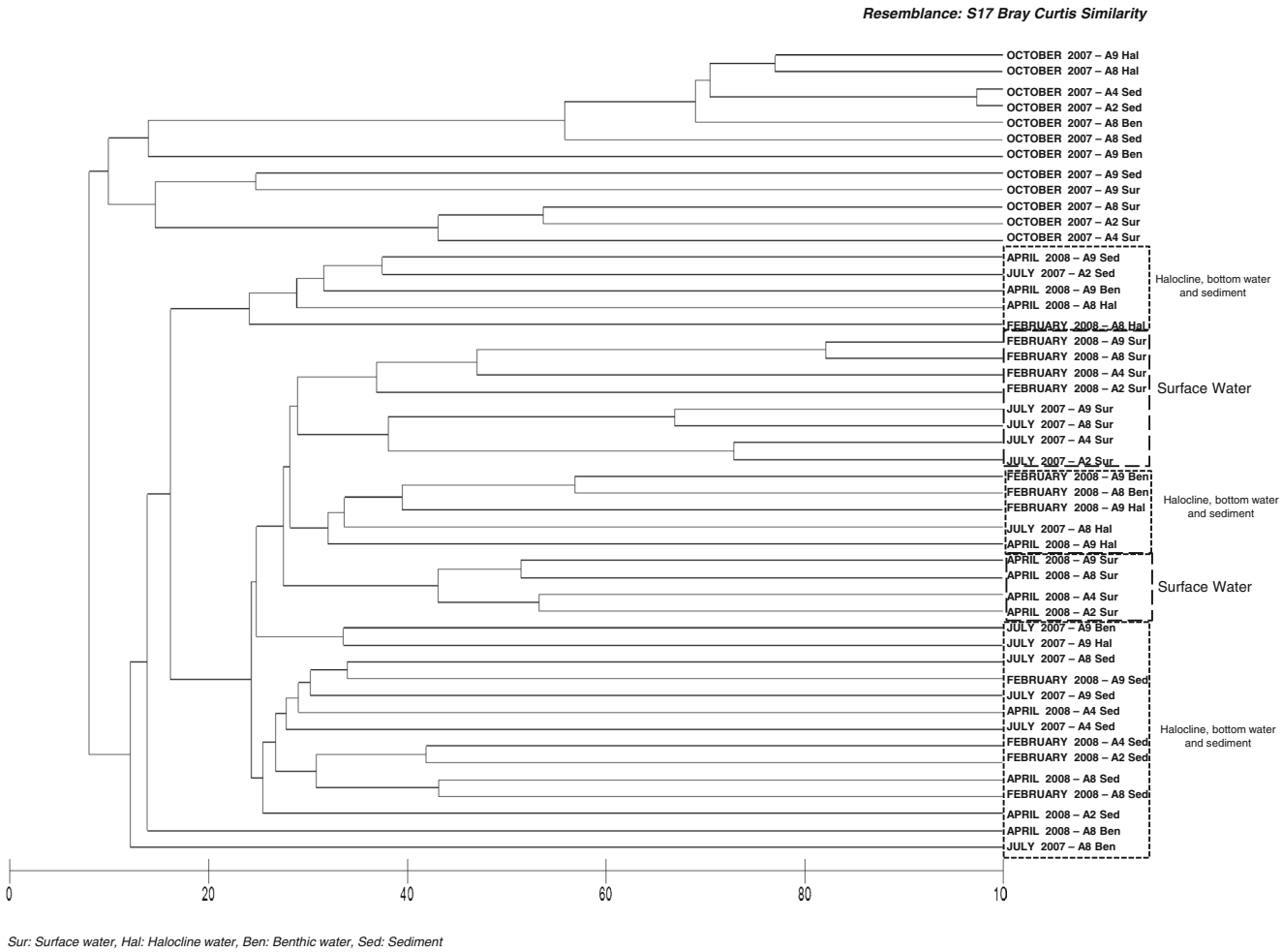
Fig. 2 **a** Seasonal profiles of density (σ-t), temperature (°C), salinity (‰) and dissolved oxygen (mg/l) in the Etoliko basin, for the period July 2007–April 2008. **b** Seasonal vertical salinity distribution of the Etoliko basin across a S–N cross-section

Table 1 Ecological diversity indices of bacterial communities, as derived from ARISA profiles

Sample	No of taxa	Shannon–Weaver	Margalef	Equitability	Temperature (°C)	Salinity (‰)	Dissolved oxygen
July 2007							
A2–A4–A8–A9 (surface)	213–223–235–242	4.38–4.46–4.51–4.60	14.42–15.13–15.92–16.48	0.82–0.83–0.83–0.84	28.24–29.86–29.96–30.65	20.81–21.91–22.13–22.52	9.29–8.62–8.71–8.59
A8–A9 (halocline)	175–231	4.09–4.48	11.94–15.82	0.79–0.82	13.54–13.42	24.12–24.31	2.45–1.27
A8–A9 (near to bottom water)	176–249	4.18–4.56	11.94–16.83	0.81–0.82	13.40–13.30	26.11–26.36	0.12–0.13
A2–A4–A8–A9 (sediment)	300–220–339–313	4.93–4.58–5.01–4.74	21.02–15.98–23.72–21.46	0.86–0.85–0.86–0.83	–	–	–
October 2007							
A2–A4–A8–A9 (surface)	87–106–109–93	3.60–3.74–3.76–3.57	6.67–7.93–8.15–7.04	0.81–0.80–0.80–0.79	13.95–23.83–15.8–15.45	21.97–22.60–23.27–24.15	11.07–11.12–10.91–10.89
A8–A9 (halocline)	87–85	3.69–3.70	6.87–6.74	0.83–0.83	15.28–16.03	25.83–25.61	1.38–1.67
A8–A9 (near to bottom water)	99–123	3.84–3.49	7.78–8.99	0.84–0.72	13.8–13.64	26.48–26.99	0.08–0.08
A2–A4–A8–A9 (sediment)	117–88–125–161	4.07–3.20–3.77–4.39	9.04–6.56–9.39–12.02	0.86–0.72–0.78–0.86	–	–	–
February 2008							
A2–A4–A8–A9 (surface)	187–167–177–223	4.35–4.18–4.15–4.43	12.75–11.69–12.02–15.04	0.83–0.82–0.80–0.82	8.52–9.11–9.31–9.56	20.10–21.11–21.28–21.34	13.02–13.17–13.13–11.75
A8–A9 (halocline)	234–221	4.47–4.32	15.88–14.94	0.82–0.80	13.18–13.73	24.22–24.80	7.96–7.64
A8–A9 (near to bottom water)	292–249	4.70–4.44	19.44–16.61	0.83–0.80	13.90–13.60	25.68–26.33	1.59–0.96
A2–A4–A8–A9 (sediment)	286–292–227–366	4.65–4.65–4.47–5.04	19.38–19.82–15.41–24.87	0..82–0.82–0.82–0.85	–	–	–
April 2008							
A2–A4–A8–A9 (surface)	313–305–278–291	4.94–4.95–4.75–4.84	21.14–20.49–18.77–19.71	0.86–0.87–0.84–0.85	15.06–15.12–15.50–15.40	20.82–21.06–21.32–21.42	10.61–10.57–10.68–10.59
A8–A9 (halocline)	217–269	4.35–4.57	14.55–17.93	0.81–0.82	12.64–12.56	24.8–24.23	0.93–2.92
A8–A9 (near to bottom water)	200–230	4.33–4.39	13.55–15.61	0.82–0.81	13.5–13.61	25.56–25.9	0.59–0.28
A2–A4–A8–A9 (sediment)	269–273–227–354	4.34–4.93–4.15–5.08	18.34–21.23–15.69–24.48	0.78–0.90–0.76–0.87	–	–	–

Diversity values calculated from electropherograms obtained from ARISA fingerprinting





Sur: Surface water, Hal: Halocline water, Ben: Benthic water, Sed: Sediment

Fig. 3 Hierarchical clustering based on Bray–Curtis similarities of ARISA fingerprints of bacterial communities from surface water at the four sampling stations (A2, A4, A8, and A9)

Fig. 4 Hierarchical clustering based on Bray–Curtis similarities of ARISA fingerprints of bacterial communities of all samples from the four sampling stations (A2, A4, A8, and A9). *Sur* surface water, *Hal* halocline water, *Ben* near to bottom water, *Sed* sediment

Hierarchical cluster analysis for all OTUs indicated that there is a seasonal variation in the surface water samples (Fig. 3). This suggests that environmental conditions play a major role in the structure of the bacterial communities of the surface water of the Etoliko basin, as has been observed in other ecosystems (Pinhassi and Hagström 2000; Schauer et al. 2003; Yannarell et al. 2003; Zwisler et al. 2003; Newton and Mcmahon 2011; Peura et al. 2012). The bacterial community structure of the sediment, together with most of the near-to-bottom water samples, appears to be stable, and these samples cluster together (Fig. 4), except the February benthic and halocline samples of the A8 and A9 stations. This is probably due to the hypoxic conditions that were observed during this month. The transition layer (halocline samples) groups together with the bottom layer of the Etoliko basin (near-to-bottom water and sediment samples), which in most cases represent the anoxic part of the ecosystem (Fig. 4). In this case, the October 2007 samples are not considered due to the high salinity observed at that time. Cluster analysis indicated the presence of two main groups. One group was formed by bacteria colonizing surface water, while the second one inhabited the transition layer (halocline), the monimolimnion (near-to-bottom water), and the sediment (Fig. 4). Similar groups have been reported for marine ecosystems like the Black Sea, and the Clipperton atoll but also for meromictic lakes like Lake Pavin (Vetriani et al. 2003; Lehours et al. 2005; Boucher et al. 2006; Galand et al. 2012).

Non-metric multidimensional scaling (MDS) was used to determine if overall bacterial community compositions changed as a function of dissolved oxygen concentrations. MDS ordinations, typically interpreted based on the distance between ordinate points where treatments appear close together, can be regarded as having similar community compositions. MDS ordinations based on Bray–Curtis similarities for each sampling station for all sampling periods can be seen in Fig. 5. The oxic samples differ from the anoxic samples. The stress values on the ordination plots for these samples are below 0.2, indicating that the observed plots are reliable representations of the data. The difference between the oxic and the anoxic part of the Etoliko basin reflects important changes of the diversity of the Etoliko basin, as has been also observed in other meromictic ecosystems, like Lake Pavin and the Black Sea (Lehours et al. 2005; Vetriani et al. 2003). Multivariable analysis (DISTLM) was performed in order to examine the influence of oxygen in the structure of the bacterial communities. Our results indicate that oxygen had a significant influence (Akaike information criterion: 394.53, $p=2.92$, $P=0.002$, df 46, percentage of variance explained 15.95 %) to the bacterial profile structure of the Etoliko semi-enclosed basin.

Interestingly, MDS plot analysis (Fig. 5) and cluster analyses (Fig. 3) demonstrate that all autumn samples form a separate group from all other samples, indicating that the high salinity water that entered the Etoliko basin had a profound effect on the structure of the bacterial community composition. The separation between the autumn high salinity sample and the other seasonal low salinity samples was confirmed by statistical analysis (PERMANOVA, $F=4.64$, $df=1$, $p=0.0001$). According to certain ecological theories, diversity should decrease along this

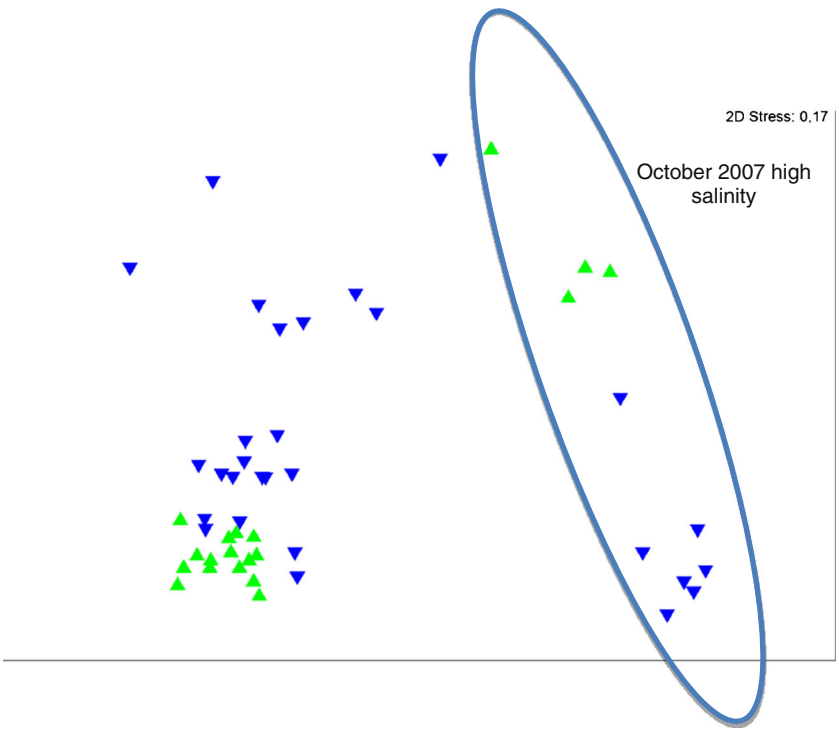


Fig. 5 Multidimensional scaling (MDS) plot, demonstrating changes in the bacterial community composition in the Etoliko lagoon. *Green triangles* represent oxic samples; *inverted blue triangles* represent anoxic samples. The Bray–Curtis measure of similarity was used to assess similarities in ribotype profiles in the dataset

salinity gradient, and this decrease is very apparent in the case of the Etoliko basin, but also in other ecosystems, in which salinity gradients and/or changes in the salinity occur suddenly (Pedrós-Alió et al. 2000; Benlloch et al. 2002).

Conclusions

The Etoliko semi-enclosed basin has a stratified water column, with the salinity being the primary factor controlling this stratification, while temperature seems to play a secondary role. During the sampling period, the Etoliko basin appeared to be homogenous at the surface, since no significant differences were observed at the four sampling stations, while seasonal variations were recorded for temperature and salinity. The microbial profile of the surface water correlates with the observed physicochemical seasonality of this layer, while the more stable ecosystem comprised of halocline, near-to-bottom water, and sediment appears to be almost constant both microbially and physicochemically. A halocline was permanently present in the Etoliko basin throughout the sampling period, but its thickness varied seasonally. Salinity distribution was highly affected by the interaction between the basin and the Messolonghi lagoon, which was apparent through the abnormal salinity distribution noticed during October 2007. Interestingly, the bacterial profile of this particular month forms a distinctly separate group from all other samples, based on non-metric multidimensional scaling (MDS) and calculated ecological diversity indices. This strong connection between salinity and composition of microbial profile supports that there are direct links between changes in environmental forcing, biogeochemical conditions, and microbial communities. The communities were able to recover within 1–2 months after the salinity peak observed in October 2007, demonstrating that the seasonal community variation is relative stable and may represent a deterministic point of return. This observation implies that changes occurring in the aquatic microbial community profiles, which exceed normal variability can: (1) correspond to stress disturbances (for example increase in salinity) or (2) with changes that are expected from gradual global climate change.

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