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Persistence of *Enterobacteriaceae* Drawn into a Marine Saltern (Saline di Tarquinia, Italy) from the Adjacent Coastal Zone

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Abstract: *Enterobacteriaceae* is present in various niches worldwide (i.e., the gastrointestinal tracts of animals, clinical specimens, and diverse environments) and hosts some well-known pathogens (i.e., salmonellas, shigellas and pathogenic coliforms). No investigation has focused on its occurrence in marine salterns, and it is not clear if these hypersaline environments could be a reservoir for these bacteria including some potentially harmful members. In this study, a two-year metabarcoding survey was carried out on samples collected from different ponds of the "Saline di Tarquinia" salterns and the nearby coastal waters. *Enterobacteriaceae* was recorded almost constantly in the seawaters feeding the saltern. Its abundance was generally higher in the sea than in the ponds, probably due to the higher anthropic impact. The same trend was evidenced for the key genus (*Escherichia/Shigella*) and OTU (OTU 5) of the *Enterobacteriaceae* community. Various parameters affected taxon/OTU abundance: *Enterobacteriaceae*, *Escherichia/Shigella* and OTU5 decreased with increasing salinity and rains; moreover, *Escherichia/Shigella* and OTU 5 were higher in autumn than in spring. Although *Enterobacteriaceae* did not seem to find the most favourable conditions for a high-abundance persistence in the saltern environment, it did not disappear. These observations suggested this environment as a potential reservoir for bacteria with possible important health implications.

Keywords: *Enterobacteriaceae*; hypersaline environment; marine salterns; Saline di Tarquinia; metabarcoding survey

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1. Introduction

Enterobacteriaceae (phylum *Proteobacteria*) is a complex family under the taxonomic point of view that includes 33 genera and 134 species (List of Prokaryotic Names with Standing in Nomenclature, https://lpsn.dsmz.de/family/enterobacteriaceae-1 (accessed on 17 May 2021), [1]). It hosts members, distributed worldwide, usually isolated from various niches, such as the gastrointestinal tracts of animals (from insects to humans), clinical specimens, and diverse environments [2,3].

The gastrointestinal tract of animals is one of their most common habitats [4], representing the environment that is historically associated with this family.

Most genera of *Enterobacteriaceae* have an intimate association as commensals with animals (including humans), being part of the gut microbiota. However, various pathogenic

variants, associated with infections, diseases, and syndromes, have been recognised [5]. In this context, the prototypical example is that of *Escherichia coli*. The vast majority of the *E. coli* population in humans has a commensal role and is adapted to colonise the host without causing disease. However, some pathogenic strains can colonise the host, often resulting in clinically significant pathologies [6]. The current classification of pathogenic *E. coli* includes enteroaggregative, enteropathogenic, enteroinvasive, enterotoxigenic, diffusely adherent and Shiga toxin-producing strains [7].

Species of *Enterobacteriaceae* are responsible for numerous animal infections, also with economic implications. For instance, *Klebsiella pneumoniae*, *E. coli*, and *Citrobacter freundii,* which cause bovine mastitis, reduce animal health and may result in considerable economic losses to the dairy industry [8,9]. *E. coli*, *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Kluyvera* spp. and *Salmonella* spp. are involved in farmed fish infections/outbreaks and are responsible for monetary losses in aquaculture [10–12].

Many *Enterobacteriaceae* are pathogens or opportunistic pathogens involved in several human diseases, from foodborne and waterborne diseases to nosocomial infections (i.e., septicaemia, meningitis, respiratory and urinary infections) [5,13–15]. *Salmonella* spp. and enterohaemorrhagic *E. coli* are among the most common foodborne pathogens, affecting millions of people annually [16]. In particular, salmonella represents the second most common causative agent of food-transmitted diseases in Europe and the United States [17,18], and the third most common cause of death among foodborne diseases worldwide [19]. Moreover, various species of *Enterobacteriaceae* (such as *Escherichia* spp., *Salmonella* spp., *Shigella* spp., *Klebsiella* spp., and *Enterobacter* spp.) are highly significant causes of intestinal and extraintestinal nosocomial infections [4]. Furthermore, some of them represent a serious public health threat due to their multidrug resistance [20,21]. Cephalosporin- and carbapenem-resistant *Enterobacteriaceae* was ranked in the category of critical priority pathogens by the WHO [22]. Infections caused by carbapenem-resistant *Enterobacteriaceae* are associated with high morbidity and mortality of hospitalised patients [23].

In addition to the clinical/health implications, another important issue is the detection of *Enterobacteriaceae* in an increasing number of environmental niches [5]. Members of this family have been found in soil [24–28], aquatic habitats [24,29–31], and associated with vegetation (fruits, vegetables, grains, flowering plants, and trees) [29,32–38]. For instance, various species (belonging to different genera, such as *Enterobacter*, *Gibbsiella*, *Klebsiella*, *Kosakonia*, *Mangrovibacter*, *Phytobacter* and *Pluralibacter*) have been reported in relationships with plants, as endophytes (involved in plant growth promotion, nutrient uptake, nodulation, defence against phytopathogens and increase in stress tolerance) [38–44] or phytopathogens [45–47].

Soil, water, and vegetation have proven to be environmental reservoirs for *Enterobacteriaceae*. In this context, some work has investigated their relationship with the environmental habitats and/or possible implications for public health [48].

Although *Enterobacteriaceae* members are found in an increasing number of environmental niches, their occurrence in some habitats is still poorly surveyed, and some environments are underestimated as potential reservoirs of these bacteria. In particular, their occurrence in marine salterns and possible variations according to the relative environmental stress conditions (such as intense salinity and temperature fluctuations) seem not to have been investigated. Moreover, it was not evaluated if these environments could represent a possible reservoir for this group, hosting bacteria with important health implications.

The "Saline di Tarquinia" (ST) are marine salterns, located ca. 80 km northwest of Rome (Italy), along a low sandy coast of the North Tyrrhenian Sea. ST, converted into a Nature Reserve in 1980, ceased salt production in 1997. This led to a dramatic reduction in anthropic activities, and also affected the management and maintenance of the pond water balance over time. However, ST is an extreme environment, featuring large and sudden variations of environmental parameters (in particular, salinity and water temperature) in the majority of the ponds. The ST salterns are located downstream from a large floodplain,

bounded to the northeast by the hilly area of the city of Tarquinia and to the northwest and southeast by the mouths of the rivers Marta and Mignone, respectively. They cover an area of about 135 ha and consist of ca. 100 shallow interconnected ponds that allow the establishment of an increasing salinity gradient, starting from the water pumped from the nearby sea which is transferred through low-salinity ponds (concentrators), to ponds where salt precipitation occurs (crystallisers) [49]. Some studies have been carried out to investigate the microbial diversity of the site [50–57]. The site algal microflora was studied both from the taxonomic point of view [50,56] and for some aspects regarding their pigment production, which is particularly interesting at the biotechnological level [52,55]. Moreover, ST bacteria were studied by culture-dependent and culture-independent methods, showing a very diversified community with members adapted to broad salinity and temperature variations [51,53,54]. The ST bacterial diversity showed increasing simplification along the gradient according to salinity increases. Moreover, the communities were structured by various factors: salinity, sampling site, and sampling year and month [57] However, investigations related to some particular bacterial groups are quite scarce. The only study considering a specific bacterial group was focused on inspecting the presence of *Vibrio* within the ST system, which was demonstrated to be a possible reservoir for members of this genus even if at very low levels of abundance [49].

The aim of the current study was to investigate the presence and distribution of *Enterobacteriaceae* within the ST marine salterns, in order to understand if this hypersaline environment can represent a possible reservoir of potentially harmful bacteria. The work (carried out by a metabarcoding approach) surveyed the *Enterobacteriaceae* occurrence over a broad time span (2 years), analysing samples taken from the coastal waters (representing the saltpan seawater inputs) and various ponds along the salinity gradient. In addition, the influence of environmental parameters on *Enterobacteriaceae* distribution within the ST system was also studied.

2. Materials and Methods

2.1. Sample Collection and Characterisation

Samplings within the ST (North Tyrrhenian Sea, Italy; 42°12′07.8″ N 11°43′17.8″ E) were carried out monthly over two years (from May 2012 to April 2014). Water samples were collected from the sea (S) and three different ponds with increasing salinities (P5, low-salinity concentrator pond; P24, intermediate-salinity concentrator pond, and P37, crystallisation pond), selected according to their annual range of salinity in order to cover the whole ST gradient (Figure 1).

Sample collection, measurement, and the determination of environmental parameters were carried out as reported by Gorrasi et al. [57].

All samples, obtained by pooling three different sub-samples [58], were stored in sterile bottles and kept refrigerated (4 °C) during transport to the ST laboratory to be processed within one hour. Then, 1 L of water was vacuum-filtered on sterile membranes (0.22 μm, Millipore, Burlington, MA, USA), which were washed twice with sterile saline (NaCl) solutions (with concentrations roughly corresponding to the salinity recorded on the sites) to remove possible nutrients from the filters [59]. The filters were kept frozen until DNA extraction. The remaining sample aliquots were used to determine BOD_5 and chlorophyll pigment concentration, which were measured by standard methods [60].

Environmental parameters (salinity, pH, conductivity, and water temperature) were recorded during samplings by common handheld probes. Daily rainfall data were obtained from a local government institution [61]. All parameters are reported in Table S1.

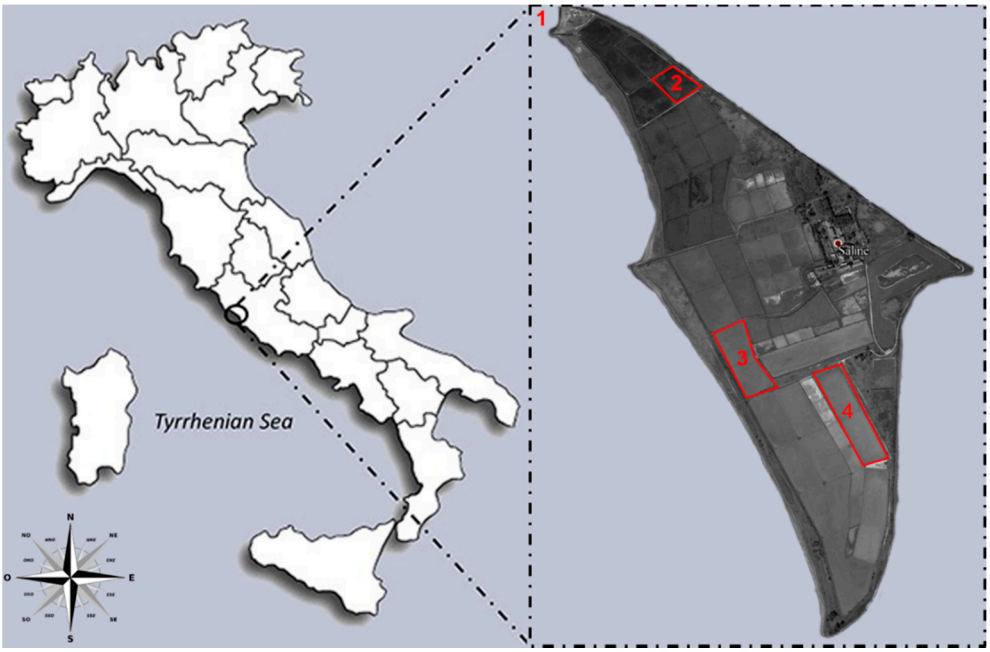

Figure 1. Detailed map of the "Saline di Tarquinia" (Viterbo, Italy) marine salterns with indication of the sampling sites. 1 = S, sea; 2 = P5, low-salinity concentrator pond; 3 = P24, intermediate-salinity concentrator pond; and 4 = P37, crystallisation pond. The map was generated using Google Earth Pro version 7.3.1 and graphically edited.

2.2. DNA Extraction, 16S rDNA Amplicon Libraries and Sequencing

Total DNA was extracted from filters using a GeneMATRIX Bacterial & Yeast Genomic DNA Purification Kit (EURx Sp. z o.o., Gdansk, Poland) as previously reported [58].

The multiplexed amplicon libraries were prepared using a dual PCR amplification protocol, amplifying the V5–V6 hypervariable regions of the 16S rDNA. The primers used, 783F (5′-CAGGATTAGATACCC) and 1046R (5′-CGACRRCCATGCANCACCT) [62,63], were modified by adding external barcodes to allow the parallel processing of multiple samples. The strategy and related protocols used to prepare the libraries have already been described in detail by Gorrasi et al. [57].

The second step of library preparation, with the addition of standard Nextera indexes (Illumina, San Diego, CA, USA), and sequencing were carried out at Nuova Genetica Italiana SRL (Monza-Brianza, Italy). Amplicon libraries were sequenced by Illumina MiSeq (Illumina, San Diego, CA, USA) using a 2 × 250 bp paired-end protocol.

2.3. Sequence Processing and Data Analysis

Sequence processing and data analyses were carried out as reported by Gorrasi et al. [57].

Reads from the sequencing were demultiplexed according to the indices and internal barcodes.

Reads were processed using the UPARSE pipeline [64]. Forward and reverse reads were merged with perfect overlapping and quality, filtered with default parameters. Suspected chimeras and singleton sequences (i.e., sequences appearing only once in the whole dataset) were removed. OTUs were defined on the whole dataset clustering the sequences at 97% similarity and defining a representative sequence for each cluster. The abundance of each OTU was estimated by mapping the sequences of each sample against the representative sequence of each OTU at 97% of similarity.

Taxonomic classification of the OTU representative sequences was obtained by RDP classifier [65], using a 50% confidence cut-off as suggested for short sequences [66].

2.4. Statistical Methods

Principal component analysis (PCA) was performed to summarise sample distribution according to *Enterobacteriaceae* genera.

Redundancy analysis (RDA) was performed to investigate the influence of environmental parameters on the *Enterobacteriaceae* community. OTU abundance data for the RDA analysis were square-root-transformed. The environmental parameters taken into account were: sampling site, salinity, water temperature, pH, chlorophyll, BOD_5, rains (cumulative data relative to the seven days preceding the sampling) and sampling month (entered as Fourier series transformed data, *sin*(Month) and *cos*(Month), to account for seasonality) [67]. Sampling site, rains and *sin*(Month) were used as predictors for the RDA analysis. The other variables were removed, being collinear (Pearson $|r| > 0.6$) with the predictors or not significant according to the forward selection. The forward selection was performed for the stepwise selection of the explanatory variables, correcting the significance according to the false discovery rate (FDR) in order to avoid Type I error inflation [68]. The RDA significance was assessed by the Monte Carlo permutation test, using 9999 permutations.

The abundance variation of *Enterobacteriaceae*, the most abundant genera, and OTUs in relation to the key environmental parameters was investigated by generalised linear models (GLMs), assuming a Poisson distribution and with a log link function. Additionally, in the GLM analysis, the periodic regression based on Fourier series transformation of months was used to account for taxa seasonal variation. The Pearson matrix was calculated using the statistical software Systat 8.0 (Systat Software Inc., Point Richmond, CA, USA), whereas the PCA, RDA and GLM analyses were performed using the CANOCO v. 5.1 software package (Microcomputer Power, Ithaca, NY, USA).

Post hoc tests were used to assess pairwise differences in taxa abundance variation among sampling sites. Non-parametric tests (Kruskal–Wallis, one-way analysis of variance, and Kolmogorov–Smirnov two-sample tests) were performed, based on data assessments obtained by Shapiro–Wilk and Levene tests (carried out to test data normal distribution and homogeneity of variance assumptions, respectively). All these tests were run using Systat 8.0.

Spearman's rank correlation coefficient (ϱ) was calculated to determine associations among the most abundant OTUs (Systat 8.0).

3. Results and Discussion

3.1. Spatio-Temporal Variation of Enterobacteriaceae

Across the 96 samples, a total of 2,566,189 bacterial sequences were obtained after quality control. Among them, 45,778 (resulting in 26 different OTUs, by sequence clustering at 97% of similarity) were assigned to *Enterobacteriaceae* using a 50% confidence cut-off.

Over the two-year survey, *Enterobacteriaceae* was always detected in the sea, except in April and June 2013. Moreover, in 13 months out of 24 it was a major taxon, with relative abundance (Ra) in the range 2.5–18.1% (Figure 2; Table S2).

Abundance of this taxon was generally higher in the sea (S) than in the various ponds, and it decreased along the salinity gradient (Table S2). The exceptions were May 2012, September 2012, December 2012, November 2013, and March 2014, when it was higher in P5 than in S. In particular, in May 2012 in the P5 and in S, it was 19.7 and 7.2%, respectively.

A high presence of *Enterobacteriaceae* in coastal waters, in particular if close to anthropized areas, could be due to various causes, possibly related to environmental contamination, such as the discharge of sewage effluents, recreational activities, and aquaculture.

As for ST, they border a rather high-impact touristic settlement that, based on seasonal issues, may contribute to the increased presence of enteric bacteria. In addition, the mouths of the two above-mentioned rivers bordering the floodplain release their freshwaters close to the site, possibly contributing with further inputs of bacteria from the upstream urbanised areas, where there are also various agricultural and animal farming activities. Sea hydrodynamics (currents and tides) can facilitate the diffusion of these bacteria toward the saltern, and their loading into the pond system by seawater pumping. These are

probably the reasons for the generally higher levels of this bacterial group in our sea samples. Moreover, the higher *Enterobacteriaceae* occurrence in the sea than in the ponds could also be due to more suitable (less stressing) conditions characterising the marine coastal environment.

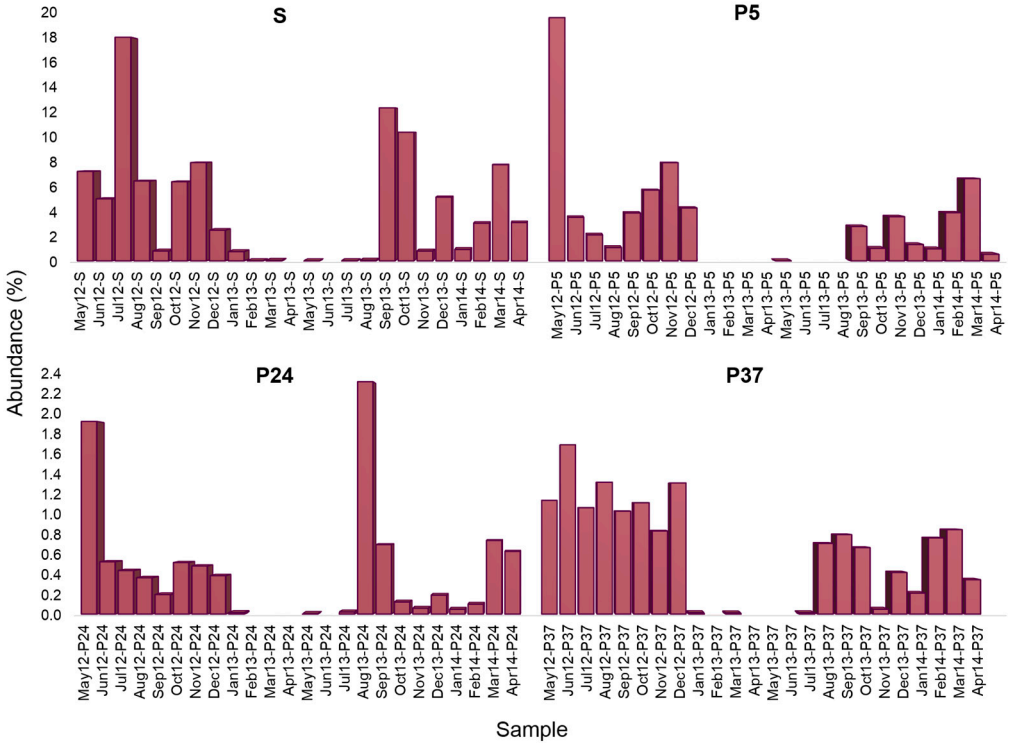

Figure 2. Occurrence of *Enterobacteriaceae* among the ST sampling sites during the two-year period, May 2012–April 2014.

In general, it was evident that *Enterobacteriaceae* driven into the ST system by the seawater influx did not seem to find the most favourable conditions for its persistence in the hypersaline environment. However, despite this, these bacteria generally did not disappear, as shown by monthly data. The presence of *Enterobacteriaceae* in the ponds could also be due to the contribution of the avifauna, which in some periods could be quite abundant.

To obtain an overview of *Enterobacteriaceae* occurrence in the ST system and in the neighbouring sea, the accumulated data were analysed (Figure 3A). Over the two years, *Enterobacteriaceae* showed mean abundances of 4.12, 2.88, 0.41 and 0.60% in S, P5, P24 and P37, respectively. However, in some periods, its abundances were notably above the average: in July 2012 in S (Ra = 18%) and in May 2012 in P5 (Ra = 19.7%).

Considering the global data, *Enterobacteriaceae* abundance was higher in S and P5 (low-salinity concentrator pond, showing salinities similar to those of the sea) than in P24 (intermediate-salinity concentrator pond) and P37 (crystallisation pond) ($p < 0.01$).

Among the 26 Enterobacteriaceae OTUs, 17 were assigned at the genus level. The genera detected were Escherichia/Shigella, Siccibacter, Buttiauxella, Atlantibacter, Yokenella, Pseudescherichia and Metakosakonia.

The genera abundances in all samples are reported in Table S3.

Metakosakonia were definitely underrepresented, being found only in two ST samples (May 2012—S and March 2014—P24) out of 96, and with very low abundances (Ra~0.01%).

Atlantibacter, *Yokenella* and *Pseudescherichia*, when detected, always represented rare taxa (with Ra values in the range 0.001–0.06%, 0.001–0.3% and 0.002–0.01%, respectively).

Over the two-year survey, *Buttiauxella* presence was revealed only in May 2012; it was found in all sites as a rare taxon (0.005–0.08%), except in P5 where it showed a Ra = 2.1%.

Siccibacter was found in 26 samples, but it showed Ra $\geq$ 1% (1.2 and 12% in May 2012—S and May 2012—P5, respectively) in only two samples.

Escherichia and *Shigella* species have essentially identical 16S rRNA sequences. Thus, the reference databases for taxonomic identification (including RDP) use the combined genus name *Escherichia/Shigella* [69]. Therefore, it is not possible to uniquely attribute the relative sequences to one of these two genera.

Escherichia/Shigella was the most represented taxon among the *Enterobacteriaceae* (82% of the *Enterobacteriaceae* sequences across all samples). It was found in 75 samples and was recorded with Ra $\geq$ 1% in 30 samples. Its mean abundances in S, P5, P24 and P37 were 3.45, 1.90, 0.34 and 0.46%, respectively. However, it showed above-average abundances in S in July 2013 (15.6 %) and September 2013 (10.7%) and in P24 in May 2012 (Ra = 1.2%) and August 2013 (Ra = 2%).

Considering the overall two-year data, as already observed for the whole *Enterobacteriaceae*, *Escherichia/Shigella* was more abundant in S and P5 (where it also showed much broader abundance variations) than in P24 and P37 ($p < 0.01$) (Figure 3B).

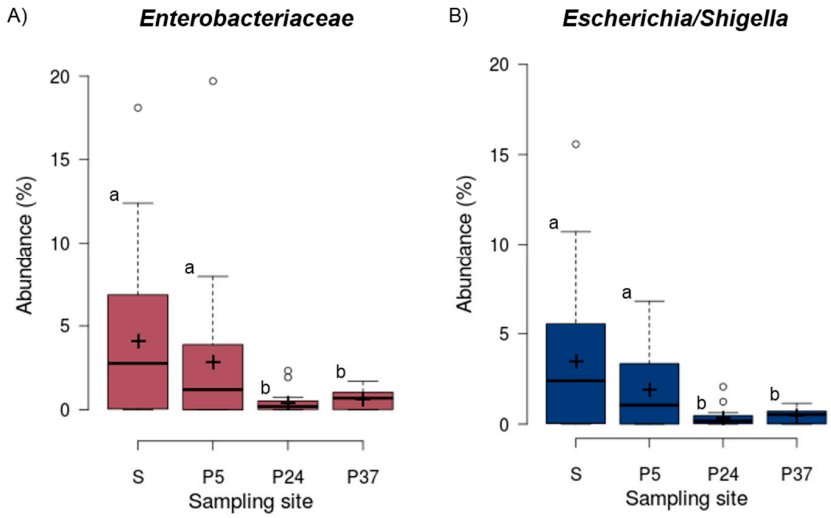

Figure 3. Box and whisker plots showing the abundance variation of *Enterobacteriaceae* (**A**) and *Escherichia/Shigella* (**B**) across the sampling sites. The thick lines represent the median; the box upper and lower limits indicate the 25th and the 75th percentiles, respectively; the whiskers indicate the data that lie beyond the 5th percentile (lower whisker) and the 75th percentile (upper whisker); and the dots represent the outliers. The different letters indicate differences between the mean values of different groups.

In addition, the PCA of sampling sites, according to the abundance of the *Enterobacteriaceae* genera, revealed *Escherichia/Shigella* as the most indicative taxon for sample variability. The PCA explained 99.58% of the total variance along the first two axes (82.56% explained by the first axis) (Figure 4).

From a general aspect, it is important to note that members of these two genera are mainly animal-adapted (including humans) enteric bacteria and, among them, some members are known human pathogens [7,70]. It is possible to speculate that their frequent detection within the ST, and in particular, with higher abundance in the neighbouring sea, could be mainly due to contamination of anthropogenic and/or animal origin in the coastal area [71].

Among the 26 *Enterobacteriaceae* OTUs, two were found as the most abundant (having a total number of mapping sequences on the whole ST dataset > 1000): OTU 5 (37,725 sequences) and OTU 206 (6714 sequences).

Most (82%) of the *Enterobacteriaceae* sequences found across all samples were ascribed to OTU 5, which was the most abundant and represented OTU (Table S4). It was assigned to *Escherichia/Shigella* and represented the main genus fraction (97.8–100% of this taxon across

all samples). Moreover, in 73 samples it represented a consistent proportion (50–100%) of *Enterobacteriaceae*. OTU 5 showed mean abundances of 3.45, 1.90, 0.34 and 0.46% in S, P5, P24 and P37, respectively. It was recorded with above-average abundances in S in July 2012 (Ra = 15.6%) and September 2013 (10.7%), and in P24 in May 2012 (Ra = 1.2%) and August 2013 (Ra = 2%).

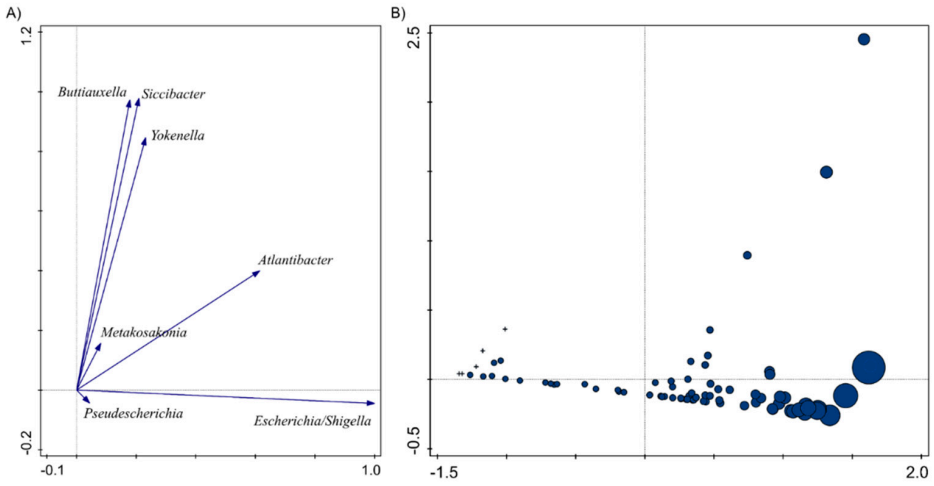

Figure 4. Principal component analysis (PCA) of sampling sites according to the abundance of the *Enterobacteriaceae* genera. The panels show genera according to their occurrence in ST samples (**A**) and sample ordering according to genera contribution (**B**). In panel B, symbol size is indicative of the *Escherichia/Shigella* abundance in each sample. The percentages of explained variation by the first and the second axes are 82.56 and 17.02%, respectively.

The cumulative data evidenced that OTU 5 was more abundant in S and P5 than in P24 and P37 ($p < 0.01$) (Figure 5A). Overall, OTU 5 abundance variation among sampling sites essentially coincided with those of *Enterobacteriaceae* and *Escherichia/Shigella* because it was the predominant fraction of both taxa.

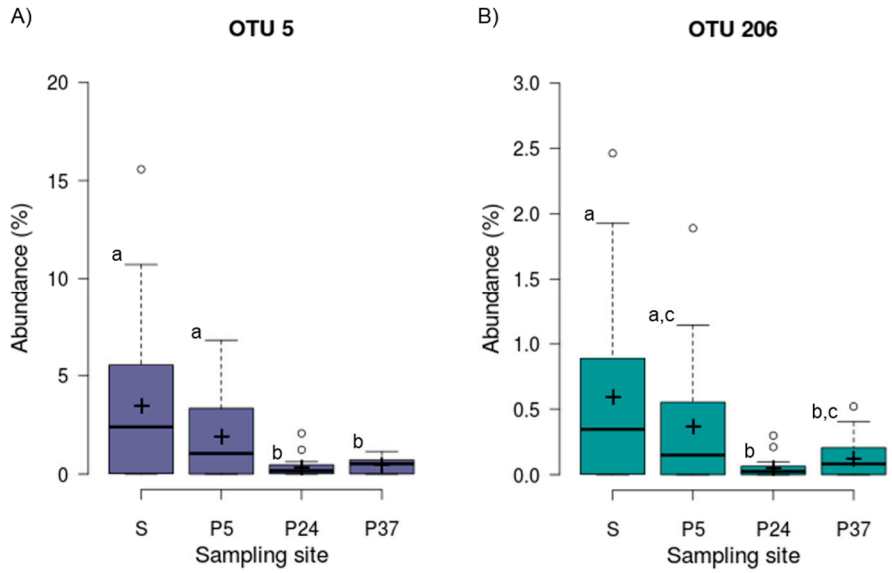

Figure 5. Box and whisker plots showing the abundance variation of OTU5 (**A**) and OTU 206 (**B**) across the sampling sites. The thick lines represent the median; the box upper and lower limits indicate the 25th and the 75th percentiles, respectively; the whiskers indicate the data that lie beyond the 5th percentile (lower whisker) and the 75th percentile (upper whisker); and the dots represent the outliers. The different letters indicate differences between the mean values of different groups.

OTU 206 (belonging to *Siccibacter*) was detected in 69 samples, but in 63 it represented a rare taxon. It was detected with Ra $\geq$ 1% (1.1–2.5%) in 6 samples: four collected in the sea (May 2012—S, July 2012—S, September 2013—S, and October 2013—S), and two in P5 (May 2012—P5 and November 2012—P5). Considering the overall data, its highest mean abundance (~0.6%) was recorded in the sea, where in July 2012 the OTU reached 2.5% (Table S4). Overall, OTU 206 was significantly ($p < 0.01$) more abundant in the sea than in P24 and P37 (Figure 5B).

3.2. Environmental Parameters Driving Enterobacteriaceae Occurrence (RDA) and Variation of Key Taxa and Most Abundant OTUs in Relation to Them

The RDA was performed to investigate the key parameters affecting *Enterobacteriaceae* occurrence and distributions and to explore the relationships between the most abundant OTUs and these parameters. Among the various environmental parameters taken into account, water temperature, pH, and conductivity were excluded from the RDA, due to these factors being collinear with other parameters (Pearson $|r| > 0.6$). The tested parameters were: sampling site, salinity, chlorophylls, BOD_5, rains and sampling month (entered as *sin*(Month) and *cos*(Month) to account for seasonality). The RDA analysis showed that the *Enterobacteriaceae* community changed significantly with sampling site, rains and *sin*(Month) (Table 1).

Table 1. Descriptive statistics of RDA.

Variable	Explains %	Contribution %	F	*p*	*p*(adj) [1]
Sampling site: S	11.26	32.9	12.0	0.00094	0.00848
Rains	8.66	25.3	10.2	0.00094	0.00424
Sampling site: P5	5.99	17.5	7.5	0.00471	0.01414
sin(Month)	5.34	15.6	7.2	0.00471	0.01060
Sampling site: P37	0.27	0.8	0.4	0.67855	0.69180
Sampling site: P24	0.27	0.8	0.4	0.69180	0.69180

[1] Corrected according to the FDR.

Among the three factors, the sampling site had the main effect on the *Enterobacteriaceae* community, explaining the highest percentage of total variance (17.79%, sum of variance percentages explained by single sampling site; Table 1).

As shown by the RDA triplot (Figure 6), a clear separation between samples collected from S and P5 and those collected from P24 and P37 was observed. A partial overlap of S and P5 sample clusters was evidenced, while a large overlap was observed between those of P24 and P37. The sample ordering was based on the OTU composition, which means that the compositions of S and P5 were rather similar, and those of P24 and P37 were very similar. Therefore, the observed overlapping of clusters was mainly due to OTU 5 contribution (represented the preponderant fraction of the *Enterobacteriaceae*), and secondly to that of OTU 6, which showed similar abundances in S/P5 and P24/P37 (box and whisker plots, Figure 3).

The RDA showed that the P24 and P37 samples collected in spring and winter were those mostly affected by rains. Actually, rainfalls had a great impact on P24 (intermediate-salinity concentrator pond) and P37 (crystallisation pond): during the rainiest periods, salinity dramatically dropped in these two ponds. This was particularly evident in January–April 2013 and February 2014, when the recorded salinity values were close to those of the sea or even lower (Table S1). As for the abundance variation of *Enterobacteriaceae*, *Escherichia/Shigella*, and the most abundant OTUs in relation to the rains, they changed significantly ($p < 0.02$) according to this parameter, decreasing with rainfall increase (Figure 7A). The decrease in these bacteria in the ponds during rainy periods was probably due to the strong brine dilution caused by the precipitation. In fact, both concentrations and the crystallisation pond were very shallow (~15 cm), and their water volume was strongly affected by rains.

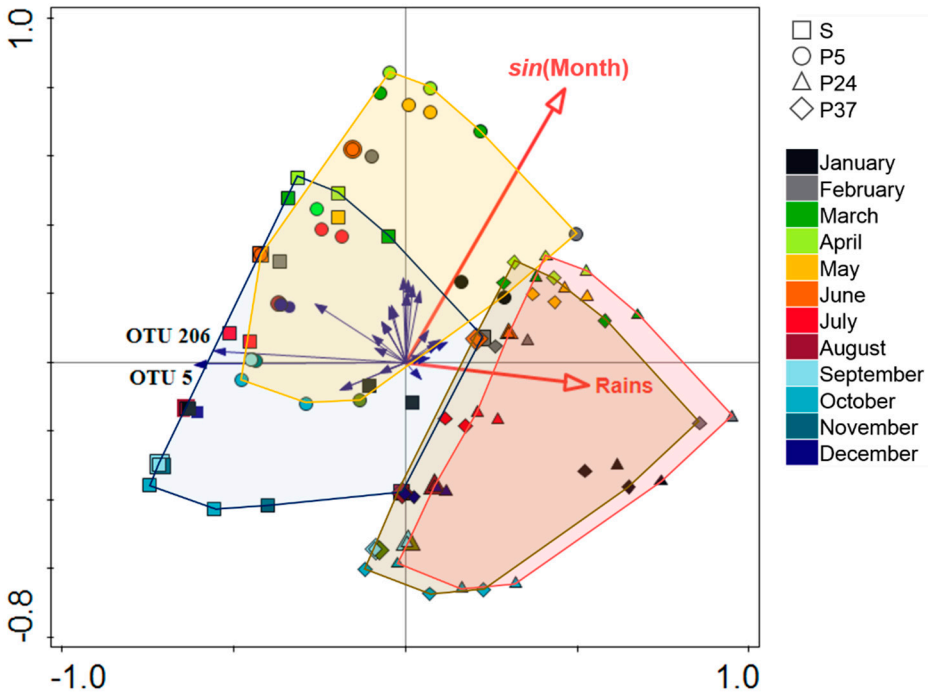

Figure 6. Redundancy analysis (RDA) triplot showing the relationships among the ST samples, the *Enterobacteriaceae* OTUs, and the environmental variables affecting the *Enterobacteriaceae* community. The first and the second axes explained 30.79% and 0.92% (F = 8.4, $p = 0.001$) of the variance in the *Enterobacteriaceae* community, respectively. The red arrows represent the variables *sin*(Month) and rains. The polygons enclose samples collected from the same site (light blue polygon: S samples; orange polygon: P5 samples; red polygon: P24 samples; brown polygon: P37 samples). The blue arrows represent OTUs; only the names of the two most abundant OTUs are reported.

In addition, samples were ordered along the *sin*(Month) vector according to the season, with those collected in spring clearly projected towards the vector tip, whereas those collected in autumn were on the opposite side. Significant abundance variations in relation to this parameter were observed only for OTU 5, and more generally for the whole *Escherichia/Shigella* taxon, being higher in autumn than in spring ($p = 0.048$) (Figure 7B).

As for the most abundant OTUs, the RDA showed that they were projected towards S and P5 and on the opposite side of the rain tip vector. In fact, as discussed above, they showed the highest abundances in S and P5 and decreased with rainfall increase. In the RDA triplot, the two vectors representing OTU 5 and OTU 206, besides having the same direction, were very close to each other. Actually, these OTUs showed a high positive correlation (as indicated by the Spearman's rank correlation coefficient, $\varrho = 0.962$), suggesting their high association and thus their co-occurrence.

As mentioned, salinity was not significant for the RDA model, based on the contribution of each single OTU. However, the GLM analyses were performed on the whole family, its key genus, and OTUs to understand whether the abundance variation in relation to this parameter could still be significant. For *Enterobacteriaceae*, *Escherichia/Shigella* and OTU 5 significant abundance variations were evidenced (Figure 7C). They showed, as expected, the same trends with an abundance decrease at increasing salinity. This was congruent with the above results, which showed that their abundance decreased from the near the sea to the ST ponds and along the salinity gradient.

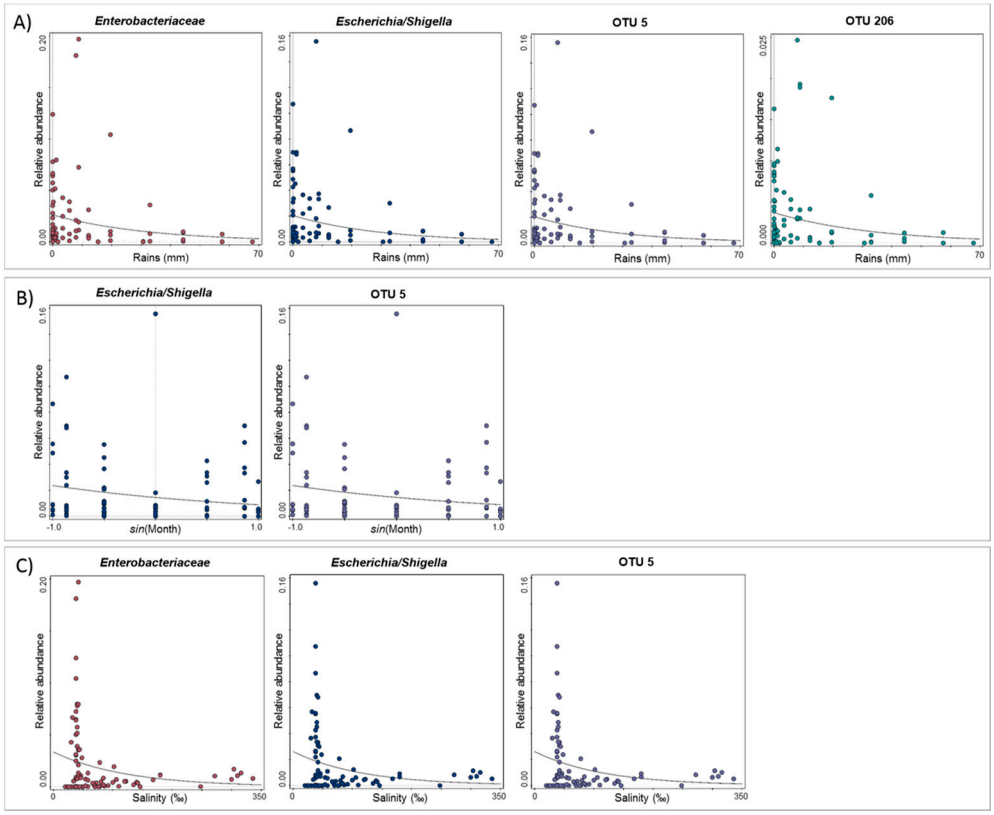

Figure 7. GLM plots showing the significant trends of the *Enterobacteriaceae* family, its key genus, and OTUs in relation to the environmental parameters. The abundance variation of *Enterobacteriaceae*, *Escherichia/Shigella*, OTU 5 and OTU 206 in relation to the rains (**A**); abundance variation of *Escherichia/Shigella* and OTU 5 in relation to the *sin*(Month) (**B**); and abundance variation of *Enterobacteriaceae*, *Escherichia/Shigella* and OTU 5 in relation to the salinity (**C**).

4. Conclusions

To the best of our knowledge, this was the first study investigating the presence and distribution of *Enterobacteriaceae* (based on monthly data recorded over two years) within marine salterns, in order to understand if these hypersaline environments can represent possible reservoirs of potentially harmful bacteria.

In the studied system, *Enterobacteriaceae* was almost always detected in the waters sampled from the nearby sea (the feeding point for the ST system). Its abundance and those of its key genus and OTU (*Escherichia/Shigella* and OTU5, respectively) were higher in the sea than in the ponds, probably due to a combination of factors: the high impact of human activities in the coastal area and the more suitable (less stressing) environmental conditions characterising the sea.

It was evident that *Enterobacteriaceae* loaded into the pond system by seawater pumping did not seem to find the most favourable conditions for its persistence at high levels of abundance in the hypersaline environment. However, despite this, it generally did not disappear, suggesting that this hypersaline environment could represent a possible reservoir for this group, hosting bacteria with important health implications.

This work also showed the importance of some parameters in determining taxon/OTU abundance. *Enterobacteriaceae*, *Escherichia/Shigella* and OTU5 decreased with increasing salinity and rain. Moreover, a seasonal pattern was evidenced for *Escherichia/Shigella* and OTU 5, which were higher in autumn than in spring.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w13111443/s1, Table S1: Environmental parameters recorded for the ST samples from May 2012 to April 2014, Table S2: Relative abundances of *Enterobacteriaceae* across the ST samples, Table S3:

Relative abundances of *Enterobacteriaceae* genera across the ST samples, Table S4: Relative abundances of *Enterobacteriaceae* OTUs across the ST samples.

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