UNIVERSITA' DEGLI STUDI DI ROMA "TOR VERGATA"

FACOLTA' DI SCIENZE MATEMATICHE, FISICHE E NATURALI

DOTTORATO DI RICERCA IN
BIOLOGIA EVOLUZIONISTICA ED ECOLOGIA

CICLO XXIV

DIVERSITY OF THE BACTERIOPLANKTON COMMUNITY IN THE MACCHIATONDA WETLAND (ROME, ITALY)

Tesi di Dottorato

Matteo Evangelisti

A.A. 2010/2011

Docenti guida: Dott. Luciana Migliore e Prof. Maria Cristina Thaller

Coordinatori: Proff. Patrizia B. Albertano e Giuliana Allegrucci

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This work is dedicated to Nonna Tuta

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1. Introduction

1.1 Wetlands

Inland wetlands include rivers, lakes, marshes, bogs and even coastal lagoons, river deltas and all the artificial wetlands, such as saline, filled tubs, artificial ponds, paddy fields etc. All of these environments play an essential role in ecological, social, cultural and economical fields (Fig. 1.1). Wetlands play a lot of important functions as: maintenance of groundwater levels, flood and erosion control, consolidation of the shores, retention of sediments, nutrient capture, mitigation and maintenance of the microclimate. (Batzer D. P. 2006; Nairn R.W. 1999), moreover, these habitats are always important as tourist and recreation centres, as a source of livelihood for local people and as special places for food and material production (Barbier E.B. 1997). The wetlands also support a high biodiversity (Hall D.L. 2004), as it happens in lagoons and coastal marshes where the productivity, in terms of biomass and number of species, is similar only to the tropical forests. The high productivity depends on both the nutrient accumulation coming from the mainland through the rivers, and the sea tides, producing a continuous mixing of the nutrients, making it constantly available. Such a rich environment is colonized for trophic and reproductive purposes by a high number of species (Mitsch W.J. 2011).

In 1972 in Italy the internal wetlands covered a total area of about 190,000 ha, accounting for the 0.6% of the country surface. This represented only a fraction of the wetlands once present in Italy as, at Ancient Roman times, wetlands covered almost 3,000,000 acres, 1/10 of the country (Ramsar Bureau, 1990).

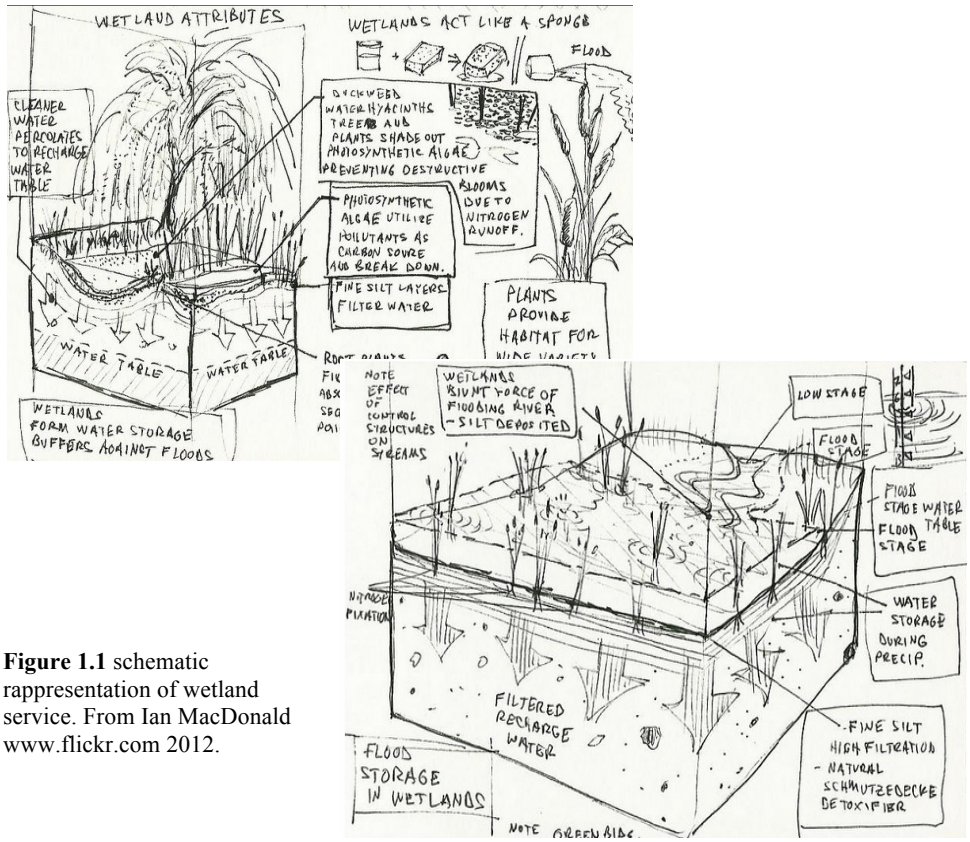

Figure 1.1 schematic rappresentation of wetland service. From Ian MacDonald www.flickr.com 2012.

This last reduction, followed a long series of draining, grinding and channelling of wetlands that were applied since antiquity. The first known retrieval interventions started with the early Roma Empire on the Pontine marshes, the Rieti valley, the Vallo di Diano and the Val di Chiana

In the XII and XIII Centuries the Benedictine monks built many monasteries and started a remarkable work of wetland clearing and drainage. However, in 1865, the

marshes, lakes and ponds still represented about the 4.36% of the national area, accounting for 1,300,000 acres (Pareto R. 1865). Thousands hectares of wetlands went disappearing over the last hundred years in Sardinia and in the Po valley; lastly, in the Fascism period, the Pontine plains were drained in order to eradicate malaria. This policy continued after the settling of the Italian Republic and still today the Italian Constitution (Article 44) promote the drainage of land, although several improvements in the administrative system have been introduced. The loss in biodiversity caused by the disappearance of wetlands is obvious and intuitive.

At the moment, the actual extension of the wetlands still staying in Italy is unknown. Although a complete list of all Italian wetlands does not exist, efforts to achieve this result has been made. The "Inventory of Wetlands of the Italian territory" (De Maria, 1992), based on specific criteria, identified 597 wetlands, including 103 areas of national and international importance. Other inventories at international, national and regional level (Pratesi Urquhart W. and Montemaggiori A., 1995) were compiled, but none of them provides an exhaustive framework.

The greenhouse effect, and the consequent global warming, is regarded as absolutely dangerous for the wetlands. Due to the shallowness of their waters some of them, such as the coastal lagoons, are very sensitive to temperature changes,that, togheter with pH and salinity fluctuations, influence also the distribution of nutrients and play a key role in the chemistry of water itself, by determining the content of dissolved oxygen in the water. In the coastal lagoons temperature and salinity are directly related: high temperature causes high evaporation rate and high salt concentration in water (Wangersky P. J. 2000).

Wetlands have a major importance because they are focal points of biological diversity (Anderson A.M. et al. 1999; Russell K.R. et al. 2002), due to the great biodiversity of the flora and fauna that are found in these ecosystems. This explains why the international Ramsar Convention, already in 1971, focussed the attention on these areas and remarked the need to preserve them. Furthermore, wetlands can easily be menaced by anthropic pressure (Castillo J.M. 2002) (Fig. 1.2) so, in order to manage and preserve them, it is necessary to collect and analyse large sets of data

so to deepen the links between abiotic measurements and biological observations. This, in turn, will allow to find the way to evaluate the ecosystems status and to set up suitable protocols to preserve them. Very little is known on the microbial component of these systems, although it drives all the nutrient recycling and, as a consequence, supports the biodiversity of the sites. It is therefore very important to understand the intimate link between the abiotic factors and the bacterial community composition (McArthur J.V. 2001, Merkley M.B. et al. 2004).

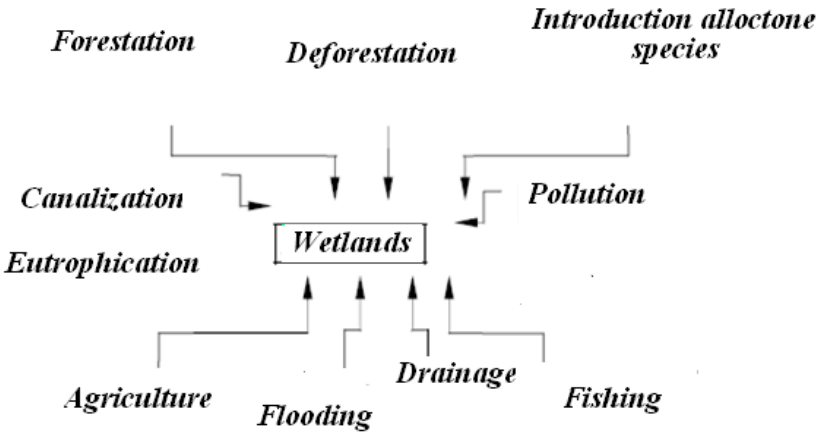

Figura.1.2 Main causes of loss and degradation of wetlands in Central and Eastern Europe (modified from Finlayson 1992).

1.2 The Macchiatonda Regional Natural Reserve

The Regional Nature Reserve of Macchiatonda established in 1983 and governed by the Santa Marinella council, represents also a site of community importance (SCI) (Fig.1.3), and special protection area (SPA). The Reserve spans over 617 acres of coastal plains, 50 Km north of Rome in the foothills of the Tolfa Mts. where they meet the Ceriti Hills.

The Reserve is bordered to the north by the Aurelia State Road, to the south by the Tirrenian sea and the Army shooting range “protects” both the west and the east

side. Three-quarters of the land are occupied by extensive farming. The remaining of the 155 acres consists of the typical environment that is found behind dunes: salted fields, fresh and salt water coastal ponds, and small woods, hence the name "Macchiatonda" that means round woods, (common in the Maremma Tuscany-Lazio region). The wood is the vestige of a very ancient forest made up of laurel and elm trees. Although the short distance from the sea, about 60 meters, conditions both the growth and the shape of the wood itself, an accurate use of windbreaks (tamarix and phragmites) has restored a dignified wood setting (ISPRA. 2008).

Before the opening of the Reserve, this fragile ecosystem was a camp-site large enough to accommodate about 3,000 people; now it can be regarded as one of the first areas where a natural environment has been successfully restored. Looking inland, the backs to the sea, the last 4 million geological years unfolds before us. In front of us a siliceous mine divides the Tolfa Mts. with its sloping sides caused from volcanic eruptions (4 – 2 million years ago), from the Ceriti Hills (its last volcanic actions dates about 25,00 years ago) with its much more rugged peaks. At the end of the eruptions the Tolfa Mts. Circeo, Ponza and Soratte Mt. were islands in a sea that stretched inland up to the Apennine Mts (Cauli F. and Ceccarelli W. 1997).

1.2.1. The environment

In the time, the land at the foot of the Ceriti Hills has been cultivated, but the natural tendency to form swampy areas on the coastal plains has helped to restore the natural environment and consequently its natural vegetation. The damp areas became bigger, making easier the growth of bird populations. The careful use of the fresh and salt water systems and the control of water level in the different periods of the year, favoured the settling of different groups of animals (mainly birds), now able to complete their reproductive cycle in the area. In fact, the Macchiatonda Natural Reserve is well known to birdwatchers, as during the year many different species can be found. Some of the wintering birds that one may observe are wild geese, bitterns, wigeons, shovelers and garganeys. Mallards, coots, moor hens and little bitterns can be seen the whole year round and also breed here; while, among the birds that may be sighted but that do not breed in the area, corsican seagulls,

herons, little egrets, bitterns and little grebes can be cited. There are also green sandpipers, sandpipers, oystercatchers, turnstones, black-winged stilts and curlews..Marsh harriers, the short eared owls and several kinds of hawks and buzzards come from the Tolfa Mts. to look for prey and are often seen in the reserve area. (Cauli F.and Ceccarelli W. 1997; Riserva Naturale di Macchiatonda 2009)

1.2.2. The origin and history

Several plants shown at the Reserve help us to follow the natural history of the landscape starting from the last glacial period (10,000 years ago). It was a coastal plain of about 3 km, further out to sea where there are reefs that outline what once was a salt-water basin spanning from the Flavia Tower to a small wood made up of elm and laurel trees that was once the starting edge of a wide forest once covering the plains and the hills of the Agro Romano and of the Latium Maremma . In the reserve plain, the water was too salty to allow the trees to grow and only gramineae (reeds) and halophyte (salt-water) plants were found. In this environment. Neolithic human populations were able to develop new hunting and fishing techniques, and to begin archaic forms of stationary farming and sheep rearing (www.riservamacchiatonda.org). The first known settling is the Pirgy one dated in the Bronze Age. This settling was the strating point for the urbanization of the aerea. Later the hydraulic engineering work made by the Etruscans widened the urbanization process, modifying the whole territory comprised within Caere, Pyrgi and Alsium, (Cervetri, Santa Severa, Palo Laziale). During the roman Republican phase, indeed, the area became a sort of holiday resort for the rich roman families. In the Imperial phase, following a new agricultural reform, it became a huge farming estate, as it was discovered that the soil was suitable for the growing of crops, especially wheat. Part of this area, indeed, is still used for farming. With the decline of the Roman Empire, and the beginning of the Saracen invasions, the coast was abandoned and nature once again took over. Meanwhile, Pyrgi came back to life: about the the year 1000 there was a a Norman defence tower, and a castle dedicated to the Christian martyr Santa Severa was later built on the on the top of the roman

walls at the sea side. Today, the castle still dominates this part of the coast. Of course, an agricultural center developed around the castle and, due to the natural tendence of the Macchiatonda area to become swampy, natural methods of extensive farming and breeding were automatically used. Today these methods of organic farming are still used and with time they have favoured a variety of ecological environments (Bandinelli A.C. 2002).

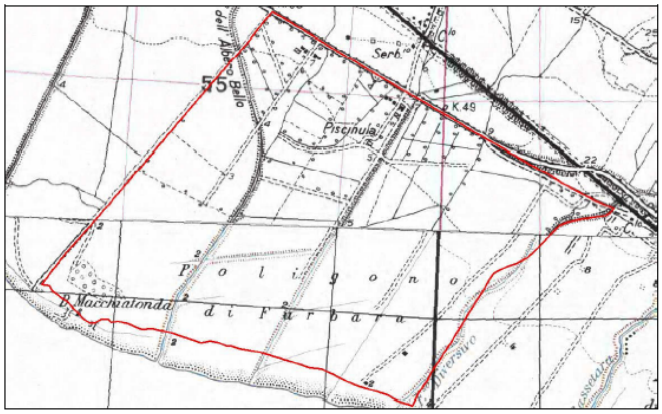

Figura 1.3 Cartography and aerial photograph of Macchiatonda Site of Community Importance (ISPRA 2008).

1.3 Bacterioplankton

The aquatic microbial communities play a very important role in ecosystem functioning. In fact, until few years ago, it was believed that matter and energy transfer in ocean happened through a simple food web made of algae, small crustaceans and large predators. Recently, this scheme has been revised since it was shown that organisms between 0.2 and 20 μM in size, represent the basis of the food web. Within this microbial community, the heterotrophic bacteria carry out pivotal functions (Ducklow H.W. 2010), such as regeneration of inorganic nutrients and secondary production of carbon. In fact, they use the dissolved organic matter (DOM), produced by autotrophic organisms and/or the metabolic activity of other heterotrophic organisms present in the environment, for their development. The biodegradable organic matter is mostly present in the form of macromolecules, which cannot be directly uptaken by the microbial cell if not splitted by exo-enzymes (that turn them into monomers). The degradation of dissolved organic matter (DOM) and particulate (POM) is carried out by groups of microorganisms (bacteria, yeasts and fungi) belonging to different taxonomic groups and species. The recovery of the dissolved organic matter by bacterial constitutes the microbial loop, which includes heterotrophic bacteria, nanoflagellates and ciliates (Munn C.B. 2004).

Although scientists are still constructing a picture of marine bacterial ecology, what is termed bacterioplankton consists of two of the fundamental domains of life: the Bacteria and the Archaea.

The microorganisms constituting the Domain of Archaea, are ultrastructurally, physiologically and genetically different from Bacteria. Marine Archaea are common in seawater but can be found also in extreme environments, including anaerobic sites, hot springs, and salt lakes. Since most of these organisms are uncultured and only known from their 16S sequence, , archeoplankton physiology

and role are almost entirely unknown. Archea account for about the 15% of the microbial plankton in the surface waters of the oceans, and increase at greater depths (Ducklow H.W. 2010),.

Bacterioplankton contains not only culturable microorganisms but also a large number of uncultured, sometimes previously unknown groups. In estuaries and other shallow near-shore habitats, Bacterioplankton is not only controlled by planktonic bacteriovores, as in ocean systems: in these productive habitats, indeed, the bacterial cells, formi biofilms on different substrate particles, so becoming vulnerable to larger predators and to a wider range of grazers and benthic suspension feeders, that find a good source of carbon, nitrogen, phosphorus, protein, nucleic acids, and iron in bacterial cells. The main function of bacteria in the microbial loop is to recover lost DOM, enrich it with macro- and micronutrients, and make it available for regeneration and resupply to primary producers. In wetlands, where bacterial abundance is greater, more of the bacterial stock is also attacked and lysed by viruses and bacterial predators as those belonging to *Bdellovibrio* genus (Ogunseitan O. 2005). This cause the release of nutrients that are immediately available to bacteria instead of entering the food web. However, the actual role of viruses and bacteriovores in the food web is still poorly understood (Fuhrman J.A. 1999, Munn C.B. 2004). Other interesting problems, such as the effect of size-selective predation, bacterial community structure, and species succession, are just beginning to be explored. The study of the marine bacterial communities by molecular probes and numerical models will lead to a new revolution. Studing and monitoring the microbial components, we will better understand the influence of environmental abiotic factors on the dynamics of the bacterial community, this will help to develop environmental indicators and strategies to preserve the aquatic environments (McArthur J.V. 2001, Merkley M.B. et al. 2004).

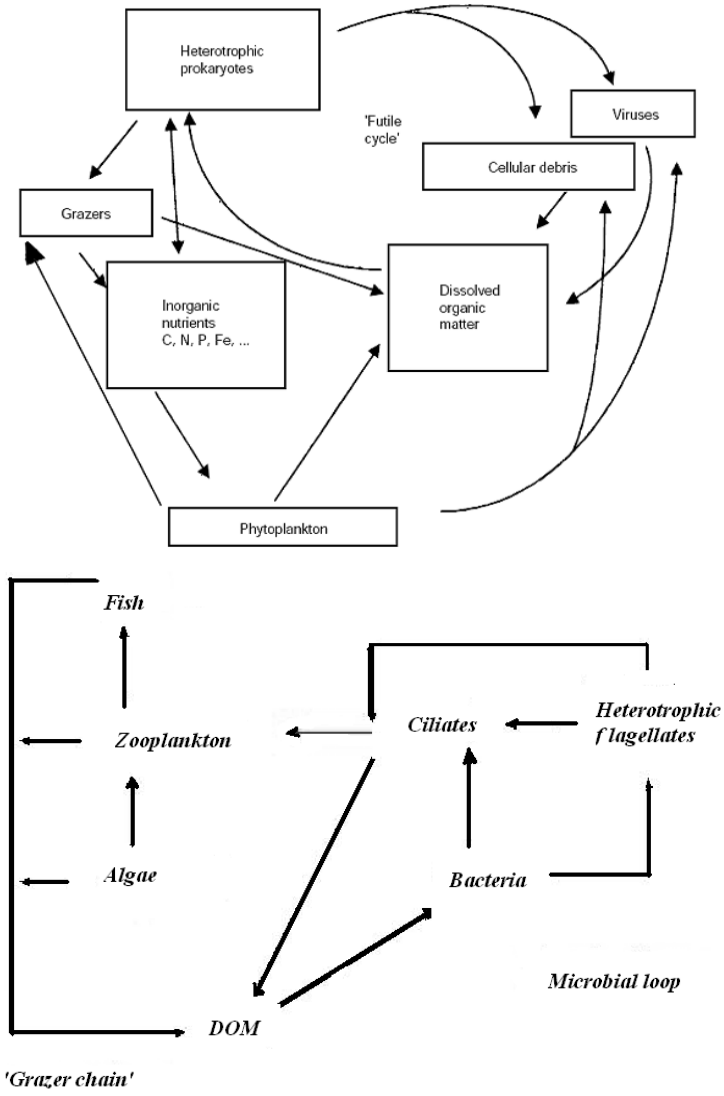

Figura 1.4 Viral shunt and microbial loop arrows represent transfer of matter (modified from Fuhrman J. and Hewson I. 2009).

Aim

Wetlands are areas of great environmental importance, as recognized by the Ramsar Convention (1971) that recommends “conservation and wise use of wetlands”. The wetland in the Macchiatonda Natural Reserve (Santa Severa, Rome, Italy) is regarded as the relic of an ecosystem once present in all Tyrrhenian coast. Coastal ponds at different salinities characterize it, and both peculiar vegetation and highly diversified migratory and sedentary avifauna can be found. To date no information on the microbial community in this or similar areas are available.

In this study a yearlong seasonal sampling has been performed in three coastal ponds: two exposed to a direct marine ingression and a third where there is no direct seawater input, but fresh water is timely introduced, as needed, to maintain a constant water level.

The aim of this work is to evaluate the diversity of the microbial community using Single Strand Conformation Polymorphism (SSCP), and to quantify the cultivable fraction of this community on both salty and no-salty ZoBell media. These results will be compared with those on salinity, temperature and pH values measured in the ponds, to relate the seasonal variation of abiotic factors to Shannon Index on the SSCP fingerprints and bacterial composition in the different ponds. This will help to understand the natural process in wetlands and will constitute the baseline to (i) identify bacterial assemblages or particular species to be used as a bioindicator and (ii) properly drive human interventions of conservation or restoration.

2. Material and methods

2.1 Sampling area

The Macchiatonda Nature Reserve is a 250 hectares-wide relict area of the drainage in the tirrenic coast, located 50 km North of Rome, within the Santa Marinella municipality. It encloses a wetland area spanning for about 1 km along the coast, consisting of three separated coastal ponds. Two ponds, *Alberobello* and *Piscinula*, undergo a direct marine ingression, whilst in the third, *Didattico* (the italian word for educational), there is not direct seawater input but freshwater filling ensures the water level, when necessary. Samples were taken from 5 stations, in June, September, December 2009 and March 2011. The sampling stations (St. 1 Didattico pond, St. 2 - St. 3 Alberobello pond and St. 4 - St. 5 Piscinula pond) were localized *in situ* by GPS coordinates and are shown in Figure 1.

Didattico is a circular pond, containing a small circular island. Thus, the water constitutes a circular ring of the same depth (about 1.5 mt if completely filled of water). Due to its homogeneous structure a single sampling station was identified (station 1).

Alberobello pond is composed by a net of channels (station 2, max depth about 60 cm), carrying water to a major water hole (station 3, max depth about 90 cm). *Piscinula* pond is a continue wetland with little islands inside, with a less deep portion (station 4, max depth 60 cm) and a deeper area (station 5, max depth 1 mt) at the SouthWestern edge of the Reserve.

PONDS	STATION	ZONE	EAST	NORTH
Didattico	St.1	32T	747143	4654014
Alberobello	St.2	32T	747302	4653953
Alberobello	St.3	32T	747661	4653870
Piscinula	St.4	32T	747889	4653900
Piscinula	St.5	32T	748049	4653815

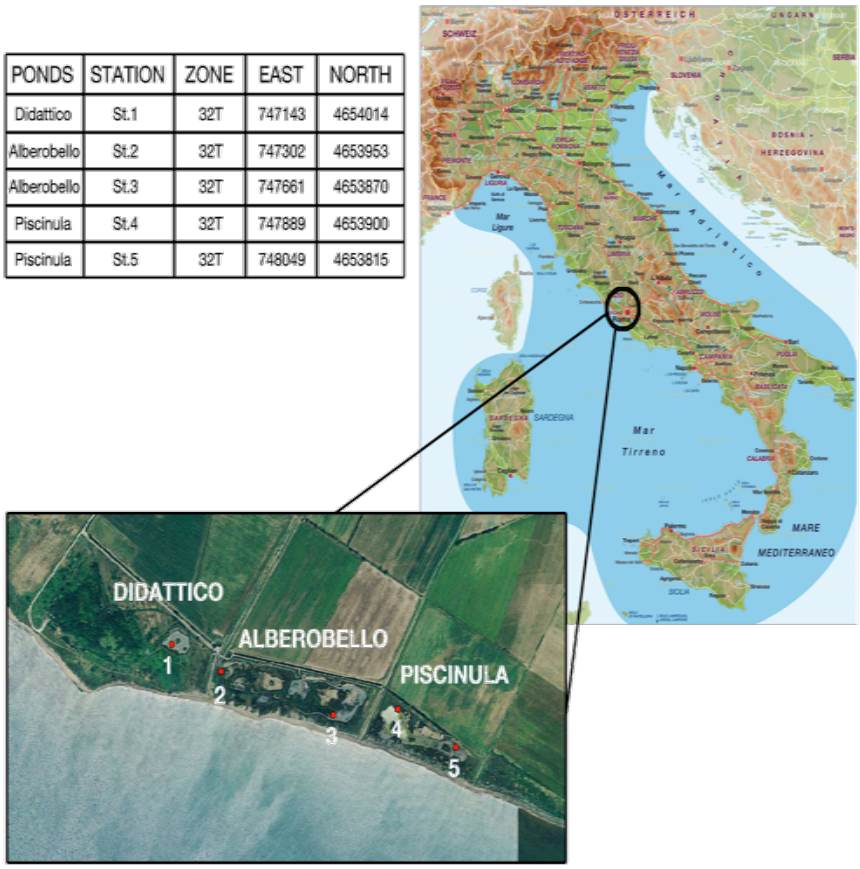

Figura 2.1 Ponds and sampling stations in the Macchiatonda wetland, their geographical localization and U.T.M. coordinates.

2.2 Sampling and field measures

In each sampling campaign and in each station, salinity, temperature and pH were measured, by utilising the multiparametric probe Multi 340i (WTW, Udine, Italy). Soon after the measure, from each sampling site, five samples of water (1 lt) were collected and brought to the laboratory. There, the water samples from each site were pooled and one litre stored at 4 °C under dark until processed for microbiological analyses (less than 6 hours).

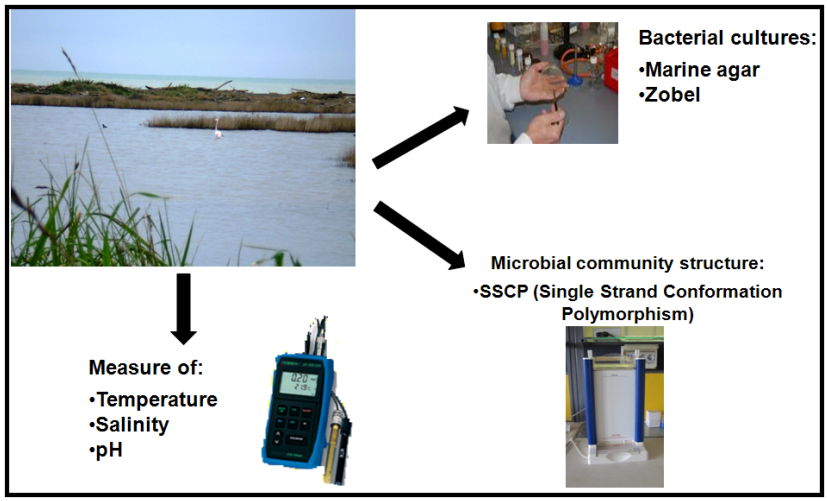

Figura 2.2. Scheme of sampling strategy.

2.3 Microbes cultivation

From each water sample, both 100 and 10 µl of water were plated on ZoBell Agar (5g/l peptone, 1 g/l yeast extract, 15 g/l agar) in order to count the limnotolerant Colony Forming Units, CFU/ml (Buller N.B. 2004, Migliore L.et al. 2006, Divya B. et al. 2009). The same procedure was applied, but plating on ZoBell medium with salts (MA, Marine Agar, Difco) to detect and count the halotolerant CFU/ml. The Colony Forming Units were counted after 48 h of incubation at *in situ* temperature (25 ± 2 °C). From each plate, all the colonies with different morphologies were picked up and isolated (to have an axenic culture), and then stored into Weaton vials

(4 ml) containing CTA to be further processed. In these conditions microorganisms are kept viable for months; for a long term preservation microorganisms are stored in BHI broth at – 80 °C.

2.4 Bacterial and Archea community DNA extraction

The DNA extraction protocol was derived from Rossolini G.M.et al. (1993) and modified according to Zhou J.et al. (1996). Each water sample was centrifuged (10.000g, 20 min), the pellet added with 1 ml of extraction buffer Solution 1 (50 mM Tris-HCl pH 8, 20% sucrose, 50 mM EDTA, 10 mg/ml lysozyme) and kept 30 min a 37 °C. Then 4 ml of extraction buffer Solution 2 (50 mM NaCl, 1% CTAB, 35 µl of 10 mg/ml proteinase K solution) were added and samples shaken by inversion for 30 min at 37 °C. After shaking, 0.5 ml of 20% Sarkosyl were added, and the samples incubated at 65 °C (2 h) under gentle inversion. After centrifugation at 6000 *g*(10 min, room temperature), the supernatants were collected and mixed with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) preheated to 60 °C. The aqueous phase was recovered by centrifugation (4000 *g*, 4 °C) and precipitated with sodium acetate 3 M pH 5.2 (1/10 volume) and ethanol (2 volume). The pellet obtained after centrifugation at 15,000 *g* was rinsed in 70% ethanol and stored in TE buffer at -20 °C until PCR amplification (Bostrom K.H. 2004, Tsai and Olson, B.H. 1992).

2.5 PCR amplification of 16S rRNA

PCR amplification targeting bacterial 16S rRNA genes was performed with forward primer Com1 (5'-CAGCAGCCGCGGTAATAC-3' positions 519 to 536) and reverse primer Com2Ph (5'-CCGTCAATTCCTTTGAG TTT-3' positions 907 to 926). Reverse primer was phosphorilated at 5' end. Both primers hybridize to phylogenetically conserved regions within the 16S rRNA genes and amplify a 407 bp fragment encompassing the two phylogenetically highly variable regions, V4 and V5 (Schwieger and Tebbe 1998;). Amplification reactions were carried out in 100

µl volume with 100 pmol of each primer in EmeraldAmp GT PCFR Master Mix (TaKaRa Shiga, Japan) and about 10 ng of total crude DNA. The following cycling conditions were used: 3 min at 94 °C, 30 cycles (1 cycle consists of 1 min at 94 °C, 1 min at 50 °C, and 70 s at 72 °C), and 5 min at 72 °C. PCR products were purified with E.Z.N.A. Cycle-Pure Kit (OMEGA bio-tek, U.S.A.).

PCR amplification targeting archea 16S rRNA gene was performed with forward primer ARC344F (5'-ACGGGGYGCAGCAGGCGCGA-3' positions 344 to 363) and reverse primer ARC915R (5'-GTGCTCCCCCGCCAATTCCT-3' positions 915 to 934; Casamayor et al., 2000). Reverse primer was phosphorilated at 5' end. Amplification reactions were carried out in 100 µl volume with 100 pmol of each primer in EmeraldAmp GT PCR Master Mix (TaKaRa Shiga, Japan) and about 10 ng of total crude DNA. The following cycling conditions were used: 5 min at 94° C, 30 cycles (1cycle consists of 0.15 min at 94°C, 0.30 min 65°C ,1 min 72 °C) and 7 min at 72 °C.

The phosphorylated strands of both bacterial and archea amplicons were removed by λ-exonuclease digest (Fermentas Vilnius, Lithuania). 30 µl of each sample were transferred into a microreaction tube and 10 µl of the master mix solution added (4 µl λ-exonuclease buffer, 0.5 µl λ-exonuclease 5U/ µl, 5 µl H2O) the tube was mixed and incubate at 37 °C for 45 minutes. The digestion was stopped according to the manufacturer's instructions. Single-stranded DNA was purified with E.Z.N.A. MicroElute Cycle-Pure Kit (OMEGA bio-tek, U.S.A.) and resuspended in 10 µl of TE buffer.

2.6 *SSCP,* principle of the method

Single strand DNA molecules fold under non denaturing condition according to the base sequence within the molecule, as complementary bases A with T and C with G will tend to form hydrogen bonds. Each conformation run differently within the gel matrix and can be separated by electrophoresis in non-denaturing polyacrilamide gels. The result is that SSCP electrophoretic run is able to separate DNA molecules having the same length but different nucleotide sequence.

SSCP was first used for diagnostic purposes, to detect mutation or allelic variation of a single gene in eukaryotic organisms. Later this method was applied without modification to characterize a simple microbial community (Dhormann A. B. and Tebbe C.C, 2010). The method cannot not be applied to complex microbial communities, because of the rehybridization of complementary single strand DNA and the formation of heteroduplexes during the electrophoretic separation. Some authors (Godon J.J. et al. 2001) tried some modifications (as a fluorescent-labeled reverse primer) to reduce the complexity of the signals amplified from a metagenome, but this approach did not eliminate the heteroduplex presence but simply avoided their detection. However, this method allowed the generation of a genetic profile for a microbial community using an automated DNA sequencer for electrophoresis. The disvantage is that a clone library from at least one sample has to be generated in parallel to the identification of the community peaks. This strategy gave problems for the identification of the smallest peaks, since this are produced by less abundant organisms which may not be easily found in a clone library.

To eliminate the presence of heteroduplex, and to reduce the number of signals generated from each single strain belonging to a microbial community, a strand removal approach was introduced by Schwieger F.and Tebbe C.C. (1998). After a PCR amplification with a regular forward primer and a phosforilated reverse primer, the reverse strand can be removed by lambda exonuclease digestion. Thus, only forward strands are separated by electrophoresies in non-denaturing polyacrylamide gels.

The SSCP gel is silver-stained, although other staining procedures can be utilized. Several bands compose the SSCP profile, each belonging to one microbial taxon. The bands are cut out of the polyacrylamide gel to identify the taxon by DNA sequence analysis. Furthermore, different SSCP profiles can be compared by digital image analysis. Silver stained SSCP profiles can be transformed into a series of densitometric values, each corresponding to a band/taxon, by using appropriate softwares (Phoretix or Gel Compare). These softwares, after the background subtraction procedure, allow to compare the different SSCP profiles and to generate

the dendogram representing the linkage level of the different profiles by the clustering method. The matrix containing the density value of each band can also be used to determine diversity indices.

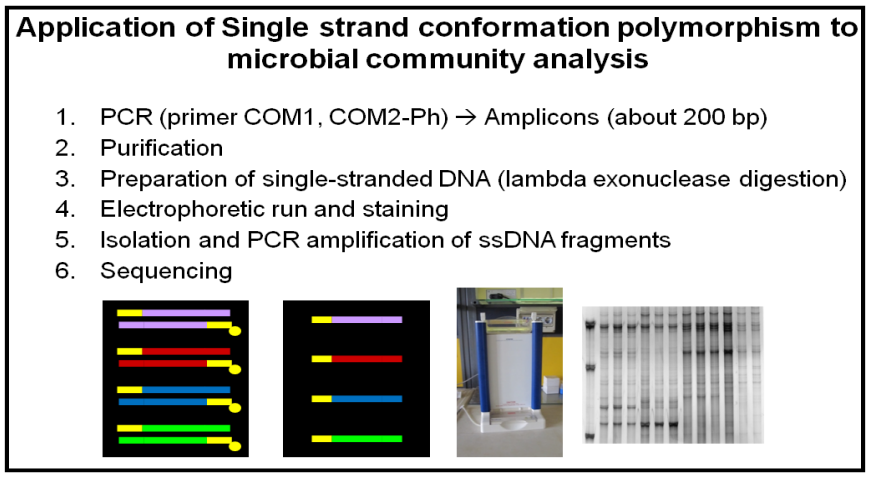

Figura 2.3. Simplified scheme of SSCP protocol.

2.7 SSCP analysis

The SSCP analysis modified by Schwieger F.and Tebbe C.C. (1998) has been utilized for this study. The gel (16 cm in length, 0.4 mm in thickness) was casted by spreading on the notched glass 1 ml of bind silan and on the other glass 1 ml of repel silan.

5 µl of single-stranded DNAs were mixed with an equal volume of denaturing loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol) before the electrophoretic run. Samples were incubated at 95 °C for 2 min and immediately cooled on ice. Then samples were run in a 0.625x MDE gel (Lonza, Switzerland) with 1 X TBE buffer (14.7 ml of water, 2.5 ml 10X TBE, 7,8 ml 2X MDE, 10 µl TEMED and 25 µl 40% APS) at 250 V, 8 mA for 17 h at 25°C in an adjustable slab gel kit (C.B.S. Scientific Co., USA). The gels were then fixed

in 10% acetic acid for 30 minutes, and silver stained according to the procedure of Bassam et al. (1994).

The bands from each lane were excised with a scalpel, transferred in microtubes containing 50 µl of elution buffer and incubated at 37 °C for 3h. The DNA was precipitate withethanol, centrifuged and resuspended in 12 µl of TE buffer. For each sample, 2 µl of DNA solution were reamplified under the same conditions described above and the PCR product was standard sequenced by Automated capillary sequencing (Macrogen Europe, The Nederland).

To identify the closest taxon each sequence is compared with those available in the Ribosomal Database Project (RDP) (Maidak B.L.et al. 1997) and subjected to BLAST analysis at the National Center for Biotechnology Information database (Altschul S.F. et al. 1990).

2.8 Statistical analysis

2.8.1 Cluster analysis

Similarity scores between the different PCR-SCCP fingerprinting from the samples were performed using the Jaccard correlation coefficient. The cluster analysis and dendrogram generation were carried out by using the Phoretix 1D (Phoretix International, United Kingdom).

2.8.2 Non metric-MDS

The different fingerprintings were also analyzed by nonmetric multidimensional scaling (n-MDS), using PAST software version 1.34 (Hammer Ø. et al., 2001). The software also calculate a score of the goodness of fit for data points, named stress, that, if < 0.2 give an acceptable representation of the relationships between samples.

2.8.3 NPMANOVA

Nonparametric multivariate ANOVA (NPMANOVA) was used to determine differences between SSCP banding profiles grups (Ramette A. 2007), using PAST software version 1.34.

2.8.4 *Shannon-Wiever diversity index*

The Phoretix 1D Pro software evaluate the density profile, by determining the relative intensity of the bands, and calculate the relative contribution of each band to the total band intensity in each lane. The rolling disk background value is subtracted. Based on band density profiles the Shannon-Weaver Diversity Index (*H*') for each line is calculated.

3. Results

3.1 Abiotic factors

In the three ponds, temperature and salinity show clear seasonal variations (Fig. 3.1). In all ponds temperature showed the lowest values in December (between 2.8 and 4.9 °C) and the highest in June/September (between 22.8 and 31.4 °C). In the *Didattico* pond salinity remains in a range of low values, varying from 6.5 ppm in winter to 22.0 ppm in summer, while in the *Alberobello* and *Piscinula* ponds, salinity showed wide variations and reach high values: the lowest values are found in March (13.9 - 8.8 ppm, respectively) and the highest in September (46.0 - 54.7 ppm, respectively).

pH values remain in a narrow range all the year round (Fig. 3.1), values ranging between 7.8 and 8.9 in the *Alberobello* and *Piscinula* ponds, with the sole exception of *Alberobello* station 2 where the pH value fall to 6.1 in September. This station, due to the sloping gradient in the area, remains almost completely dry from July until the autumn rains, abundant in November/January (Tab. 3.1). In the *Didattico* pond, pH ranged from 8.2 to 9.0.

	•2009									•2010		
•Month	•A	•M	•J	•J	•A	•S	•O	•N	•D	•J	•F	•M
•mm rain	•40.2	•31.0	•38.2	•0.0	•0.4	•48.0	•46.2	•85.4	•116.8	•69.6	•58.0	•40.2
•Rainy days	•6	•5	•3	•---	•---	•6	•5	•7	•12	•11	•10	•5
•Air temperature	•16.2	•20.8	•22.6	•25.6	•26.9	•24.1	•18.1	•15.5	•11.2	•9.5	•10.5	•12.1

Table 3.1. Montly rainfall, rainy days and mean temperature from April 2009 to March 2010. Data from Regione Lazio, "Ufficio Idrografico e Mareografico" http://www.idrografico.roma.it [data accessed Decembre 30th, 2011].

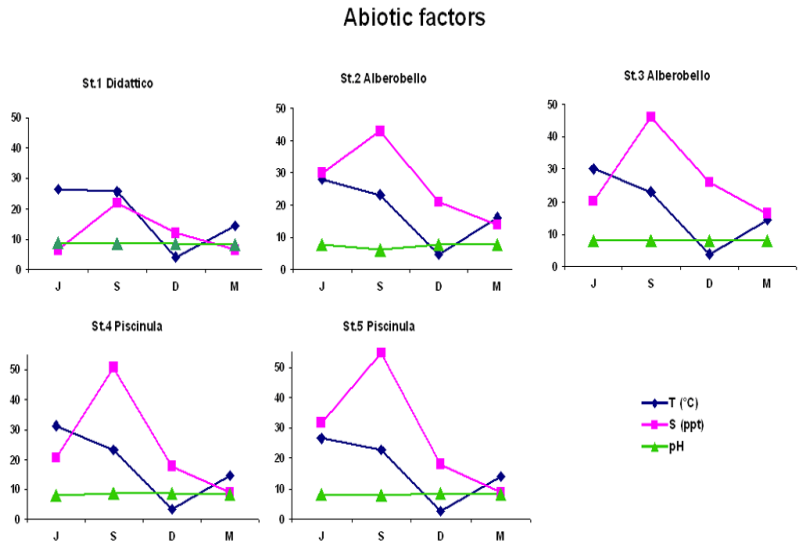

Figure 3.2 Temperature, salinity and pH values measured in the five sampling stations from July 2009 to March 2010 (J=July, S=September, D=December and M=March).

3.2 Bacterial abundance

Counting of Colony Forming Unit (CFU/ml) from ZoBell (limnotolerant bacterial component) and salty added ZoBell (MA, Marine Agar; halotolerant bacterial component) showed differences among stations and season (Tab. 3.2). As a total, the highest CFU number was found in March, the lowest in December, everywhere. Halotolerant microbes were more abundant from June to December in all the stations. Limnotolerant microbes are more abundant in March in all stations (always higher than $1x10^6$ CFU/ml). The *Alberobello* pond counting (September sampling) is not included in the table because microbes were really scarce due to the dryness during the summer season.

	CFU/ml							
	June		September		December		March	
	MA	Zb	MA	Zb	MA	Zb	MA	Zb
St1 Didattico	9.0*10³	5.1*10³	4.0*10⁴	3.0*10³	3.3*10³	7.0*10²	1.95*10⁴	>10⁶
St2 Alberobello	6.4*10³	1.6*10³	ND	ND	2.7*10³	2.0*10³	2.1*10⁴	>10⁶
St3 Alberobello	6.5*10³	3.0*10²	4.0*10³	3.0*10²	1.0*10³	3.0*10²	2.1*10⁴	>10⁶
St4 Piscinula	9.1*10³	9.0*10²	5.9*10⁵	1.0*10²	1.2*10³	4.0*10²	3.2*10⁴	>10⁶
St5 Piscinula	4.8*10³	3.0*10²	1.0*10⁵	2.0*10²	1.8*10³	5.0*10²	2.2*10⁴	>10⁶

Table 3.2 Aerobic heterotrophic bacteria (as Colony Forming Unit, CFU) in the water column of the five sampling stations sampled from June 2009 to December 2010. Data refers to limnotolerant isolates, (grown on Zobel medium) and halotolerant isolates (grown on Marine Agar, MA).

3.3 Bacterial SSCP profiles

The SSCP bacterial profile of all the samples is shown in Fig. 3.2. Each lane represents the fingerprint of one sample (one station, one season) and shows the presence/absence of each taxon (band).

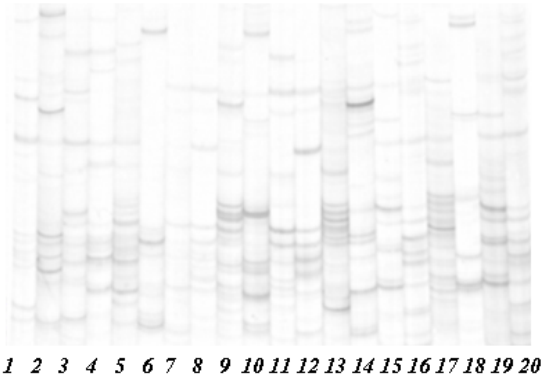

Figure 3.3 SSCP profiles of the bacterial communities. 1-4 Didattico St. 1 (June, September, December, March), 5-8 Alberobello St. 2 (June, September, December, March), 9-12 Alberobello St. 3 (June, September, December, March), 13-16 Piscinula St. 4 (June, September, December, March), 17-20 Piscinula St. 5 (June, September, December, March).

The image analysis (Phoretix 1D Pro software) evaluates the presence or the absence of each band and their intensity, on this base diversity in the fingerprints can be evaluated. The Shannon index was quantified for each lane. In the *Didattico* pond the highest diversity values were found in September (H' = 2.43) and December (H' = 2.24); lower values were found in June and March. In the *Alberobello* and *Piscinula* ponds the highest diversity values were found in June (H' = 2.48 - 2.54, respectively). Lower diversity values were found in December and September (Tab. 3.3).

	H' value			
	June	*September*	*December*	*March*
St.1 Didattico	*1.93*	*2.43*	*2.24*	*2.04*
St.2 Alberobello	*2.18*	*1.65*	*1.80*	*2.03*
St.3 Alberobello	*2.48*	*2.18*	*2.14*	*2.08*
St.4 Piscinula	*2.27*	*2.06*	*2.10*	*2.25*
St.5 Piscinula	*2.54*	*2.01*	*2.13*	*2.59*

Table 3.3 Aerobic, heterotrophic bacterial diversity. as Shannon index (*H'*) values, measured in different sampling stations and seasons.

Furthermore, the image analysis (Phoretix 1D Pro) demonstrated that *Alberobello* and *Piscinula* showed similar fingerprints (NPMANOVA $p > 0.05$), while *Didattico* fingerprints are significantly different *vs* both *Alberobello* and *Piscinula* fingerprints (NPMANOVA, $p < 0.05$). The overall patterns of the five stations were further analysed by cluster analysis and by n-MDS analysis, the results reported in Figs. 3.3 and 3.4. Although the *Alberobello* and *Piscinula* ponds cluster together according to the season, results are not clear-cut (stress value > 0.2) and *Didattico* pond is clearly separated by the other two ponds.

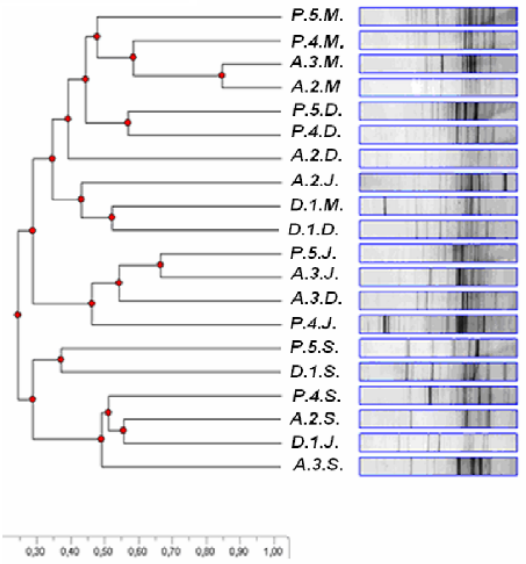

Figure 3.4 Cluster analysis of SSCP profiles from the three ponds. Each sample is identified by a 3 letters code: the 1st letter represents the site (D=*Didattico*, A=*Alberobello*, P=*Piscinula*), the 2nd the sampling station (1=St. 1, 2=St. 2, 3=St. 3, 4=St. 4, 5=St. 5) and the 3rd the season (J=June, S=September, D=December, M=March).

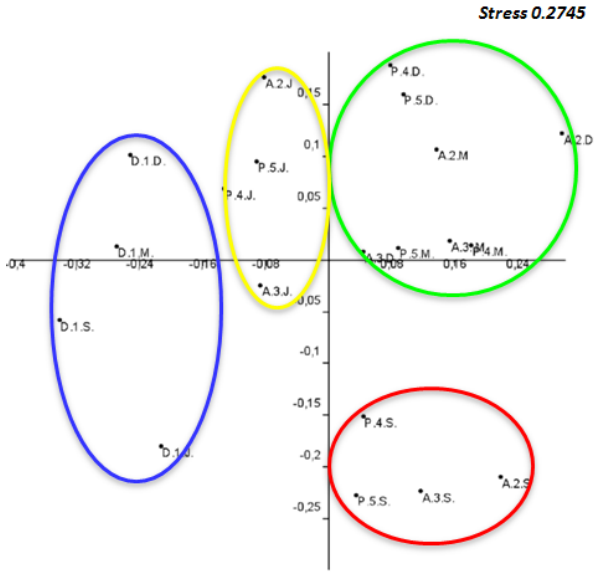

Figure 3.5 Non-metric multidimensional scaling of SSCP profiles from the three ponds. Each sample is identified by a 3 letters code as explained in Fig. 3.3. Grouping is highlighted by colour circles; Alberobello and Piscinula cluster togheter in the different seasons: June (yellow), September (red), March_December (green); Didattico cluster alone (blue).

Thus, the *Alberobello* and *Piscinula* fingerprints were processed without *Didattico* by cluster analysis and by n-MDS analysis the results reported in Figs. 3.5 and 3.6. The *Alberobello* and *Piscinula* ponds cluster in three well separated groups according to the season, one including the June samples, one the September ones and a last group including March and December samples. A robust clear-cut grouping is found (stress value = 0.178).

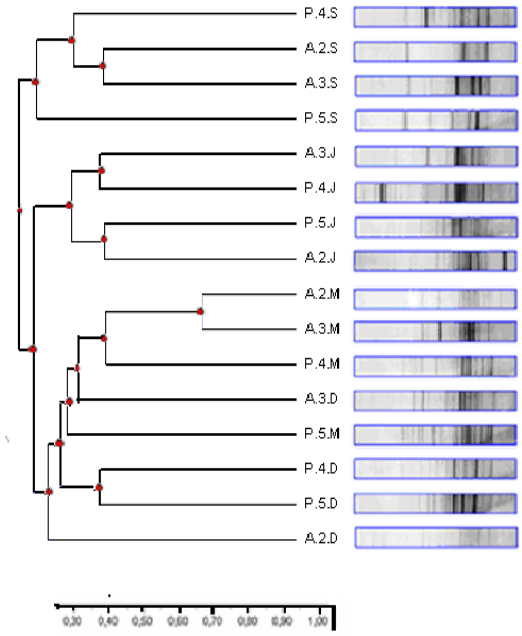

Figure 3.6 Cluster analysis of SSCP profiles from *Alberobello* and *Piscinula* ponds. Each sample is identified by a 3 letters code as explained in Fig. 3.3.

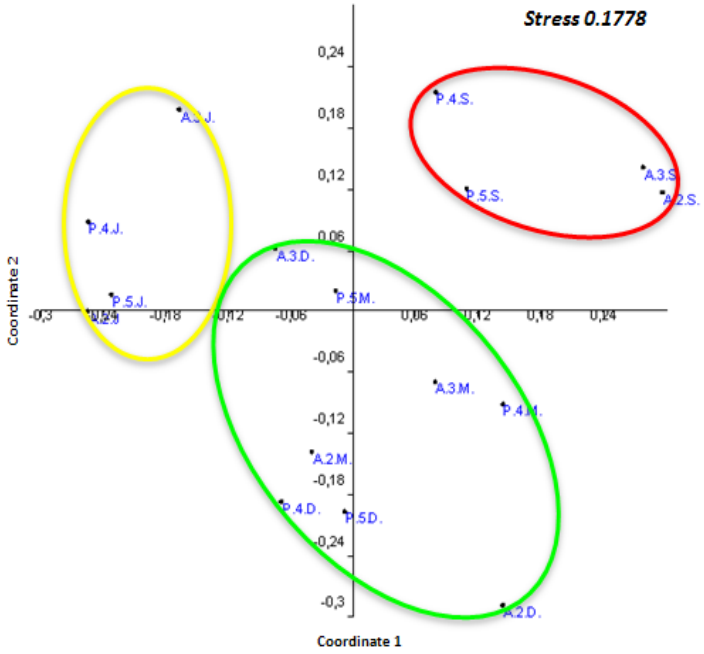

Figure 3.7 Non-metric multidimensional scaling of SSCP profiles from the *Alberobello* and *Piscinula* ponds. Each sample is identified by a 3 letters code as explained in Fig. 3.3. Grouping is highlighted by colour circles: June (yellow), September (red), March_December (green).

3.4 Archaea SSCP profiles

The SSCP Archea profile of all the samples is shown in Fig. 3.7. Each lane represents the fingerprint of one sample (one station, one season), and shows the presence/absence of each taxon (band).

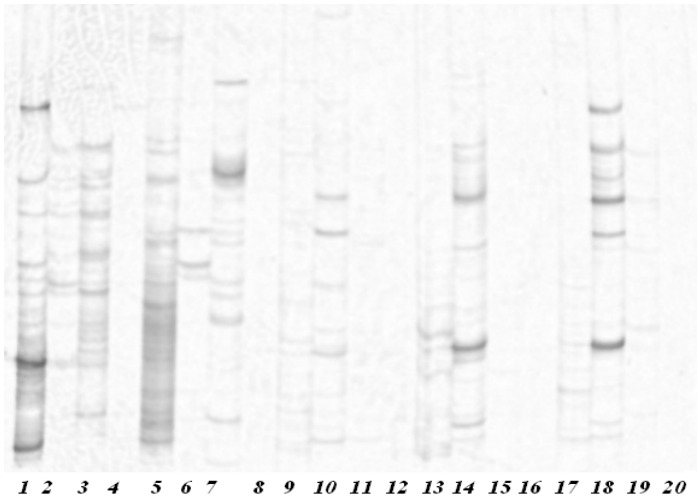

Figure 3.8 SSCP profiles of the Archea communities. 1-4 *Didattico* St. 1 (June, September, December, March), 5-8 *Alberobello* St. 2 (June, September, December, March), 9-12 *Alberobello* St. 3 (June, September, December, March), 13-16 *Piscinula* St. 4 (June, September, December, March), 17-20 *Piscinula* St. 5 (June, September, December, March).

Also in this case the image analysis (Phoretix 1D Pro software) was used to evaluate the presence or the absence of each band and their intensity, on this base, diversity in the fingerprints was evaluated. The Shannon index was quantified for each lane (Table 3.4).

In the *Didattico* pond the highest diversity values were found in June (H' = 2.17). In the *Alberobello* and *Piscinula* ponds the highest diversity values were found in September (St. 3, H' = 2.04 and St. 4, H' = 2.16, respectively). During March and December a very low diversity of archaea was found (except for St. 2 *Alberobello* and *Didattico* were in December diversity values were high). The *Alberobello* St. 2 showed the lowest long year diversity.

	H' value			
	June	*September*	*December*	*March*
St.1 Didattico	2.17	1.65	1.71	NP
St.2 Alberobello	1.56	0.45	1.47	0
St.3 Alberobello	2.00	2.04	NP	0
St.4 Piscinula	1.86	2.16	NP	0
St.5 Piscinula	1.54	1.76	0.40	NP

Table 3.4 Archea diversity as Shannon index (H') values, measured in different sampling stations and Seasons. (NP = not present 0= one band).

The image analysis (Phoretix 1D Pro) demonstrated that *Didattico* fingerprints are not significantly different *vs* both *Alberobello* and *Piscinula* fingerprints (NPMANOVA, $p < 0.05$), although differences among seasons were found ($P<0.05$). The patterns of the five stations, all seasons, were analysed by cluster analysis; the results are reported in Fig. 3.7.

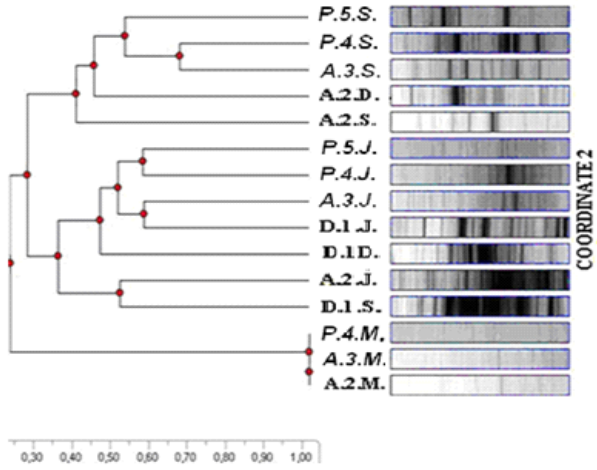

Figure 3.9 Archea cluster analysis of SSCP profiles from the three ponds, in the different sampling stations and seasons. Each sample is identified by a 3 letters code: the 1st letter represents the site (D=*Didattico*, A=*Alberobello*, P=*Piscinula*), the 2nd the sampling station (1=St. 1, 2=St. 2, 3=St. 3, 4=St. 4, 5=St. 5) and the 3rd the season (J=June, S=September, D=December, M=March).

The clustering of the five stations in all seasons highlighted the separation of the samples in which only one taxon was found (P.4.M, A.3.M, A.2.M). To clarify the possible differences among the other samples, December and March data were escluded. The SSCP profiles from the three ponds in June and September were analysed by cluster analysis and by n-MDS analysis; the results are reported in Figs. 3.8 and 3.9.

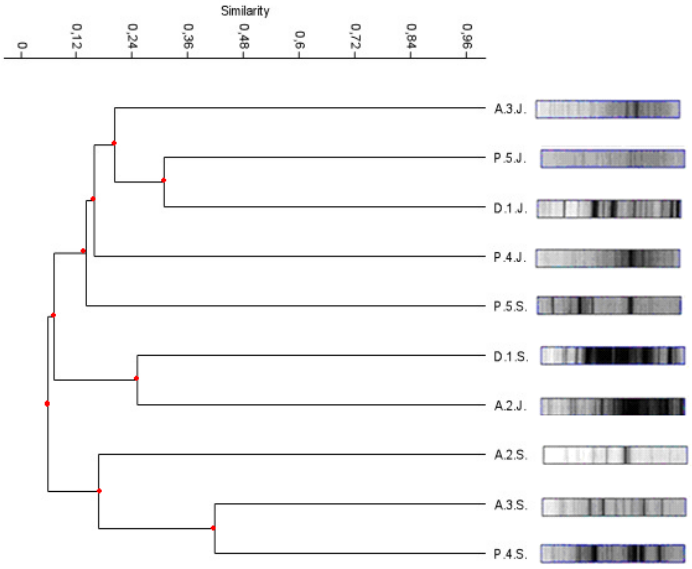

Figure 3.10 Archea cluster analysis of SSCP profiles from the three ponds in June and September. Each sample is identified by a 3 letters code: the 1st letter represents the site (D=*Didattico*, A=*Alberobello*, P=*Piscinula*), the 2nd the sampling station (1=St. 1, 2=St. 2, 3=St. 3, 4=St. 4, 5=St. 5) and the 3rd the season (J=June, S=September).

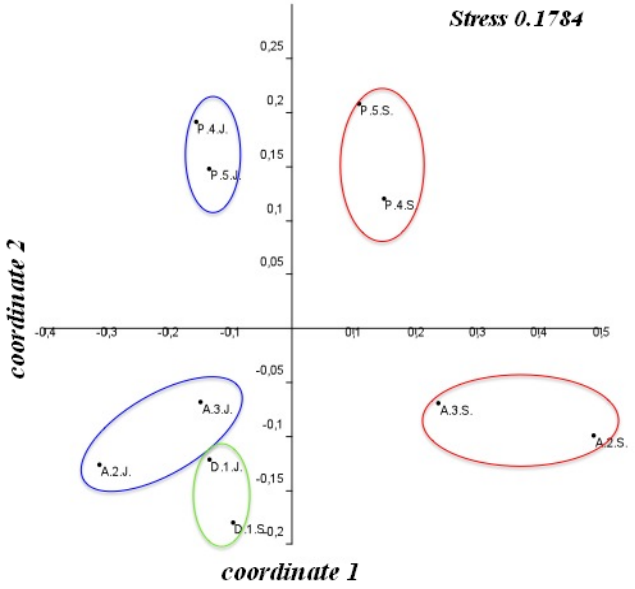

Figure 3.11 Non-metric multidimensional scaling of SSCP profiles from the three ponds in June and September. Each sample is identified by a 3 letters code as explained in Fig. 3.8. Colour circles highlight grouping: June (blue), September (red); *Didattico* June and September (green).

Cluster analysis and n-MDS showed the robust seasonality (stress 0.1784) of the Archea in the *Alberobello* and *Piscinula* ponds, although *Didattico* seemed to have lower seasonal variation.

3.5 Sequence analysis of selected bacterial and archeal SSCP bands

The more intense bands in the bacterial and archea SSCP gels were re-amplified and sequenced to get the identification of the prevalent microbial taxa. The bacterial identification reached the order level. The identified orders are listed in Table 3.5.

In the *Didattico* pond the more represented orders within the bacterial community were three, the order Burkholderiales being present in all the examined samples. In the Archea community the prevalent populations were found to be unknown taxa

belonging to the Euryarchaeota phylum. In September the class of Methanomicrobia was found.

In the *Alberobello* and *Piscinula* ponds the prevalent bacterial populations belonged to three Phyla: the Proteobacteria, the Actinobacteria and the Bacteroidetes/Chlorobi group. The mainly observed orders were six: Rhodobacterales (α-Proteobacteria) were found in all seasons;. Micrococcineae (Actinobacteria-suborder of Actinomycetales) were evident in March, September and December samples, while Alteromonadales (γ-Proteobacteria), Bacteroidales, and Sphingobacteriales (both belonging to the Bacteroidetes/Chlorobi group) were only found in June. Although not one of the prevalent bands, a well represented band was seen only in September and was identified as Bdellovibrionales (δ-Proteobacteria). In these ponds the Archeal community could be observed in the samples taken in the March - September period (other than *Piscinula* St. 5) and the prevalent components were unknown taxa of Euryarchaeota. In September the Halobacteria and Methanobacteria classes were found.

	June	September	December	March
Didattico	***Bacteria*** Burkholderiales Micrococcineae	Burkholderiales	Burkholderiales Micrococcineae	Burkholderiales Flavobacteriales
	Archea Euryarchaeota§	Euryarchaeota§ Methanomicrobia		Euryarchaeota§
Alberobello ***Piscinula***	***Bacteria*** Alteromonadales Bacteroidales Rhodobacterales Sphingobacteriales	 Micrococcineae Rhodobacterales	 Micrococcineae Rhodobacterales	 Micrococcineae Rhodobacterales
	Archea Euryarchaeota§	Euryarchaeota§ Halobacteria Methanobacteria	Euryarchaeota§	Euryarchaeota§

Table 3.5. List of prevalent bacterial/archeal orders found in the different sampling sites. § = Unknown taxa

4. Discussion

In this study the microbial dynamics in the Macchiatonda Natural Reserve wetlands (Rome, Italy) were analysed from June 2009 to March 2010. In particular, three ponds, named *Didattico*, *Alberobello* and *Piscinula*, were studied. The *Didattico* pond receives freshwater input, timely introduced, as needed, to maintain the water level; the other two ponds, *Alberobello* and *Piscinula*, have direct marine ingression. In the last two ponds the salinity depends on the evaporation rates, occurring in different ways and periods in each basin and, obviously, on the rainfall. This yearlong study of the bacterial community was performed by both cultural and molecular methods; temperature, salinity and pH were measured at every sampling time in each sampling site,.

The analysis of the microbial cultivable fraction seemed to indicate a similar structure of the microbial community in the three ponds. Indeed, The seasonal variation of the bacterial abundance is comparable among the ponds; the halotolerant component is dominant everywhere and all the year long, other than in Spring, and both salinity and temperature are pivotal in shaping the bacterial assemblages. In particular, high salinity values favour the growth of the halotolerant strains *vs* the limnotolerant ones, while low temperature values reduce the abundance of both the halotolerant and the limnotolerant component.

When analysed by molecular analyses, the bacterial and archaea assemblages found in the three ponds showed clear differences between ponds and seasons.

Once digitally analysed, the bacterial community SSCP profiles - obtained for each sampling station and season - showed different diversity indices (H'): the bacterial composition in the two more salty ponds had analogous trends in SSCP profiles, Shannon Index, and 16S composition, while the entire set of results was different for the *Didattico* one. In fact, the *Alberobello* and *Piscinula* ponds showed similar profiles, samples clustered in 3 different group, (Figs 3.6 3.7) one represented by the

June samples, characterized by high temperature and salinity, one by the September samples, characterized by very high salinity and low water levels, and a last group including March-December, characterized by lower temperature and salinity, clustering together due to a low interseasonal variation.

In this case, in fact, salinity is the driving force of the microbial community, as described also in several other environments (Lozupone C.A. and Knight R. 2007; Jackson C.R. and Vallaire S.C. 2009;. Wu Q. L et al., 2006, Crump B.C. et al., 2004). This abiotic factor is responsible for differences in bacterial composition among the different basins considered and plays an important role, not alone but coupled with T, in changing the composition of the microbial community during the different seasons. In fact, temperature is not responsible for the community differences between the basins but plays an important role together with salinity, in the community interseasonal changes in all the basins (Panswad T. et al. 2003, Lipson D. A. 2007, Henriques I.S. et al. 2006) .

pH values have been observed to influence the microbial soil communities (Lauber C. L. et al. 2009, Rousk J. et al. 2010; Fierer N. et al. 2006) ; in our case strong variation of pH was registered only in Alberobello (station 2) during September (Fig 3.2) when pH, decreases until a value of 6; in that sample a rapid diversity decline was observed but temperature was high and the water content very low, so that the actual influence of pH cannot be stated. During September, in that particular station, abiotic factors, were those of an extreme environment especially regarding salinity as witnessed by the presence of Halobacteria. Bacterial diversity values also show different trends (Table 3.3) in the Didattico basin, respect to Alberobello and Piscinula where trends are similar: in the latter we find the highest diversity values in June, whereas in September and December these values are drastically reduced in some cases (St.2 Alberobello), while diversity values in Didattico have not such strong oscillations and they reach higher values of diversity during September, instead to decrease.

This is due to the fact that during September, the salinity values in Didattico basin are around 20 ppm whilst inthe other two basins the water level is very low and

causes salinity to rise to up to 40 ppm,(Fig. 3.2) in Didattico salinity and the water level is higher: this evidently favors a more diversified community in Didattico during September, than in the other seasons (Wu Q. L.et al. 2006,. Jackson C. R and. Vallaire S. C 2009) While, when salinity exceed certain values (> 40 ppm) and water content falls markedly, diversity begins to decrease (Nielsen D.L. 2003).

This variation of diversity along a salinity gradient could also be explained in term of 'Intermediate Disturbance Hypothesis'(Grime J.P. 1973; Horn H.S. 1975; Connell J. 1978; Sousa W.P. 1979). In this case the species diversity will vary in the salinity gradient, as shown in Fig. 4.1. This is similar to what happens in the Macchiatonda wetland. In fact while during September in the other two basins the diversity begins to decrease because the water level is very low and the salinity is very high (around 40 ppm); in Didattico salinity is around 20 ppm and the water level is higher: this evidently favors a more diversified community in Didattico.

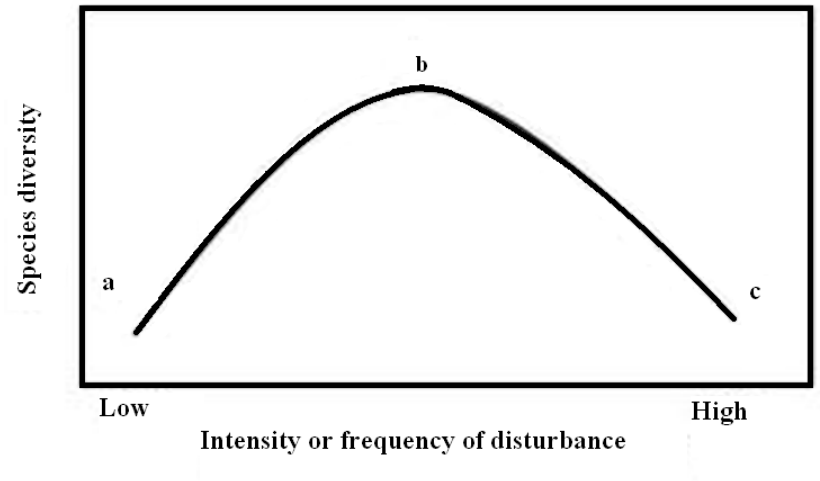

Figure 4.1 Simple representation of intermediate disturbance hypothesis

The dissimilarity from the different pond are also confirmed by the results obtained from the archaea community. In fact also in this case the fingerprinting profile (Fig

3.10 3.11) of the three ponds are different with the *Didattico* basin that cluster alone and the June sample and September sample of the other two basin that cluster respectively together. Also the diversity show differences between the ponds (Table 3.4) with the *Didattico* basin that show highest diversity during June And a *Aberobello* and *Piscinula* that show highest diversity during September with the exception of *Alberobello* station 2 due to the very low level of water during September .

Thanks to sequencing of the 16S we identified the dominant order in different costal basins and in different seasons.

The identifications support the existence of a diversity between *Didattico* vs *Alberobello* and *Piscinula* (Table 3.5). In fact, the most common bacteria in the first basin belongs to the orders of the Burkholderiales, whereas in the other two Rhodobacteriales order is more common. This is because orders which predominate in *Didattico* (Burkholderiales and Flavobacteriales) are those more easily found in fresh water ponds, rice paddies, etc. (Sun L. et al. 2008, Bouvier T. C. and Del Giorgio P.A. 2002, Lemes G.A.F. et al. 2008, Spring S.et al. 2000). While the order of Rhodobacteriales is more frequently found in shallow coastal sea waters (Dang H. Et al. 2008, Brinkhoff T. 2008). These changes are consistent with that seen also by Bouvier T.C. 2002, Rappé M.S. 2000). Obviously, there are community changes also during the different seasons: for example, there is a greater presence of Micrococcinae in all basins during cold months. Interesting is the occurrence of Bdellovibrionales in salty station during September, when this basin is almost completely dried up.

Bdellovibrio is a small spiral member of the delta-Proteobacteria which has the unusual property of preying other gram-negative bacteria. *Bdellovibrio* attaches to its prey, burrows through the cell wall and replicates in the periplasmic space, causing the eventual lysis of the host cell and the release of up to 30 progeny daughter cells. This organism is widespread in the marine environment, and is probably important in controlling other bacteria, although its full ecological role is unknown (Munn C. B. 2004). The Bdellovibrio presence is most probably due to the

environmental conditions observed in Septeber: these organisms are indeed often been observed during periods of low water-amounts , high temperature and salinity (Sánchez Amat A. and Torrella F. 1990, Kelley J. I. et al. 1997; Williams H. N. 1988); triggering ecological dynamics details that are still poorly understood (Marbach A. 1975). It has been hypothesized that they could exert a top-down control as phages do (Chauhan A et al.. 2009, Shemesh Y., and Jurkevitch E. 2004; ; Rice T.D. et al. 1998).

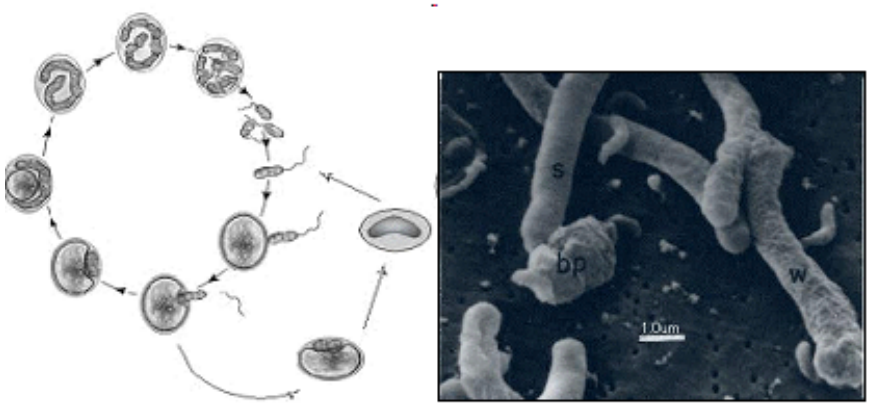

Figure 4.2 Bdellovibrio life cycle and SEM image of Bdellovibrio attack from http://faculty.plattsburgh.edu

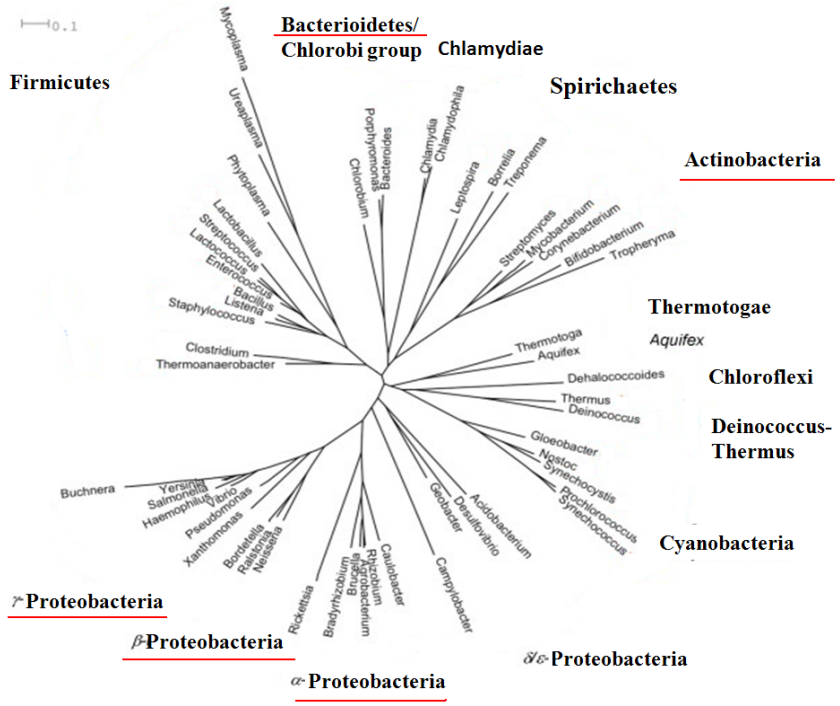

Figure 4.2 Bacterial phylogenetic trees from From www.biomedsearch.com

Rather few information on the archaea community are available in literature:in the past they were thought to be uncommon in the environments other than the strictly anaerobic or the extreme ones, and the conservation that these microorganism are, on the contrary, quite abundant in nature is recent (Munn C. B. 2004). The few data available in the databases often do not allow a precise classification of the archaea found in the Macchiatonda wetland, and most of them have been classified just as belonging to the Euryarchaeota phylum, according to their similarity to uncultered unclassified sequences. This phylum collects the majority of the known Archaea and has an high level of internal heterogeneity.

During September when the environmental conditions become unfavorable, the filogenetic level of archaeal identification is lower (class level), maybe due to the larger availability of data on extremophyle archaea. In fact the archaea found in

Didattico belog to the class of Methanomicrobia, and those found in *Alberobello* and *Piscinula* belong to the classes of Methanobacteria and Halobacteria. This finding remarks the importance of salinity in driving the microbial community in this environment because, also as far as archea are concerned, the communityin *Didattico* is clearly other than the one in *Alberobello* and *Piscinula.* The low archaea incidence during the cold period underlines also the importance of temperature,. As regard the Archaea, the Macchiatonda wetland can be considered also an interesting model system to study halophylic microorganisms in the field. This is important to know also because this organisms have a biotechnological potential for the production of compatible solutes, hydrolytic enzymes or exopolysaccharides (Arias S. et al. 2004,; Yeon et al., 2005; Ventosa et al., 2008).

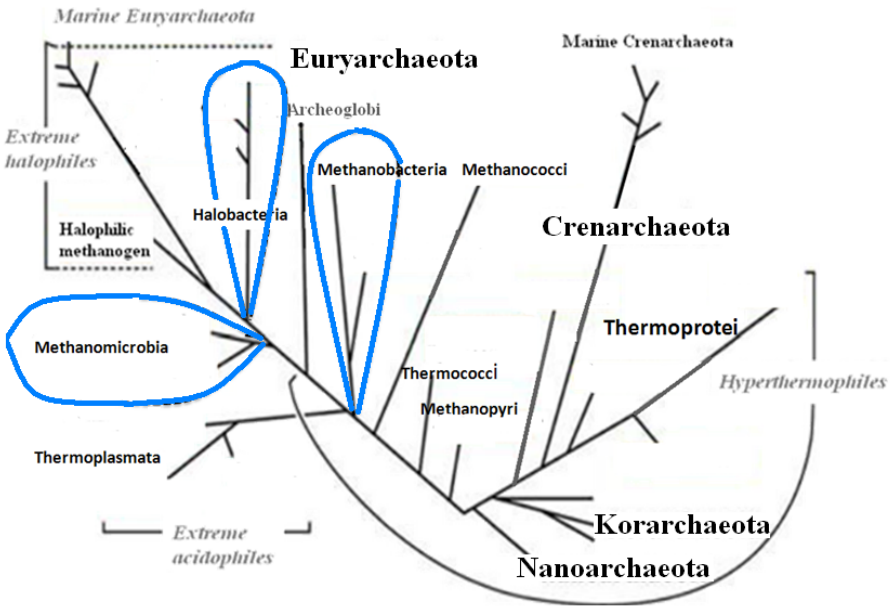

Figure 4.3 Archaea phylogenetic tree from brock biology of microrganism 2006

5. Conclusions

In this study it has been found that basins that are close one another and could therefore be mistakenly grouped, can differ widely in composition of the microbial community in terms of molecular fingerprinting, prevalent microbial taxa and biodiversity values. Cultural and molecular data gave different results: cultural analyses highlighted the overall importance of salinity and temperature in shaping the bacterial assemblage while molecular analyses showed the differences in the composition of the bacterial assemblage between ponds and seasons. The information obtained in this study allows us to correlate the variation of the community to the measured abiotic factors, so enabling us to understand the community's trends in the various basins during the seasons. In our case, the clear-cut differences between Alberobello and Piscinula ponds it has been seen that salinity and water level are the main responsible of differences between the communities of the basins. This information will allow us to monitor community changes in the future using bacterial assemblage as a bioindicator, as suggested by McArthur (2001). It is especially important to the aim of plan human interventions in these fragile areas (relict wetlands), such as water inputs during the dry periods in summer, used to preserve the habitat for migratory birds. In addition, this work reinforces the effectiveness of using biological information, in this case molecular profiles of microbial community, to classify wetlands, especially when there are small differences between neighbouring basins, that otherwise would be grouped together by the classical classification. This information is useful not only to know the extent of microbial diversity in different areas and at different times, but also to speculate about the factors that drive them. The obtained results will be useful also to implement the data for numerical environmental models. For this reason the still poorly information on microbial communities structures, should be more and more

implemented, being an important issue for the conservation and management of these important ecosystems.

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