UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II

Dottorato di Ricerca in Biologia Applicata

XXIII ciclo

Water acidification: effects on the macroalgal community

Tutor:

Dott. Carmen Arena

Co-Tutor:

Dott. Maria Cristina Buia

PhD student

Dott. Lucia Porzio

Coordinator:

Ch.mo Prof. Ezio Ricca

Anno Accademico 2010-2011

A mio padre e mia madre

Ragione e Passione sono Timone e Vela della Nostra Anima Navigante…

~ Kahlil Gibran ~

CONTENTS

1. General introduction

1.1. Algae

Algae, even if the group is unnatural, are very diverse photosynthetic plants that have neither roots nor leafy shoots and which also lack vascular tissues. Different size, morphology, life cycle, photosynthetic pigments, chemical nature of the storage products and cell wall play an important part in the definition of the various algal groups.

In 1753 the algae were placed By Linneo in the Cryptogamia along with other non-flowering plants and since that time scientists have been intrigued by this heterogeneous group of photosynthetic organisms with an estimated 350,000 known species (Brodie & Lewis, 2007). The number of algal groups has varied over the years, with as many as 16 phyla (Van den Hoek *et al*., 1995). The latest classifications place algae into four of the five supergroups of eukaryotes (Keeling, 2004) (Figure 1).

1.1.1. Origin and Classification

A debate is over the question of monophyly or poliphyly in the algae. Many scientists fall on the side of the algae being a heterogeneous group that are not monophyletic, but there are others who argue for monophyly of all photosynthetic organisms, based on the origins of the plastids.

Common ancestor of eucaryotic cells was a phagotroph (already having a endocellular membranous system) which established an endosymbiotic relationship with a bacterium that evolved in a mitochondrium (Figure 2). Another similar endosymbiotic event but with a cyanobacterium occurred for the ancestor of algae; in this case cyanobacterium evolved in plastid. In mitochondria as in plastids the bacterian wall disappeared during evolution. Endosymbiotic bacteria had originally a double membrane, one of them fused togheter with phagocytosis vacuole membrane; for this reason the inner membrane of mitochondria and plastids has procaryotic origin, whereas the external membrane has mixed composition, although almost of procaryotic origin. After separation from the phagocytosis vacuole, plastids as mitochondria were situated in the cytoplasm. Both organelles of eucaryotic cell are of procaryotic origin and derive from a primary endosymbiotic event. Chlorophyta and Rhodophyta also originated from a primary endosymbiosis. Rhodophyta hereditated phycobilisomes from the endosymbiotic cyanobacterium but loose flagella, whereas Chlorophyta loose phycobilisomes. On the contrary Heterokontophyta originated by a secondary endosymbiotic event; in fact it has been hypothesized that a flagellate phagotroph captured an eucaryotic alga which already had a plastid of primary endosymbiotic origin and then the flagellate established an endosymbiotic relationship. For

that endosymbiotic algal external membrane (the phagocytosis vacuole membrane) fused with the endoplasmic reticulum of the phagotroph while its cytoplasm regressed until disappear. As a result Heterokontophyta have four plastidial membrane: the most external is of phagotroph origin while the one immeditely below was the endosymbiotic alga plasmalemma; finally the two inner membrane represent those of the primary plastid that was in the endosymbiotic alga.

Recently, Delwiche (2007) supported the monophyletic origin of algae focusing on the plastid and how endosymbiosis events unite the algae and the land plants as a monophyletic group. On the opposite, according to Cavalier-Smith (2007), algae are considered polyphyletic because the eukaryotic algae arose as chimeras of a bikont protozoa and a cyanobacterium and that there were at least four secondary symbiosis that generated the diversity of the meta-algae.

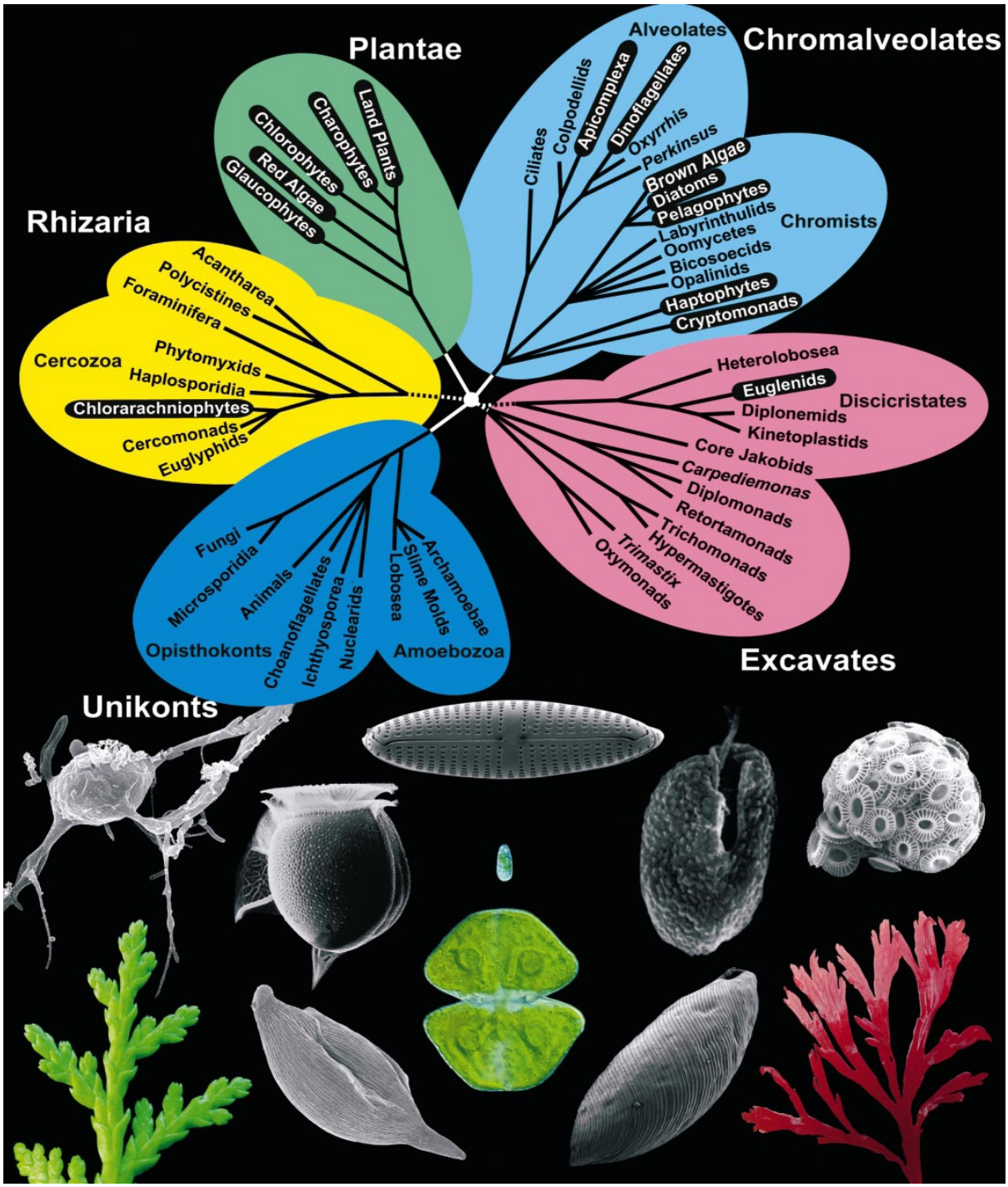


Figure 1: Tree of eukaryotes and diversity of plastid-bearing eukaryotes. Top: an unrooted hypothetical phylogeny of eukaryotes based on a synthesis of many gene trees, protein insertions and deletions, and cellular and biochemical characters. In this tree, eukaryotes are divided into five large groups, of ''supergroups,'' within which representatives of the major lineages are shown with their interrelationships as we know them. Dotted lines are plausible but more weakly supported parts of the tree. All groups in which plastids are known from at least a large number of species are indicated by white text on black. Bottom: a small taste of the diversity of plastid-bearing eukaryotes can be seen from one representative of each of the major ''algal'' lineages. (Keeling 2004)

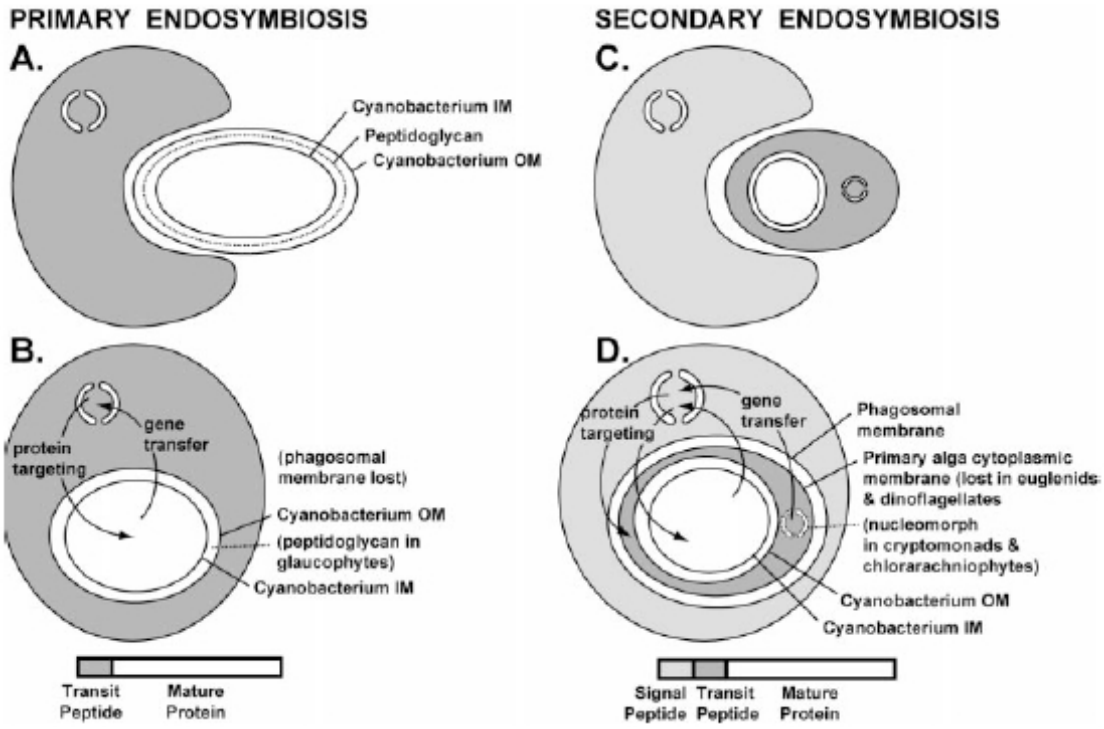


Figure 2: Primary and secondary endosymbiosis. A–B. Primary endosymbiosis. A heterotrophic eukaryote eats a Gram-negative cyanobacterium (A), which is retained rather than being digested (B). The cyanobacterial endosymbiont is substantially reduced, and a large number of genes are transferred to the nuclear genome of the host. The protein products of these genes are targeted to the plastid by way of a transit peptide. The primary plastid is bounded by two membranes derived from the inner and outer membranes of the cyanobacterium. The presumed phagosomal membrane is lost, as is the peptidoglycan wall (except in glaucophyte algae). B–C. Secondary endosymbiosis. A primary alga (either a red or green alga) is eaten but not digested by a second eukaryote (C). This eukaryotic endosymbiont degenerates and genes encoding plastid-targeted proteins are moved from its nucleus to the secondary host nuclear genome. Some genes may also move from the plastid genome to the secondary host nucleus. These plastids would originally be bounded by four membranes derived as indicated (Keeling, 2004)

Rhodophyta are a very large and diverse group of microscopic algae and macroscopic algae. Their plastids contain chlorophyll a, phycobilins, and phycobilisomes and they are characterized by the absence of any flagellate cells (including gametes). Because of the presence of phycobilisome thylacoids lie singly inside the cloroplast which is enveloped by a double unit membrane while one thylacoid run along the inner membrane. The principal reserve compound is represented by floridean starch, an a-1,4-glucan polysaccharide. Grains of this compound are located in the cytoplasm. Another unique feature of this group is the presence of pit connections (open protoplasmic connections) that are junction between daughter cells closed by a proteinaceous stopper (the pit plug) (Van den Hoek *et al*., 1996).

Red algae are divided in two classes: Bangiophyceae and Floridophyceae. The first class, preserves characters found in ancestral red algae and comprises also unicellular

organisms. Macroalgae of this group are either filamentous or sheet-like thalli. The second class includes morphologically and functional more complex species.

For a review of red algae, see Saunders and Hommersand (2004).

Heterokontophyta are an extremely diverse group of photosynthetic and nonphotosynthetic groups that were once classified separately as protozoa, algae, and fungi. Heterokont plastids, where they occur, are structurally similar to those of haptophytes and also contain chlorophyll a and c. Heterokont algae include microscopic forms of great ecological significance (e.g., diatoms) as well as macroalgae (e.g., kelps).

Mostly marine, this Division is characterized by the presence of two different flagella on one cell (unicellular organism or gametes); one is a mastigonemate, plum-like flagellum which points forward during swimming while the other is shorter, lying along the cell, directed backwards. Heterokontophyta have both chlorophyll *a* and *c* although carotenoids dominate on chlorophylls. The principal accessory pigments are b-carotene and fucoxanthin. Stacks of threee thylacoids are called *lamellae* one of which runs along the periphery of the chloroplast, called *girdle lamella*. The chloroplast are enclosed by double membrane and externally also by endoplasmic reticulum. The main storage compund is chrysolaminarin, a b-1,3-glucan, stocked inside special vacuoles in the cytoplasm. Phaeophyceae represent the Class grouping brown macroalgae, which diversify from branched filaments to more complex structures as kelps have. For a review of heterokont algae, see Andersen (2004).

Green Algae are a large and diverse group roughly divided into Charophytes and Chlorophytes. Charophytes are the ancestors of land plants, which share a great number of similarities to Charophytes and green algae as a whole. Chlorophyta is the most ubiquitous and heterogeneous group by a morphological point of view. It includes unicellular flagellates and multicellular thalli arranged in different ways. Flagella are isokont which means that they are morphologically similar but different in length.

Chlorophylls dominate on carotenoids and other accessory pigments as xantophylls; both chlorophyll *a* and *b* are present also with b- and g-carotene. Chloroplast are enclosed by a double plasmatic membrane and contains stacked thylacoids forming *grana*. Pyrenoids are present within the chloroplast, embedded to thylakoids; these structures can be ovoidal or spherical but their function is still unknown (de Reviers, 2002). Starch represents the most important reserve compound and it is stored inside the chloroplast as a grain.

For a review of green algae, see Lewis and McCourt (2004)

In this work the attention will be focused only on pluricellular marine algae, generally named *macroalgae* or *seaweeds*, distributed among three Divisions: Rhodophyta, Heterokontophyta (or Ochrophyta according to Cavalier-Smith (2007) which contain the class of Phaeophyceae, and Chlorophyta (Van den Hoek *et al*., 1995).

1.1.2. Algal Morphological features

As they are multicellular organisms, macroalgae can have diverse thallus structure organizations, distinguished according to Barsanti & Gualtieri (2006) in:

Filamentous algae. Filaments are the result of a series of cellular division perpendicular to the filament axis forming cell chains. Uniseriated filaments have only one layer of cells whereas multiseriated filaments consist of multiple layers of cells. Both uniseriated and multiseriated filaments can be simple or branched.

Siphonous and siphonocladous algae. Siphonous algae are characterized by ceonocytic tubular filaments. These algae undergo repeated nuclear divisions without forming transversal cell walls; siphons generally have a filamentous (e.g. *Briopsis spp*.) or vesicular (e.g. *Derbesia spp*.) appearance but, differently by unicellular algae, they have multinucleated cytoplasm envelopping a big centrale vacuole. Even if the term “siphonous” is wrongly it is widely accepted for some red algae to indicate multilayered filaments of multinucleated cells characteristic of the *Polysiphonales* Order.

Siphonocladous algae are constitued by filaments divided in a series of multinucleated articles. They can be also branched as it occurrs in *Cladophora spp*. (de Reviers, 2002).

Parenchimatous and pseudoparenchimatous algae. This kind of algal structure derives from a cell division in three dimensions. *Ulva spp*. (Chlorophyta) and many phaeophycean algae (Heterokontophyta) have this “tissue” organization. In pseudoparenchymatous algae filaments can be distinguished; they can be loosely or closely aggregated, twined each others and branched, held by mucilage, especially in red algae. Vegetative apparatus can reach an high complexity as in the genus *Sargassum* where a *medulla*, the inner part of the thallus, and a *cortex*, the external part, can be distinguished. In this complex taxon, thallus is divided into a *phylloid* which have foliose branches bearing air

vesicles (bladders) and in a *cauloid* which looks like a stipe with rhizoidal apparatus (de Revier, 2002).

1.1.3. Reproduction of macroalgae

Macroalgae and algae in general have different reproduction cycles according to the succession of generations: a) monogenetic haplontic life cycle (with a haploid generation only); b) diplontic or gametic life cycle, with a diploid generation only; and 3) diplohaplontic or sporic life cycle with two (digenetic) ot three generations (trigenetic).

According to Feldmann (1978): "A generation is a phase of the development of an organism which begins from a reproductive cell (spore or zygote) and then resulting in the production of other reproductive cells that can be different or not from those produced by the previous generation". A haploid or a diploid status can dominate during an algal life cycle.

Along with these fundamental classification of life cycles there is obviously a large number of variants (de Reviers, 2002).

<u>*Monogenetic haplontic life cycle: example of Acetabularia acetabulum*</u>

Acetabularia acetabulum develops from the zygote into a single cell (gametophyte) in summer and produces 2 o 3 whorls of hairs. In winter only the stalk persists with a giant nucleus in the rhizoidal part. In the following spring the stalk continues to growth reaching winter season again just with its stalk.

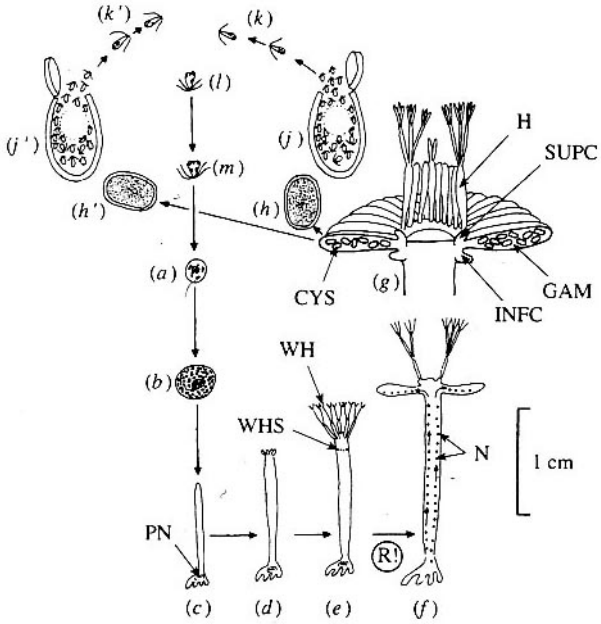


Figure 3: Life cycle *Acetabularia acetabulum* (Van den Hoeck *et al.*, 1995)

During the third year the thallus develops the reproductive *umbel,* encrusted of calcium carbonate and divided into radial secti. Each sectus of the umbel is a gametangium connected with the axis. When the umbel is formed, the nucleus undergoes to a cromosomic reduction (meiosis) followed by other numerous mitotic divisions leading to a large number of small haploid nuclei. They are then brought through citoplasmic currents to the whole algal axis comprising the umbrella. Finally they concentrate into the umbrella where they differentiate into ovoidal gametangial cystseach containing a haploid nucleus. Cysts are finally released through the thallus kink and spend the winter season within the sediment where they become constituted of numerous haploid nuclei. The next spring nuclei will differentiate in biflagellate gametes and released through the opening of an operculum once mature and will form a new mononuclear diploid zygote; only the zygote is diploid and this cycle is interpreted as *monogenetic haplontic cycle*. (Figure 3).

Monogenetic diplontic life cycle: example of Sargassum sp.

Sargassum sp. exhibits cavities known as *conceptacles* which contain gametes; *receptacles* are groups of conceptacles; they origin from a meristodermic cell. *Sargassum* species are monoecious: mature gametes, oospheres and spermatia, are released and they can fuse forming a zygote which settles down on the substrate forming rhizoid (a root-like filament of cells). So diploid thalli produce through meiotic events haploid gametes and then zygote germinates in a new thallus similar to parents (Figure 4). Life cycle seems to be monogenetic diplontic and thalli occurring in the field are gametophytes.

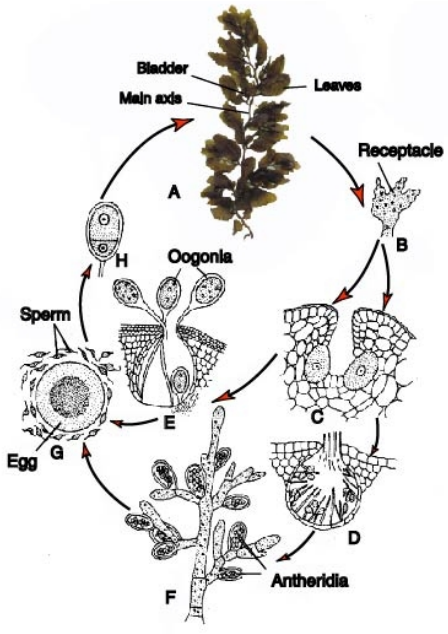


Figure 4: Life cycle of *Sargassum sp.* (from www.niobioinformatics.in)

Isomorphic digenetic diplohaplontic life cycle: example of Dictyota

Dictyota sp. gives an example of isomorphic digenetic diplohaplontic life cycle. In fact gametophytes bring groups of oogonia (female) or groups of spermatangia (male). Spermatangia release the spermatia while each oogonium release only one oosphere. The zygote develops into a sporophyte, morphologically similar to the gametophyte but bringing unilocular sporangia where meiotic processes occur. Finally, haploid spores germinate into new haploid gametophytes (see Chapter 4).

Eteromorphic digenetic diplohaplontic life cycle: example of Laminaria

Diploid sporophyte thalli produce spores within sori, groups of sporangia. Mature sporangia release, through an apical pore, spores with two heterokontic flagella called zoospores. Zoospores settle on the substrate loosing their flagella and germinate into small creeping filaments. Some of these microscopic thalli produce male gametes (spermatia) within spermatangia while some others generate female gametes (oospheres) in reproductive structure called oogonium or oocysts. Spermatia are released whereas oospheres are not bulging out from the oogonia and waiting for the male gamete. The zygote will growth in a new big sporophyte, generally found in the field.

In conclusion *Laminaria sp.* presents a succession of two morphologically different generations: one macroscopic, the sporophyte, and the other microscopic, the gametophyte.

Eteromorphic trigenetic diplohaplontic life cycle: example of Polysiphonia

This life cycle characterizes the Floridophycean group to which *Polysiphonia* genus belongs (Figure 5). In *Polysiphonia sp.* there are generally male and female individuals and they form spermatangia (which bring male gamets called spermatia) and carpogonium (which contains the oosphere, female gamete). Aflagellate spermatia, passively transported by water movements, reach the carpogonium and, through the trichogyne, fecondates the oosphere constituting the gonimoblast; it contains carposporangia that will release diploid carpospores. All the gonimoblastes represent the carposporophyte, a parasitic generation of the gametophye. Then carpospores develop into diploid individuals, the tetrasporophyte, that produce by meiotic divisions four haploid spores (tetraspores) Each spore develops into a gametophyte completing the life cycle. In conclusion, there is a succession of three generations: gametophytic, carposporophytic and tetrasporophytic generations. Gametophyte and tetrasporophyte are morphologically similar so they are difficult to distinguish into the field; on the opposite, the carposporophyte is microscopic and parasitic of gametophyte.

Two types of morphologies occur and the cycle is called dimorphic. Natural populations of *Polysiphonia* has two types of distinct and indipendent gametophytes: carposporophyte and tetrasporophytes. For that reason these populations are *diplobionts*. This term is opposite to *haplobiont* which means that there is only one kind of individual.

Other Florideophyceae have a trimorphic cycle because gametophyte, carposporophyte and tetrasporophyte are morphologically different. *Asparagopsis-Falkenbergia*. gives an example; *Asparagopsis* is the macroscopic erect gametophyte, *Falkenbergia* is the microscopic tetrasporophyte of the same species. These different morphologies explain the term trimorphic.

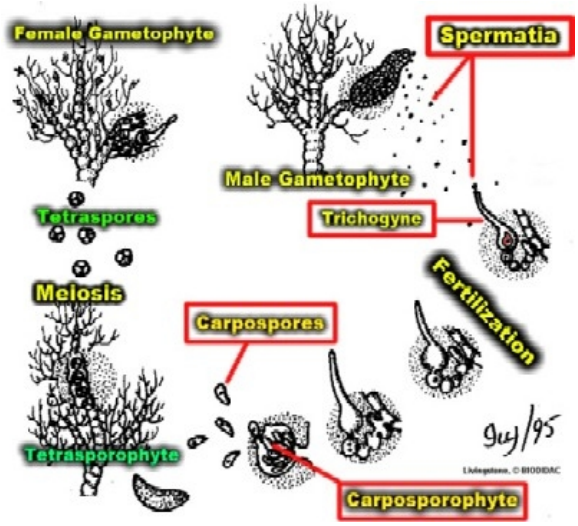


Figure 5: Life cycle of the genus Polysiphonia (from www.jochemnet.de)

1.1.4. Macroalgal communities

According to Karl Möbius (1877) *communities* are species populations that coexist in a particular time and geographical area, in which dominant environmental conditions are homogeneous. According to a phytosociological approach (Braun-Blanquet, 1915), community represents a predictable species association, that is "a stable species group, living in balance with an homogeneous environment, in which characteristic species reveal, with their presence, a determined ecological situation".

Communities are characterized by:

- Composition: presence of species and their relative abundance;
- Nature and shape of the relationships among these species (community structure *sensu strictu*);
- Dynamics: fluxes in time and space (i.e. successions).

To study communities, three kind of factors should have to be analyzed:

I. Habitat factors (called abiotic factors in the past);
II. Niche factors (e.g. competition, predation);
III. Population factors (e.g. natality and mortality rates).

Abiotic factors select species constituting the community whereas biotic factors control community within itself.

In extreme environments, where physico-chemical parameters have strong variations, competition is reduced and only few species are dominant because they have to face up to a physiological stress (Begon *et al.*, 1998). In these environmentally controlled communities, the structure is similar to that present during early stages of colonization, where pioneer species (*r*-species), characterized by high abundance, high turnover and wide dispersion capacity, are dominant. These kind of communities are present in marine ecosystems at low depths, in coastal zones, lagoons, intertidal zones, estuaries, where some variables such as temperature and salinity vary a lot (Sanders, 1968).

On the opposite, biologically controlled communities are usually present where environmental conditions are more stable and constant. This kind of community is mainly controlled by "biological stress" drivers as competion for space, light and food (Littler *et al*., 1975). Such communities are characterized by a high specific diversity with few *dominant* species (named K-competitive dominant species, characterized by long life cycle, low turnover and low dispersion ability) and by several *rare* species with low covers.

In nature a combination of both kind of communities is usually present. Each community does not present a fixed ratio in species occurrence and abundance (both in time and space), due to the continuity of the water marine environment and to the presence of transtional zones called *ecotones* (Cognetti *et al*., 1992). A single community can change according to variations in irradiance, pH and hydrodinamic state and others.

It is clear that community structure and functioning may change through a stress gradient in presence of detrimental conditions derived by human activities, which can lead to a loss of preexistent species and replaced by new ones, more tolerant (Cognetti *et al*., 1992).

1.1.4.Macroalgal community stratification and vertical zonation

Marine macroalgae form stratified communities. The upper layer, named canopy, is formed by erect and frondose algae covering the understorey layer, formed by the turf compartment. Finally, crustose algae grow below the turf, directly on the substrate (Lüning, 1990). Due to this stratification, many canopy species can directly and indirectly modify the local environment to create conditions that facilitate some species (e.g coralline algae) but

exclude other (e.g. filamentous turf) taxa (Bruno *et al*., 2001). Negative effects of abrasion on articulated coralline algae and filamentous turf may augment effects of shade provided by canopies, which appear primarily responsible for the maintenance of extensive covers of encrusting coralline algae beneath canopies. Moreover, negative effect oa abrasion may have indirect positive effects on the abundance of other taxa by excluding competitors for space (Irving & Connell, 2006)

Each species has its own *range* of tolerance and resilience to environmental changes to which its ecological and physiological *optimum* takes place. Besides, more than one species could have similar tolerance degree and therefore interspecific competitions regulate their coexistence. In the same area, benthic marine vegetation can change in relation to three principal environmental factors: light, hydrodynamic regime and nature of substrate. It can be distinguished photophilic and sciaphilous vegetations in relation to irradiance regimes, calm environments and exposed environments vegetations in relation to hydrodynamic forces, and hard and soft substratum vegetation in relation to the bottom nature (Cormaci *et al*., 2003).

In particular both light and water movement differ with depth, generating a *vertical zonation* of macroalgal communities. Vertical zonation is based on two model system, one based on irradiance, the other on hydrodynamism.

The first one, devised by Pérès and Picard (1964), divides littoral system into five zones:

- *Supralittoral zone*, which is reached by spray water;
- *Eulittoral zone*, the intertidal zone, which is periodically emersed and submersed;
- *Sublittoral zone*, which is always submersed and that can be subdivided in an upper zone, the *infralittoral zone* and a lower zone, the *circalittoral zone*. The infralittoral zone is characterized by the presence of photophilic species whereas the circalittoral one is characterized by sciaphilous algae.

The second one (Riedl, 1971) distinguishes five critical depths according to changes in water movements:

- *Splash zone* between +0.3 m and -0.3 m depth with splashing waves and tide effects;
- *Surf zone* between 0.3 m and 1-4 m depth where particles move without a dominant direction and with high energy;

- *I critical depth*, or *oscillation-zone*, between 1-10 m and maximum 4-20 m depth. This zone is dominated by oscillating and orbiting movements. The diameter of the orbits are equal to the height of the waves;
- *II critical depth*, or *drift-zone*, between a minimum of 10-20 m and maximum 30-40 m. Oscillations decrease and finally disappears. Mechanical stress is minimal and comes from one side;
- *III critical depth* where flatten water movements become dominant, characterizing the circalittoral zone.

Since subtidal rocky shore vegetation has been considered in this work, subtidal sciaphilus and photophilous communities of hard substrate will be described, according to Giaccone *et al.* (1994a).

Photophilous vegetation of hard substrata infralittoral zone

Marine vegetation is dominated by large brown algae such as *Sargassum spp*. and *Cystoseira spp*. These form complex structured communities: an upper photophilic stratum, subdivided in a *suprastratum* composed by large brown algae, and a *mesostratum*, formed by medium size upright algae; an epiphyte stratum or *epistratum*, which is made up by several species directly living on other macroalgae; a sciaphilous stratum, constituted by species shaded by those of the upper stratum. Marine vegetation of this zone is influenced both by the light intensity and hydrodinamism

Associations succed on the batymetric profile from the top downward as follows:

a) ***Cystoseiretum strictae* Molinier 1958** whose characteristic species are *Cystoseira amentacea* (C. Agardh) Bory and its varieties as *amentacea*, *spicata* (Ercegovic) Giaccone and *stricta* Montagne, and *Feldmannia paradoxa* (Montagne) Hamel. This community is characteristic of exposed environments while in slightly shaded, with reduced water movements, *Cystoseiretum strictae* is replaced by ***Sargassetum vulgaris* Mayhoub 1976** characterized by *Sargassum vulgare* C. Agardh and *Sargassum trichocarpum* J. Agardh (Mayhoub, 1976).

b) ***Cystoseiretum crinitae* Molinier 1958** whose characteristic species are *Cystoseira crinita* Duby, *Sphacelaria cirrosa* (Roth) C. Agardh, *Stypocaulon scoparium* (Linnaeus) Kützing, *Cladostephus spongiosum* (Hudson) C. Agardh f. *verticillatum* (Lightfoot) Prud'Homme van Reine and *Anadyomene stellata* (Wulfen) C. Agardh. In particular ecological conditions, this community has one or, rarely,

more different species, called differential species, forming *facies* which are considered by Giaccone *et al.* (1994a) and Marino *et al.* (1999) as sub-Associations:

- *Alsidium helminthochorton* (Schwendimann) Kützing, where there is reduced water movements and high sedimentation;
- *Cystoseira compressa* (Esper) Gerloff et Nizamuddin, in calm and a little polluted biotopes;
- *Stypocaulon scoparium*, often with *Padina pavonica* and *Dictyota fasciola* (Roth) J.W. Lamouroux in unstable enviroments due to their sedimentary nature;
- *Halopithys incurva* (Hudson) Batters and *Dipterosiphonia rigens* (C. Agardh) Falkenberg where water transparency is variable;
- *Gelidium spinosum* (S.G. Gmelin) P.C. Silva v. *hystrix* (J. Agardh) where intense grazing on subvertical substrata.

c) ***Cystosereitum sauvageauanae Giaccone 1994*** in which *Cystoseira sauvageauana* Hamel, *Cystoseira foeniculacea* (Linnaeus) Greville f. *tenuiamosa* (Ercegovic) Gòmez Garreta *et al*., *Stilophora tenella* (Esper) P.C. Silva are characteristic species living in the oscillating zone of Riedl 1971.

d) ***Cystoseiretum spinosae Giaccone 1973*** where *Cystoseira spinosa* Sauvageau, *Cystoseira foeniculacea* (Linnaeus) Greville f. *latiramosa* (Ercegovic) Gomez Garreta *et al*., *Valonia macrophysa* Kützing, *Halopteris filicina* (Grateloup) Kützing, *Dictyota linearis* (C. Agardh) Greville are considered as characteristic species and it is established in monodirectional currents flowing zones.

Other plant Associations could be found in particular environmental conditions:

- ***Cystoseiretum barbatae*** **Pignatti 1962** in reduced light and hydrodinamism environments with *Cystoseira barbata* (Stackhouse) C. Agardh, *C. compressa*, *Halymenia floresia* (Clemente) C. Agardh, *Gracilaria bursa-pastoris* (S.G. Melin) P.C. Silva, *Hypnea musciformis* (Wulfen) J.W. Lamouroux, *Nemastoma dichotomum* J. Agardh, *Ceramium siliquosum* v. *siliquosum*, *C. deslongchampsii* Chauvin ex Duby, *C. siliquosum* v. *lophophorum* (Feldmann-Mazoyer) Serio, *Bonnemaisonia asparagoides* (Woodward) C. Agardh.
- ***Herposiphonio-Corallinetum elongatae*** **Ballesteros 1988** in slightly deep with high hydrodinamism biotopes with *Herposiphonia tenella* (C. Agardh) Ambronn and *Corallina elongata* J. Ellis *et* Solander

- ***Pseudobryopsidetum myurae*** **Mayhoub 1976** in high irradiance and low hydrodinamism biotopes with warm affinity (deep *cuvettes*) grouping *Pseudobryopsis myura* (J. Agardh) Berthold, *Ganonema farinosum* (J.V. Lamouroux) Fan *et* Wang, *Hydroclathrus clathratus* (Bory ex C. Agardh) M. Howe.

<u>*Sciaphilous vegetation of hard substrata infralittoral zone*</u>

Sciaphilous vegetation is subdivided in *surface* and *middle* vegetation according to their distribution depth.

Surface sciaphilous vegetation

a) ***Schotteretum nicaeensis*** **Berner 1931** with Schottera nicaeensis (J.V. Lamouroux ex Duby) Guiry et Hollenberg and Gymnogongrus crenulatus (Turner) J. Agardh which are at less than 1 meter depth with low light and high hydrodinamism.

b) ***Rhodymenietum ardissonei*** **Pignatti 1962** present in holes and rock cracks with low hydrodinamism in which Chondracanthus acicularis (Roth) Fredericq and Rhodophyllis divaricata (Stackhouse) Papenfuss are characteristic species whereas Rhodymenia ardissonei Feldmann is considered a dominant species.

c) ***Pterothamnion-Compsothamnietum thuyodis*** **Boudouresque, Belsher *et* Marcot-Coqueugniot 1977** developing on artificial substrata in little polluted harbours. *Pterothamnion crispum* (Ducluzeau) Nägeli and *Compsothamnion thuyoides* (J.E. Smith) Nägeli are characteristic species of this Associacion.

<u>Middle sciaphilous vegetation</u>

d) ***Flabellio-Peyssonnelietum squamariae*** **Molinier 1958** with *Peyssonnelia squamaria* (S.G. Gmelin) Decaisne, *Flabellia petiolata* (Turra) Nizamuddin and *Osmundaria volubilis* as caracteristic species (Linnaeus) R.E. Norris. This Association is characteristic of *Posidonia oceanica* (Linnaeus) Delile rhizomes. In high sendimentation condition *O. volubilis* may develop as *facies*.

e) ***Halymenietum floresiae*** **Giaccone *et* Pignatti 1967** which develops in salty and eutrophic environments. Its characteristic species are *Halymenia floresia* (Clemente) C. Agardh, *Boergeseniella fruticulosa* (Wulfen) Kylin, *Sebdenia dichotoma* Berthold, *Cladophora prolifera* (Roth) Kützing, *Scinaia furcellata* (Turner) J. Agardh, *Sphaerococcus coronopifolius* Stackhouse, *Chrysimenia ventricosa* (J.V. Lamouroux) J. Agardh, *Halarachnion ligulatum* (Woodward) Kützing, *Thuretella schousboei* (Thuret) F. Schmitz, *Alsidium corallinum* C. Agardh.

f) ***Rhodymenio-Codietum vermilarae*** **Ballesteros 1989** which develops in sheltered environments of the lower infralittoral zone with high concentration of nitrogenous compounds, especially in spring-summer time. Characteristic species are *Codium vermilaria* (Olivi) Delle Chiaje, *Aglaothamnion tripinnatum* (C. Agardh) Feldmann-Mazoyer and *Spermothamnion flabellatum* Bornet. Different from Ballesteros (1989) *Rhodymenia ardissonei* Feldmann and *Mesophyllum lichenoides* (J. Ellis) Me. Lemoine are considered by Giaccone *et al.* (1994) as dominant species rather than characteristic species since they are widly distributed along the depth.

1.1.5.Importance of macroalgal species and communities as ecological indicators

Community's structure and function can change in relations to environmental variations. The presence of species with different pollutant tolerance can be used as indicator of water quality (*biological indicators*) (Pergent, 1991).E*ecological indicators* are assemblages of species that inform on the ecological status of the system through modification of its quantitative and/or qualitative features (Blandin, 1986) (Figure 6).

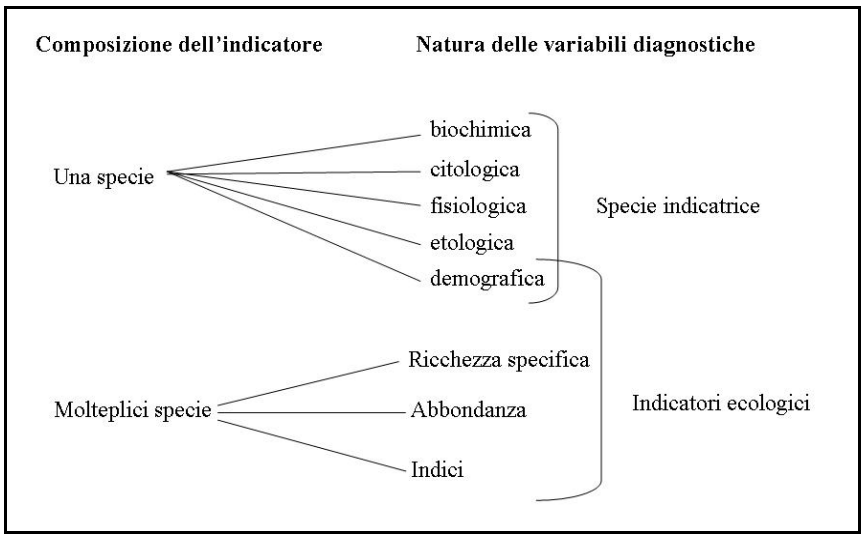


Figure 6: Different kind of biological indicators according to Blandin (1986).

Bioindicators utilization in environmental monitoring studies allows to determine the presence in time of chemical pollutants even when physico-chemical analysis are not able to detect them in seawater (Cognetti *et al*., 1992; Della Croce *et al*., 1997).

Among marine flora, macroalgae are considered good indicators of water quality According to several authors (littler and Murray, 1975; Levine, 1984; Diez et al 1999), benthic macroalgae, because of their sedentary condition, integrate the effectssof long-term exposure to nutrients or other pollutants resulting in a decrease or even disappearance of the most sensitive species and its replacement by higly resistant, thionitrophilic or opportunistic species. *Cystoseira* spp. (i.e. *Cystoseira amentacea* var. *stricta*) are considered very sensitive indicators. They form relevant ecological facies where many organisms find shelter, habitat and nursery and for that they are considered habitat formers. Several authors reported a decrease all over the Mediterranean basin of the presence of this genus in relation to the increasing anthropogenic impact and as a consequence a decrease in the associated communities (Pergent, 1991; Rodriguez-Prieto *et al*., 1996; Diez *et al*., 1999; Cormaci and Furnari, 1999; Soltan *et al*., 2001; Thibaut *et al.*, 2005; Mangialajo *et al*., 2008). On the contrary, other macroalgae are used to indicate an increase in organic matter and nutrients such as Ulvaceae (Chlorophyta).

Community is the most utilized organization level to assess ecological changes on long time series. Since natural communities develop on time in a way to reach a dynamic equilibrium, each disturbance shifts this equilibrium, modifying the whole structure.

Borowitzka (1972) showed how species diversity moves unidirectionally along neither a stress gradient or the environmental stability. Decrease in severity of conditions and increase of stability from intertidal to subtidal habitats, leads to higher biodiversity. In contrast, communities in proximity of an increase in severity of pollutants decreased their biodiversity (Borowitzka, 1972; Panayotidis *et al*., 1999).

Dominant macroalgae living in natural sites have got lower productivity, indicative of a quite mature community; in this case species diversity and relative abundances are higher and species have more complex thalli and life cycles (K-species). Their populations are regulated by biological interactions as competition for space and light (Littler *et al*., 1975).

Thanks to achieved knowledge, some statements on algal assemblages in polluted areas have to taken into account:

- *Original associations*. Vegetative populations are both original and diversified in contrast to animal populations.

- *Eterogeneity*. Algal population of polluted environments has to be considered in its diversity without looking for authentic characteristics in every cases as it happens for sciaphilus and photophilus biotopes, for inter- and subtidal assemblages, harbours and open waters, warm and cold sectors in Mediterranean (Belsher, 1974).

- *Species decline*. Decrease in species number is usual compared to homologous biotopes, even though it is not so evident as expected.

- *Phaeophyceae and Bangiophyceae dominance*. Qualitative dominance of Chlorophyceae is considered as absolute principle of pollution; however it does not always occur everywhere and, when it is the case, Chlorophyceae do not became dominant whereas Phaeophyceae and Bangiophyceae they do in the harbours and open water respectively. By a quantitative point of view, increase in nutrients determines an increase in dominance of Chlorophyceae, principally *Ulva spp*., and a contemporary strong decrease of Phaeophyceae and Rhodophyceae, sensitive to high concentration of phosphate, limiting growth factor into the Mediterranean Sea (Basson *et al*., 1976; Belsher & Boudouresque, 1976).

Colonization processes carry on differently in polluted and not polluted environments. In both sites in the first stages of succession Cianobacteria, filamentous Ectocarpaceae (Phaeophyceae), colonial diatoms and *Ulva spp*. occur. In polluted area, colonization time is faster because of the efficiency of recruitment of pioneer species (also called opportunistic species) even in presence of high mortality (Murray *et al*., 1978). In unpolluted areas, more complex development patterns have been evidenced leading to a more structured community in time. Simpler temporary dominant species, as *Ralfsia sp*. and *Scytosiphon sp*., are indeed substituted by more structural complex species with recruitment strategies optimized in determined seasons as it happens in Fucales *Cystoseira sp*. and *Sargassum sp*. When a subtidal mature community is submitted to not treated drainage for a long time, its restoration takes a lot because of different reasons. One of them may be due to gametes dispersal strategy; in fact female gametes of Fucales are quite big, adapted to sink rapidly with very low dispersal range, about 3 meters far from their parents. Even though this distance could be higher in presence of a major hydrodynamic force, Fucales recolonization is strictly dependent by their parent neighbourhood (Soltan *et al*., 2001).

For their ecological importance and high sensitivity, macroalgae have been introduced as biological quality elements into the Water Framework Directive 2000/60/CE with the goal of maintaining and improving the aquatic environments. Up to now, different indices have been proposed for this purpose (Orfanidis *et al*., 2001; Panayotidis *et al*., 2004; Ballesteros *et al*. 2007; Falace *et al*., 2009; Ivesa *et al*., 2009).

1.2. Global Climate Change

Climate system is a complex system that involves atmosphere, oceans, emersed lands, cryosphere and biosphere. Climate is generally defined as "mean weather" on long-term time scale (decades) or as Earth's energetic balance response. This balance can be modified with:

- ✓ Changes in solar radiation due to terrestrial orbit around the Sun;
- ✓ Changes in *albedo* that is the solar radiation reflected by snow cover, forests, deserts and aerosol particles;
- ✓ Changes in greenhouse effect.

Terrestrial climate is affected by these changes and by their feedback mechanisms (IPCC, 2007). *Positive* feedbacks (increase in water vapour and decrease of snow cover with consequently less albedo) (Soden *et al*., 2002) enhance global warming, whereas *negative* ones cause cooling. However everything begins from the Sun with its radiant activity on the Earth. About 30% of the solar energy, emitted as electromagnetic radiation, is scattered back to the space while 20% is absorbed within the atmosphere, and about 50% is absorbed by the Earth's surface. Energy absorbed by the planet is re-emitted as long-wave radiation. Some of these long-wave radiations are scattered back to the space while an other part some are absorbed by the overlying atmosphere and re-emitted downwards as heat; this is the so-called natural *greenhouse effect*. In fact atmosphere contains absorbers and emitters known as *greenhouse gases* (GHGs), among which the most important is water vapour that contributes 50%. Water vapour, in comparison to carbon dioxide and other gases (i.e. methane), is not a well mixed gas within the atmosphere. Without the greenhouse effect life would not be possible on the planet; in fact if atmosphere was absent it would be a mean surface temperature of about -18 °C instead of +15 °C. The balance between absorption and emission of radiation is called global radiative balance. Some factors called *radiative forcings* have the power to change this balance. Positive forcings and negative forcings can rise or fall the global temperature, respectively, shifting the radiative balance. Carbon dioxide is the most important positive forcing while aerosol (sulphate, organic carbon, dust, black carbon and nitrate), associated with scattering and absorption of solar radiation in the atmosphere can be classified as negative forcing, even if its magnitude is still uncertain (Serreze, 2010).

Net anthropogenic radiative forcings result positive (global warming effect) and they are more important than natural radiative forcings.

Anthropic activities (i.e. burning fossil fuel, agriculture) contribute to climate change with GHGs emissions and aerosol and with land use (i.e. deforestation causes decrease in CO_2 uptake and changes in albedo). Increasing in GHGs began during the industrial time (1850) (Figure 7).

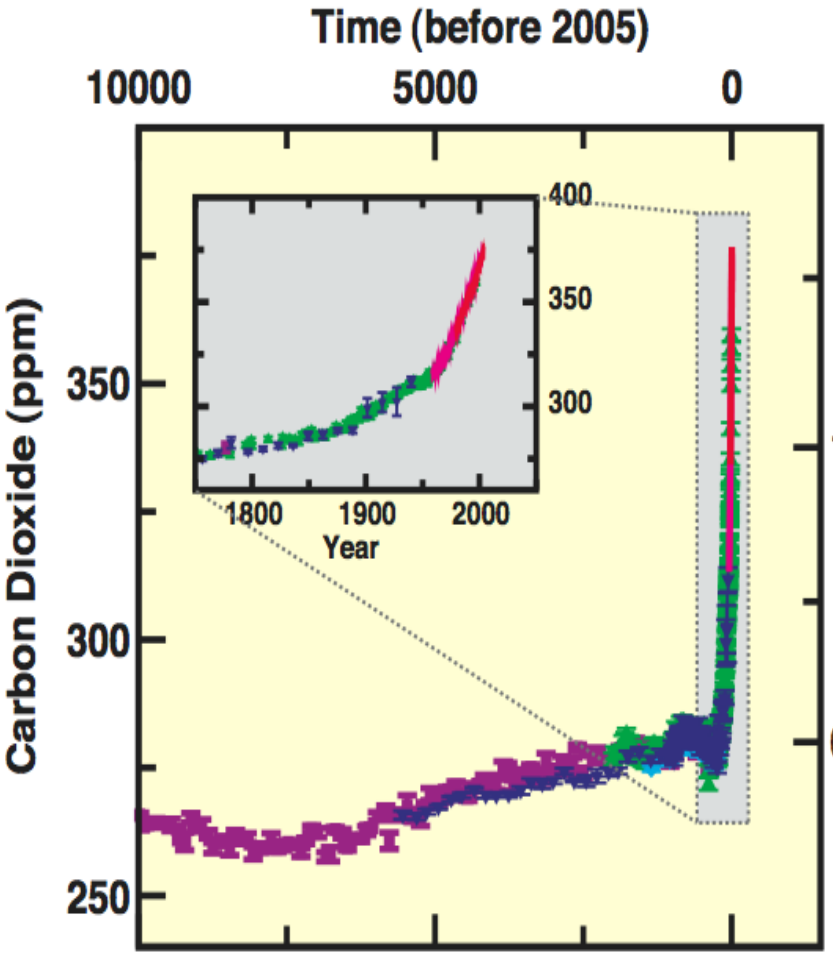


Figure 7: CO_2 changes within the last 10000 years (from IPCC, 2007).

CO_2 is the most important anthropogenic GHG (77% of the total GHGs). Its annual emission has grown during 1970-2006 by about 80% (from 280 ppm to 380 ppm) and its growth rate has been much higher during the period 1995-2006. Future perspectives, based on increased CO_2 concentration into the atmosphere, causes two fundamental impacts on Earth's system:

- ✓ Global warming
- ✓ Ocean acidification

These perspectives are based on different model scenarios that consider different CO_2 concentrations in the future, due to different urbanisation and technological progress levels.

Information collected by climate indictors (tree rings, ice cores, marine sediments) confirm that CO_2 concentration in 2006 exceeds values range (180-300 ppm) of the last 650.000 years within glacial and interglacial periods (IPCC, 2007). During the last two centuries, data showed that CO_2 concentration have increased very fast (about 100 ppm); differently increases of 80 ppm at the end of glacial period in the Quaternary have occurred in thousands of years.

1.2.1. Global warming

CO_2 effects will continue for more than a millennium, due to the time scale required for the removal of this gas from the atmosphere. In fact even if the concentrations of all GHGs had been kept constant at year 2000 level, a further warming of about 0.1 °C per decade would be expected (IPCC, 2007).

Global temperature has increased of 0.74 °C during last century and temperatures have been the warmest during 1996-2006 and a temperature increase of 0.5 °C would still be expected by 2200. Temperature increase causes a decrease of the glaciers cover with consequent sea-level rise. Satellites and mareographs have registered an increase of 1.8 mm of the global sea-level per year during 1961-2003 and it would be expected a further rise of 0.3 to 0.8 m by 2300. Oceanographic observations have evidenced oceans temperatures increase until 3000m depth (IPCC, 2007).

Among the effects of global warming we can remind:

- Alteration in atmospheric circulation with increase of extreme weather events (inland flash floods);
- Coastal erosion and floods due to climate change and sea-level rise;
- Glacier retreat with extinction of species and winter tourism
- Reduced water availability with increased health risk and frequency of wildfires
- Increased invasion by non-native species, particularly on mid- and high latitude.

The resilience of many ecosystems is likely to be exceeded this century by a combination of unprecedented human disturbances (climate change, ocean acidification,

pollution). It has been calculated that about 20-30% of species are at increased risk of extinction. Moreover global change will likely cause changes in ecosystem structure and function, species' ecological interaction and shifts in species' geographical range with negative consequences for biodiversity.

1.2.1. Ocean acidification

Ocean plays a fundamental role in the exchange of CO_2 with the atmosphere. Over the past 200 years, since pre-industrial times, the ocean has absorbed about half of the carbon dioxide produced from burning fossil fuels and cement manufacture. This demonstrates the integral role that oceans play within the natural processes of cycling carbon at global scale. In the oceans CO_2 dissolved in seawater exists in three main inorganic forms collectively known as dissolved inorganic carbon (DIC). These are: (i) aqueous CO_2 including carbonic acid (H_2CO_3) (about 1% of the total), and two electrically charged forms, (ii) bicarbonate (HCO_3^-, about 91%) and (iii) carbonate ions (CO_3^{2-}, about 8%) (Figure 8).

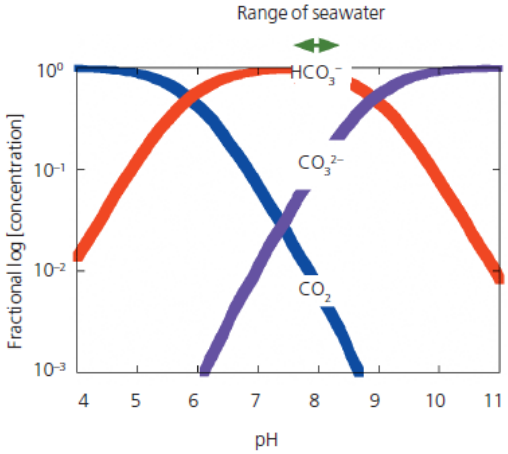


Figure 8: Relative proportions of the three inorganic forms of CO2 dissolved in seawater. The green arrows at the top indicate the narrow range of pH (7.5–8.5) that is likely to be found in the oceans now and in the future. Note the ordinate scale (vertical axis) is plotted logarithmically (from The Royal Society, 2005).

Thus under current ocean conditions, bicarbonate is the most abundant form of CO_2 dissolved (Royal Society report, 2005). In fact CO_2 present in the atmosphere, when in contact with superficial water (up to 100 m depth), produces this reaction:

$[CO_2] + [H_2O] \rightarrow [H_2CO_3]$ 1.The carbonic acid is formed

$[H_2CO_3] \rightarrow [H^+] + [HCO_3^-]$ 2.The carbonic acid splits up into its constituents; the pH decreases

$[HCO_3^-] \rightarrow [H^+] + [CO_3^{2-}]$ 3.The bicarbonate ions react releasing carbonate ions and Hydrogen ions.

Because of the dissolution of CO_2 decreases concentration of carbonate ions (CO_3^-) which reacts with hydrogen ions (H^+); this reaction moves to the right precluding the formation of carbonates minerals following the reaction:

 mineral formation
$CaCO_3 \rightarrow [Ca^{2+}] + [CO_3^{2-}]$
dissolution

The carbonate buffer acts to diminish pH changes through two principal processes: the uptake of CO_2 from the atmosphere, and the interaction of seawater with deep oceanic sediments rich in $CaCO_3$.

As CO_2 obeys to Hanry's law, its concentration in seawater is proportional to partial pressure in the atmosphere. So the large increase in atmospheric CO_2 is leading to move the carbonate system toward the formation of H^+. Because $CaCO_3$ is abundant in deep sediments the pH cannot change over timescales of 10000 years. As CO_2 is absorbed at the sea surface, it is the surface ocean which is the most affected.

Thanks to its carbonatic system, the ocean has got a buffering power which maintains an almost constant pH around 8.2 but it can vary of ±0.3 units depending on local and seasonal factors. Temperature and up-welling of deep waters rich of CO_2 regulate spatial distribution of ocean pH. Higher temperature decreases CO_2 uptake by seawater promoting its release. CO_2 produced by biological decomposition sinks into the deep oceans increasing there its concentration. Moreover, seasonal temperature variation and daily biological activity (i.e. photosynthesis and respiration) are the major factors driving pH fluctuations in seawater (Gonzales-Davila, 2003). In particular coastal water is more affected by wider pH variation compared to open ocean, due to the terrestrial system influences (e.g. run off from rivers) (Hinga, 2002).

Nevertheless continuous increasing in carbon dioxide into the atmosphere defies the oceanic buffer. Excessive CO_2 absorption by ocean system causes a decrease in pH; in fact since 1750 absorption of CO_2 by oceans had already produced a decrease of 0.1 units of pH and a further decrease up to 0.5 units of pH is foreseen by 2100 on the global ocean surface (Caldeira & Wickett, 2005).

1.2.2. Impact of acidification on marine organisms

Ocean acidification may have important effects on marine organisms because of the abundance of both CO_2 and H^+ interfering with normal physiological processes.

Many studies have been done on different groups of marine organisms: corals, pteropods, foraminifera, coccolithophorids, molluscs (for a review see Fabry *et al*., 2008).

All the three forms of dissolved CO_2 are important for the biological processes of marine organisms. These include photosynthesis and calcification, providing structures such as $CaCO_3$ shells.

Impact on photosynthesis

Also changes in carbonate chemistry and pH due to absorption of CO_2 by ocean will happen firstly in superficial layers of the ocean, namely into the photic zone where are dominant autotrophic organisms. Many studies on photosynthetic organisms take in count just phytoplankton and very little has been done for macroalgae. However photosynthetic response result different in accordance with different species considered. It has been demonstrated that doubled present today concentration of dissolved CO_2, the photosynthetic rates of phytoplanktonic communities increase up to 10% because of carbon concentration mechanisms (Royal Society report, 2005). Different results have been showed for macroalgae; Giordano *et al.* (2005) found a significant increase in photosynthetic activity whit increasing of CO_2 concentration. More studies have been carried out to study photosynthesis at increased pH values and they outlined how only few species increase their photosynthetic rate with increased pH value, as they can convert bicarbonate (the most abundant inorganic carbon at higher pH value) into CO_2. Other species have not developed this conversion mechanism and photosynthesis is not saturated; rather more species increase their photosynthetic rate at lower pH (Beer *et al.*, 1996; Johnston *et al*., 1992; Raven, 2003; Menéndez *et al*., 2001). Middelboe et al. (2007) have remarked, at compared carbon concentration, a decreased of photosynthetic rates at higher pH as a consequence of the membrane transport mechanism or with cellular pH homeostasis.

Impact on calcareous organisms

Calcification is a well controlled process and it generally requires a biological input even thought seawater can be considered $CaCO_3$ saturated today. Cacification mechanism is still nowadays bad known. It is either intracellular or extracellular. In the first case, the precipitation is realised into specific cellular compartments, whereas in the second one

calcium carbonate precipitates onto external cellular surfaces. Calcareous species differ one another both for the structure and for chemical composition of mineral crystals. Benthic macroalgae offer an example of that: either high Mg-calcite or aragonite can precipitate.

Supersaturated water of Ca^{2+} and CO_3^- ions is needed to set up and maintain calcareous structures. Low pH values reduce carbonate saturation and therefore the calcification process too, weaking the organisms' resilience.

During calcification processes, water alkalinity decreases and organisms actively increase pH in the medium that determine an increase in carbonate ions. If environmental pH is already low, calcification mechanism will require more energy which is normally used in metabolic processes with consequent fall of both growth and fitness (Royal Society report, 2005).

Calcium carbonate exists in two mineral structures: calcite and aragonite, the former less soluble than the second. However different intermediate states are present into the calcareous organisms. Macroalgae precipitate mostly aragonite or calcite with high abundance of Magnesium (high Mg-calcite is with >4% MgCO3) (Stanley *et al.*, 2002). They deposit calcareous structures outside the cell even though background studies have demonstrated in Corallinaceae a strong relationship between calcification and their cell wall (Wray, 1977) In fact calcification begins from the external side of the cell wall through vesicles derived from Golgi apparatus; then it extends toward inside leaving just a not-calcified layer around the protoplast (Wray, 1977). Although calcification is a quite widespread mechanism in biological systems, its function is not clear yet (protective, metabolic, adaptive, etc…) and its lost may have long-term consequences on physiological and ecological fitness still unknown.

A helpful parameter to determine the critical concentration of carbonate ions for biological systems in seawater is the *omega factor* (Ω) that increases decreasing the temperature and increasing the pressure:

$$\Omega = [Ca^{2+}]\ [CO_3^{2-}]\ K_{sp}$$

(when the mineral is at equilibrium (K_{sp}), that is, when the mineral is neither forming nor dissolving) (Atkinson & Cuet, 2008). When Ω is < 1 $CaCO_3$ dissolves.

Published data on corals, coccoltiphorids and foraminifera suggest a reduction in calcification of 5-25% with doubling CO_2 atmospheric concentration compared to pre-industrial values (Feely *et al*., 2004) and that occurs even when omega is over 1. This

response is valid both for intra- and extra-cellular calcification and concerns both aragonitic and calcitic organisms. However it should be outlined that these studies are carried out on short term periods and on species only. For the same reason it is very difficult to predict and to study community responses to ocean acidification (Royal Society report, 2005).

Because the $CaCO_3$ mineral calcite is less soluble than the form aragonite, saturation horizon (the depth at which $CaCO_3$ begin to dissolve) is shallower.

Macroalgae has both high Mg-calcite and aragonite. Any changes in calcification will have also important feedbacks both on biodiversity and on the global carbon cycle.

1.3. General aim

Global changes will lead biological systems to face up to new environmental scenario both in the terrestrial and aquatic environments; for this reason serious interest has been addressed to study acidification effects on marine organisms.

Particular attention has to be focused on aquatic primary producers, as macroalgae, because trophic webs are strictly dependent on them. Macroalgae are a crucial component of coastal ecosystems: they provide both habitat and nursery areas for numerous species including those belonging to microbial loop, fundamental for the nutrients recycling. Moreover, macroalgal communities are sensitive to pollution derived from coastal anthropogenic activities and for that they are used as biological indicators.

Even though more and more studies have been carried out during last years on the responses of calcareous marine organism to acidification (see Fabry *et al*., 2008 for a review), little have been achieved on non-calcareous macroalgal species and more less on changes in macroalgal communities structure and functioning. Physiological approaches have been performed on the effects of higher pH values (Beer *et al.*, 1996; Johnston *et al*., 1992; Raven, 2003; Menéndez *et al.*, 2001) but very few in relation to an increase in water acidification (Barott *et al*., 2009; Hurd *et al*., 2009)).

It needs to extend ecological studies from individual organism to communities and systems in order to predict future coastal marine scenario. Although temperature is known to have ubiquitous effects on rate of physiological processes and the integrity of macromolecular structures, water chemistry is changing rapidly and greatly affecting the physiology of marine organisms, calcareous and not. In order to assess the capacity for a given genotype to produce different genotypes in response to different environment, it needs to integrate comparative physiology and evolutionary biology. For this reason, this PhD project, focused on the effects of water acidification on shallow benthic algae, has been carried out with an integrated approach by using taxonomic, ecologic, physiologic and molecular tools.

The thesis is therefore subdivided in the following chapters:

Chapter 2: Changes in the algal community structure along natural pH gradients.

The study of the community composition is fundamental to understand how organisms walk the tightrope between stability (normal pH) and change (water acidification). Moreover, changes in the colonizing pattern along a natural pH gradient has

been invistigated. Alterations in the settlement of species succession can contribute to the final structure and functioning of the community

Chapter 3: Different responses in ecophysiological traits in target species along a pH gradient.

The functional diversity of organisms is responsible of their ability to compete and overcome the water acidification.

Chapter 4: Changes in genetic diversity along a natural pH gradient.

Molecular diversity can represent the window to look at the genome variability in stressed environments.

2. Changes in the algal community structure along natural pH gradients

2.1. Introduction

The oceans currently absorb over 25 million tons of anthropogenically produced CO_2 every day causing unprecedented changes to ocean chemistry (IPCC, 2007). Observations and models indicate that the average pH of the surface ocean has declined from 8.2 by 0.1 units since pre-industrial times due to CO_2 emissions and is projected to be around mean pH 7.8 by the end of the century (The Royal Society, 2005). As well as lowering pH, increasing CO_2 levels are lowering calcium carbonate saturation states but increasing the amounts of bicarbonate ions (HCO_3^-). Laboratory and mesocosm experiments have shown that these changes in carbonate chemistry can significantly affect carbon fixation by photoautotrophs which may cause global ecological disturbances over the coming decades (Kroeker *et al*., 2010) although there are still too few data to confirm that (Hendricks & Duarte, 2010).

Benthic photoautotrophs exhibit mixed responses to ocean acidification, indicating that there will be changes in their settlement, competition and dominance that will have knock-on effects on coastal ecosystems (Connell & Russell, 2010). However, it is very difficult to scale-up the observations undertaken to date to predict the effects of increasing CO_2 emissions at the ecosystem level since most studies have been short-term (<1 year) on single or small groups of species. Some cyanobacteria grow well with CO_2 enrichment and their increased N_2 fixation may alter ocean biogeochemistry (Fu *et al*., 2008; Kranz *et al*., 2009). Some marine algae also benefit from higher CO_2 levels, enhancing their growth (Gao *et al*., 1999, Kübler *et al*., 1999; Riebesell *et al*., 2007) and calcification (Iglesias-Rodriguez *et al*., 2008), although the effects can vary between closely related species and even between strains of the same species (Langer *et al*., 2009). In general, seagrasses seem able to thrive under high CO_2 conditions (Palacios & Zimmerman, 2007; Hall-Spencer *et al*., 2008), although the associated epiphytic algal cover falls as CO_2 levels increase (Martin *et al*., 2008). Coralline algae appear to be amongst the most sensitive photoautotrophs as they have a skeletal mineralogy that dissolves easily at predicted levels of calcium carbonate saturation (Gao *et al*., 1993; Martin & Gattuso, 2008) and this is a particular concern as these algae are of fundamental ecological importance in a range of coastal habitats worldwide (Nelson, 2009).

Macroalgal communities perform a range of ecosystem services in shallow coastal systems such as providing food, forming substrata for settlement, offering protection from predators and shelter from disturbances, other than breeding and feeding for many commercially important species (Choi *et al*., 2002). Macroalgal communities are sensitive to

anthropogenic disturbances and so are used to assess the status of coastal ecosystems, for example under the European Water Framework Directive (Orfanidis *et al*., 2001; Ballesteros *et al*., 2007). The sensitivity of Mediterranean macroalgal communities is well documented: Ulvales can become dominant in nutrient enriched areas (Pergent, 1991); habitat-forming *Cystoseira* spp. are lost from large stretches of polluted coastlines (Rodriguez-Prieto *et al*., 1996; Thibaut *et al.,* 2005; Mangialajo *et al*., 2008); ecosystems have been degraded by the invasive behaviour of introduced species such as *Caulerpa* spp. (Balata *et al*., 2004).

In addition to species analysis, a morpho-functional approach has been also applied. The first analysis was carried out to assess which taxonomic units can survive in acidified seawater and therefore how communitiies change their structure. However, a taxonomic unit may be composed by species possessing very different structural and functional properties. To overcome these problems, ecologists have tried to group organisms with similar structural and functional characteristics with the aim to obtain a better understanding, and possibly generalization, of the functioning of the ecosystems. The functional groups may be defined utilising elements that bear a certain set of common structural and/or functional features. These include quality criteria (i.e. size/form, physiological and life strategy characteristics), temporal appearance and distributional characteristics, whereas, at the species level, the functional groups may include taxonomic units as well (Salmaso & Padisak, 2007) Functional groups were used in different fields of ecological research, including vegetation studies (Leishman & Westoby, 1992), prediction of effects of global climate change (Gitay *et al*., 1999), conservation biology (Pressey *et al*., 1993), studies of microorganisms (Meyer, 1993), fungi (Oberwinkler, 1993), macrophytes (Shipley *et al*., 1989) and macroinvertebrates (Usseglio-Polatera *et al*., 2000). In this work classifications based on the morphological and functional characteristics of benthic macroalgae were used to evaluate the effect of water acidification on the functional richness of the shallow communities.

Algal morpho-functional groups were used by Steneck and Watling (1982) to study susceptibility of some thallus structures to grazing pressure by molluscs. Later, there have been made many attempts to use algal group classification in community analysis and prediction according to diverse environmental and ecological factors (Littler & Littler, 1982; Phillips *et al*., 1997; Lavery & Vanderklift, 2002; Guidetti, 2006; Bulleri & Benedetti-Cecchi, 2008).

Physical and chemical parameters interfer with the development process of macroalgal species which may lead to significative changes in their community structure

(Falace & Bressan, 1994; Branco *et al*., 2005) and consequently the loss of services they offer.

Also, differences in susceptibility of macroalgae to ocean acidification may completely alter coastal ecosystems as changes in algal communities lead to changes in herbivore diversity and abundance (Benedetti-Cecchi *et al*., 2001; Darcy-Hall & Hall, 2008). Such changes in species distributions and abundances are expected to propagate up through the trophic levels of marine food webs (Guinotte & Fabry, 2008; Fabry *et al*., 2009) but little research has been carried out on the effects of ocean acidification at the ecosystem level (Wootten *et al*., 2008).

To improve knowledge regarding responses of marine ecosystems to ocean acidification we assess macroalgal community changes at the Castello Aragonese (Ischia) site, already described by Hall-Spencer *et al.* (2008), where increasing CO_2 levels cause a natural pH gradient at ambient temperature and salinity. Changes in seaweed species diversity and abundance along a pH gradient have been examined and also changes in morphological groups to help link changes in community structure to ecosystem functioning (Micheli & Halpern, 2005).

Finally, early colonization stages and processes have been investigated in order to assess which could be the effects of water acidification on macroalgal community development when new substrata, derived both by natural and anthropogenic perturbation, are available.

2.2. Material and Methods

2.2.1. pH monitoring

pH was continuously monitored at Castello by means of a prototype pH-meter created by SCRIPPS, Institution of Oceanography, University of California (Figure 9). It is constituted by an automated data-logger which records every hour. They were moved periodically between the Southern and the Northern side.

Figure 9: pH-meter

2.2.2. Macroalgal community: natural substrate

In order to characterize Macroalgal community sampling was carried out during autumn 2007 and autumn 2008. Samples were collected from 0.70-1.0 m below mean sea level along the pH gradient removing all organisms from the bedrock using a hammer and chisel from 20*20 quadrats, according to sampling techniques already developed for this Mediterranean rocky shore habitat (Ballesteros, 1986). In 2007 a hierarchical experimental design was used on the Southern side of Castello: three 30 m wide sectors where chosen at least 50 m apart one another: sector S1 was at normal pH (8.1); S2 had mean pH 7.83 and S3 had mean pH 6.72. In each sector three 5 m wide zones were chosen at least 6 m apart (A-I, Figure 10).

Figure 10: Experimental sampling design in 2007.

In each zone, three randomly placed quadrats were sampled. Sector was a fixed factor with three levels, each zone (A-I) was a fixed factor with three levels nested in each Sector whereas quadrats were random replicates (Figure 10). In order to investigate the combined effect of water acidification with coastal exposition, a new sampling was performed in autumn 2008, adding the Northern side of the Castello and a new control site Sant'Anna (C3) having the same exposition of S3 but normal pH (8.1). Four replicates were randomly chosen in S1, S2, S3 on the South, in N1, N2, N3 on the North, and in C3 (Control) (Figure 11).

Figure 11: Experimental sampling design in 2008.

Samples were preserved in 4% formalin:seawater. Algae were identified to species level and their coverage was quantified as percentage of horizontal surface measured after spreading the algal thalli in a laboratory tray (Ballesteros, 1986). Coverage of 0.01% was assigned to species with negligible abundance. Light microscopy was used to detect the presence of reproductive structures: their occurrence was compared only in those species recorded at the three pH conditions. A taxonomic list was compiled. Later, species were grouped into three morphological categories, namely 'crustose', 'turf' and 'erect' according to the thallus size and form. Algal turfs consist mostly of tiny filaments with canopy height of less than 10 mm; erect algae are larger (>10mm in height), canopy-former and can be either fleshy or calcareous; crustose algae are prostrate forms, calcareous and non-calcareous (Diaz-Pulido & McCook, 2008).

Another classification was achieved according to structural and functional thallus complexity (Table 1).

Table 1: Classification and description of each Agal Group (AG) with respect to the thallus structure

ALGAL GROUP (AG)	DESCRIPTION
1	Uniseriate filamentous algae
2	Thinly corticated and polysiphonous algae
3	uni- or multilayered foliose algae
4	Globular algae
5	Non-calcareous encrusting algae
6	Corticated Laminar algae
7	Corticated terete algae
8	Leathery Macrophytes
9	Semi-calcareous laminar algae
10	Uni-layered Calcareous algae
11	Calcareous crustose algae
12	Articulated calcareous algae

Each identified species has been assigned to one of these Algal Groups (AG). Quantitative dominance has been calculated for each Algal group as DR%, that is:

$$DR\% = \Sigma Ri/Rt \times 100$$

where *Ri* is the species cover and *Rt* is the total cover in ~~a~~ the quadrat.

Multivariate analysis (nMDS) using Primer-E software (Clarke & Warwick, 2001) were performed to examine community structure, at both species and algal groups levels. Finally, differences in species and group composition among sectors have been evaluated by means of similarity analysis (ANOSIM, Clarke & Warwick, 2001).

2.2.3. Macroalgal community: artificial substrata early colonization experiment

In order to determine the effect of acidification on early algal colonization, 15*15 lavic stone tiles were used. The tile material was chosen according to the volcanic origin of the rocky substrate at the Castello area. They were fixed on the rock by means of screws in their center.

Nine tiles were set up in each sector (S, N, and C). Algal colonization was followed from April to July 2008. Apart the first month, three replicates were removed in each sector after two, three, and four months (May, June and July, respectively).

Once removed, the tiles were preserved in 4% formalin:seawater for 1-2 days and then mantained in ethanol 70° until laboratory observations.

By using a grid net (2.5 cm), three sub-quadrats randomly selected were observed under the stereomicroscopy (Figure 12). Sub-quadrats of the edge and the inner part were not included in the analysis.

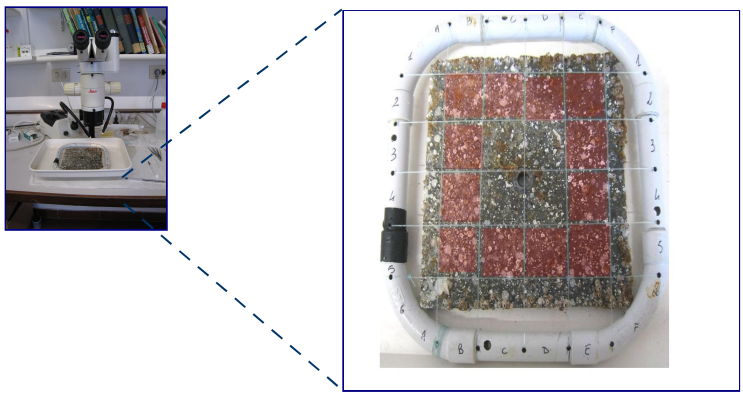


Figure 12: Highlighted red area on the tile from which sub-qudrats were randomly chosen.

2.2.4. Community data analyses

For natural communities, differences among sectors were determined by calculating species number, percent cover of each species, species diversity (estimated as $\log_e$ based Shannon-Weaver diversity Index, H'), percent cover of the crustose, turf, erect categories and Algal Groups. K-dominance curves of species coverage data were plotted and Principal Component Analysis (PCA) was performed using Syn-tax software (Podani, 2001). Species with <0.03 percentage cover were excluded from this analysis.

ANOVA and the *Post hoc* Tukey's test (GraphPad Prism package) were applied to test any differences among the three sectors. Homogeneity of variances was always tested; when not met, data were log-transformed.

Differences in species, algal groups, and abundances among sectors of both natural communities and early colonization were evaluated by means of non- Multi Dimensional Scaling (n-MDS), similarity analysis (ANOSIM), and SIMPER analysis. K-Dominance, ANOSIM, SIMPER and nMDS analyses were performed with PRIMER 5 (Clarke & Warwick, 2001).

2.3. Results

2.3.1. pH monitoring

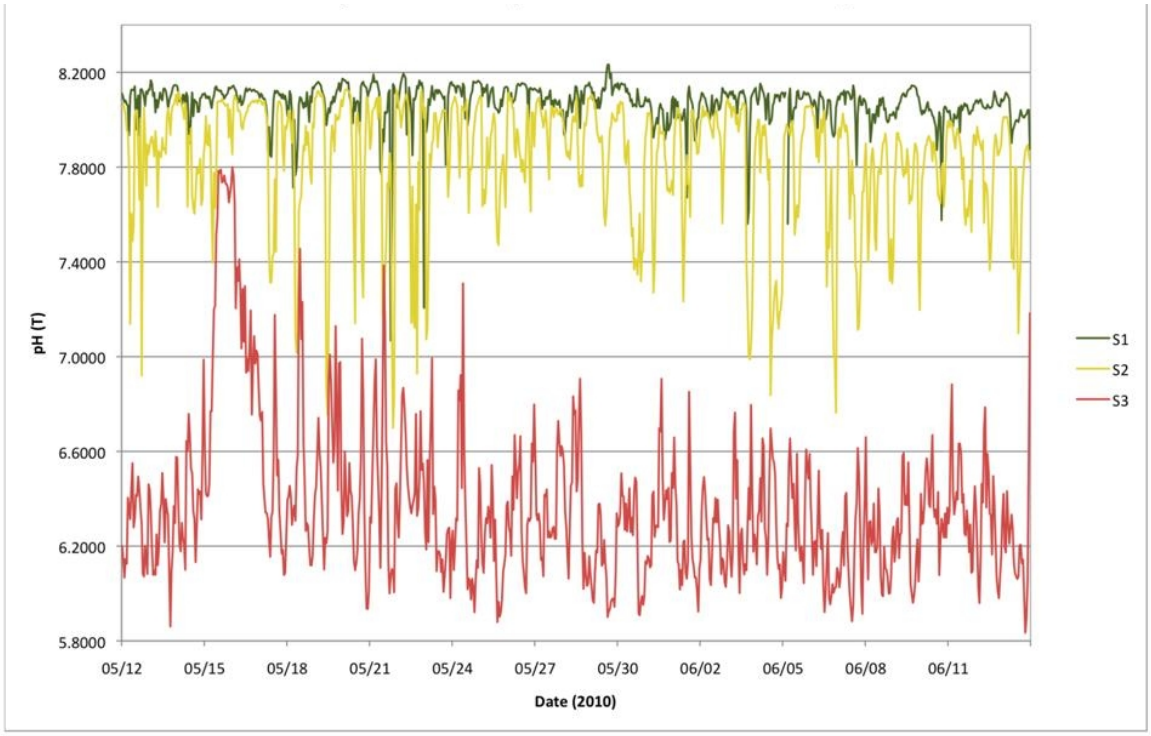


Figure 13: Variability in seawater pH on the Southern side of Castello (Kroeker et al., in prep.)

Daily changes in pH seawater were clear on the South side and on the North side (Figure 13 and Figure 14 respectively). Sector were well characterized by different mean pH values. Higher pH variability were reported in more acidified seawater.

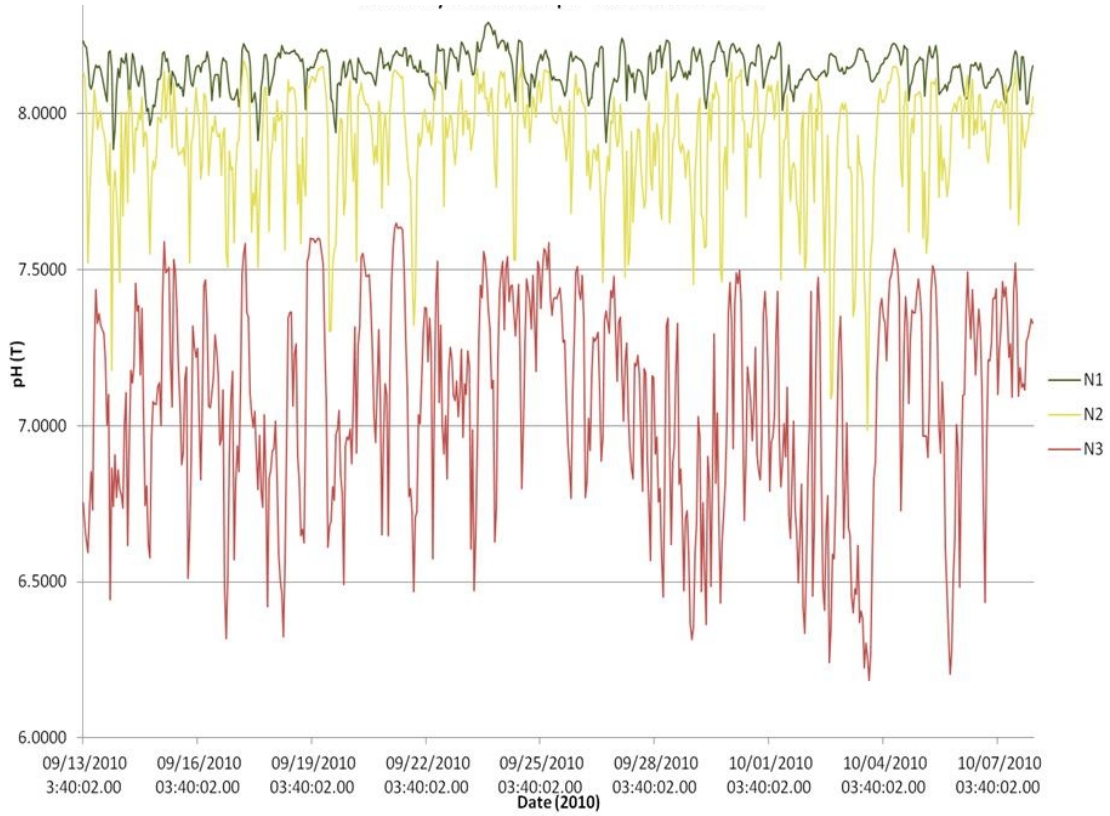


Figure 14: Variability in seawater pH on the Northern side of Castello (Kroeker *et al.*, in prep.).

2.3.2. Macroalgal community: natural substrata

In 2007 a total of 101 macroalgal taxa were recorded in 27 quadrats, including 71 Rhodophyta, 16 Ochrophyta and 15 Chlorophyta (Table 2).

Table 2: Total number of Rhodophyta, Ochrophyta and Chlorophyta species and total number of erect, crustose and turf species collected in 2007.

	S1 pH=8.1	S2 pH=7.8	S3 pH=6.7
Rhodophyta	50	48	10
Ochrophyta	12	11	6
Chlorophyta	12	11	3
Erect	17	14	7
Crustose	19	20	2
Turf	38	36	10
Total	74	70	19

The highest species richness (Table 2) was sampled at S1 (pH=8.1); 5% fewer species were sampled at S2 (pH=7.8) and 72% fewer species were sampled at S3 (pH=6.7). ANOVA and a *post-hoc* Tukey test showed highly significant reductions in species diversity (H') as

CO_2 levels increased (H'_{S1}=2.29 ± 0.07; H'_{S2} =1.88 ± 0.07 and H'_{S3}, =1.18 ± 0.05, respectively) (F=25.62; p<0.0001, S1=S2>S3) in 2007.

In the 2008's sampling a total of 113 macroalgal taxa were identified in 26 quadrats, including 80 Rhodophyta, 19 Ochrophyta and 14 Chlorophyta (Table 3). As regard as species diversity, the same pattern was observed in 2008, compared to 2007 in both Southern and Northern side (F=9.68; p<0.0001): H'_{S1} =2.68 ± 0.06; H'_{S2} =2.31 ± 0.16 and H'_{S3} =1.36 ± 0.12, whereas H'_{N1} =1.69 ± 0.48; H'_{N2} =1.52 ± 0.30 and H'_{N3} =1.66 ± 0.22, revealing the higher diversity for the Southern side compared to the Northern. However, the highest diversity was observed in C3 (H'_{C3} =2.44 ± 0.21) even though there were no statistical differences with S1 (p>0.05).

Table 3: Total number of Rhodophyta, Ochrophyta and Chlorophyta species and total number of erect, crustose and turf species collected in 2008

	C3 pH=8.1	S1 pH=8.1	S2 pH=7.8	S3 pH=6.7	N1 pH=8.1	N2 pH=7.9	N3 pH=7.09
Rhodophyta	33	48	44	14	32	29	24
Ochrophyta	11	11	11	6	6	9	9
Chlorophyta	9	11	11	4	7	6	6
Erect	13	13	17	10	9	12	11
Crustose	13	17	14	2	17	15	6
Turf	27	40	35	12	20	17	22
Total	53	70	66	24	45	44	39

Clear shifts were evident in species distribution along the CO_2 gradient. The kite graph (Figure 15) shows the distribution of the most abundant species along the different zones (A-I) identified in the 2007 sampling. The same pattern has been recorded one year later, on both side of Castello (Figura 16).

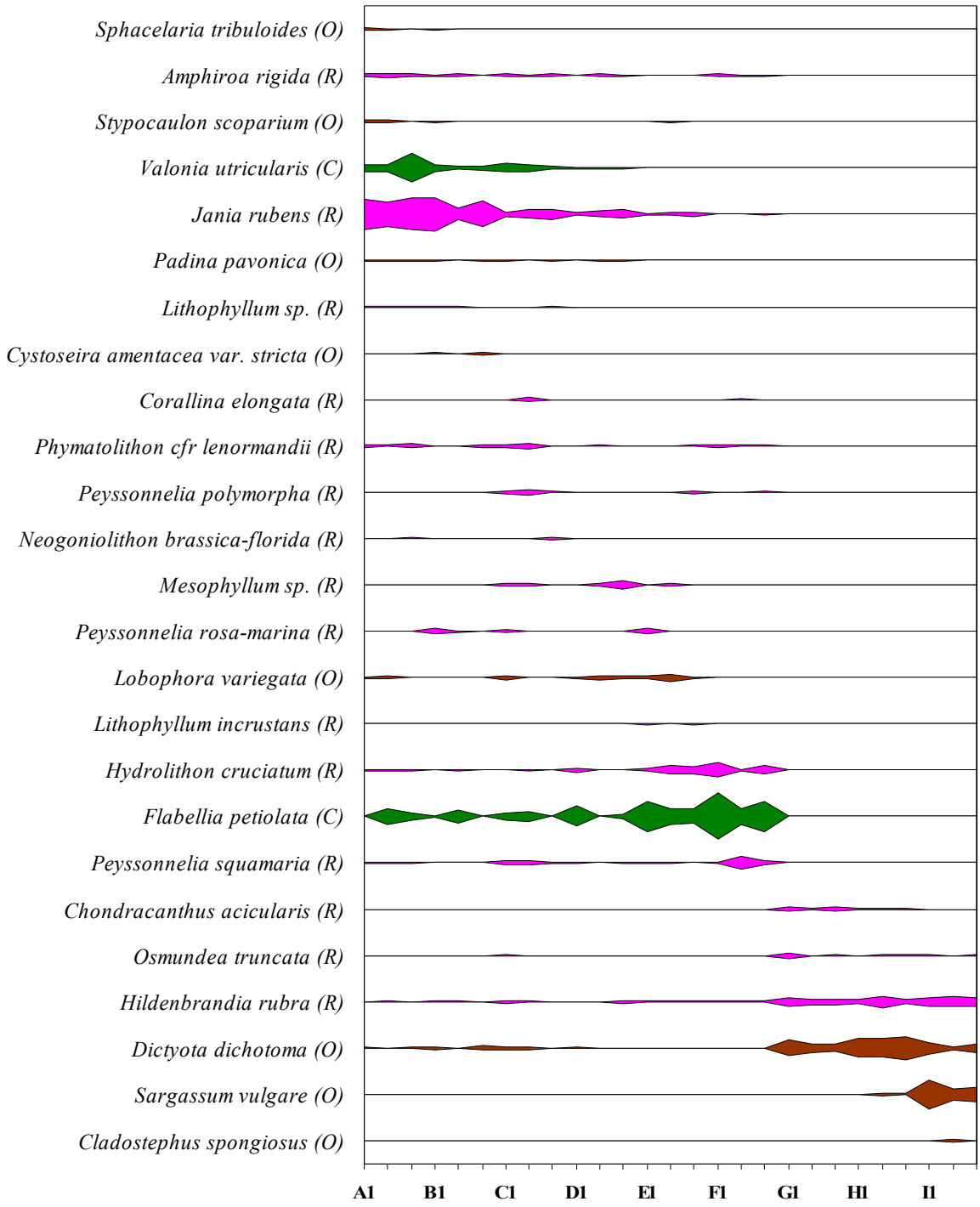


Figure 15: Kite diagram showing distribution of the most abundant macroalgal species (>3% coverage) in 27 20*20 cm quadrats along a pH gradient from S1 (pH=8.1), S2 (pH =7.8) and S3 (pH =6.7) in Autumn 2007. R = Rhodophyta, O = Ochrophyta, C = Chlorophyta.

Errore. Non si possono creare oggetti dalla modifica di codici di campo.
Figura 16: Kite diagram showing distribution of the most abundant macroalgal species in 26 20*20 cm quadrats along a pH gradient on South (S), North (N), and Control (C) sites (S1: pH=8.1; S2: pH=7.8;

S3: pH=6.7; N1: pH=8.14; N2: pH=7.87; N3: pH=7.09; C3: pH= 8.1). R = Rhodophyta, O = Ochrophyta, C = Chlorophyta.

In 2007, fourteen species of calcareous algae were sampled at normal pH, but only 7 at mean pH 7.8 and none at mean pH 6.7, where both calcite and aragonite saturation states were <1 (Figure 17A and Figure 17B). The same trend was observed in 2008 (Figure 17C and Figure 17D). In this year no calcareous species were recorded in the most acidified site on the South side (S3) while they were present (only four species) on the North side (N3).

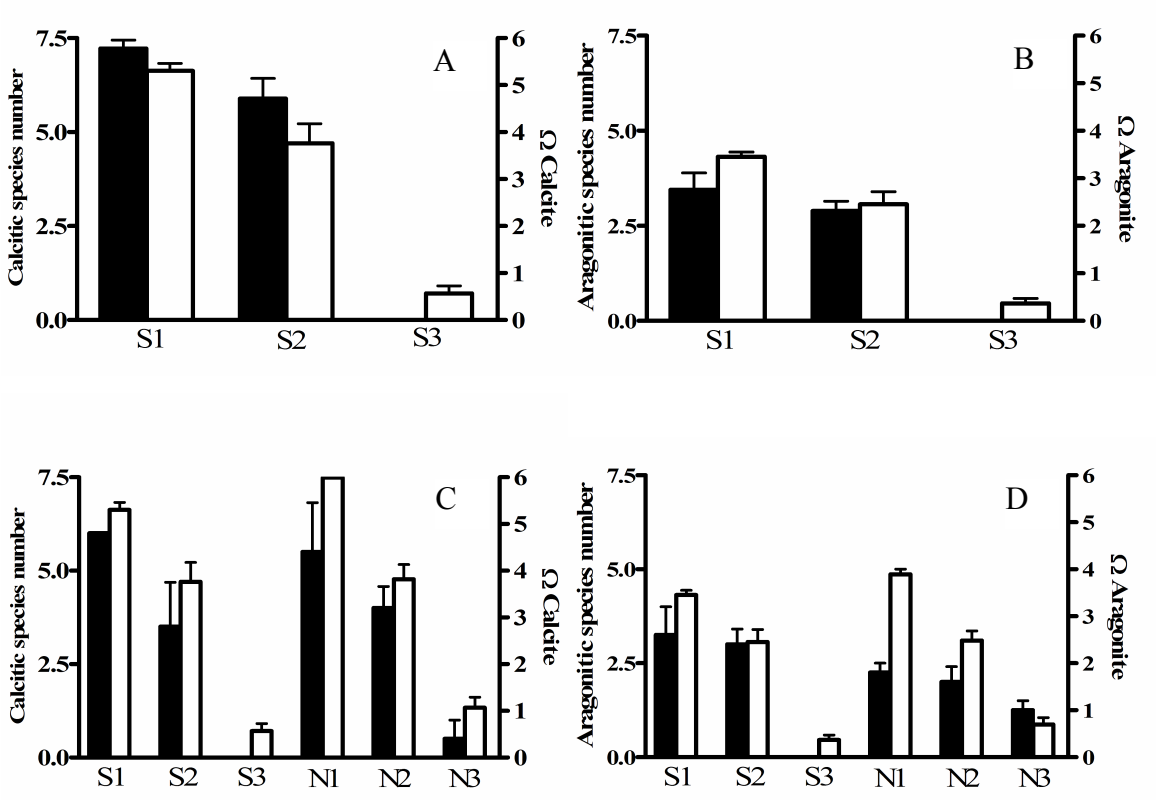


Figure 17: Calcitic (A) aragonitic (B) macroalgal species number (black bars, n=9) in 2008 along the Southern side pH gradient (from pH=8.1, to pH=7.8 and pH=6.7) and along the Northern side gradient (from pH =8.14, to pH=7.87 and pH=7.09. Calcitic (C) aragonitic (D) macroalgal species number (black bars, n=4) in 2008 along both the Southern and the Northern side pH gradient. Corresponding Ω values (white bars) are reported. (Mean ± s.e.).

ANOVA results (Table 4) show a significant decrease in calcitic species number at mean pH 7.8 where the overall cover of calcareous algae was much lower than at mean pH 8.1. Most calcitic (e.g. *Jania rubens*, *Amphiroa rigida*, *Phymatolithon* cf. *lenormandii*) and aragonitic species (e.g. *Peyssonnelia* spp., *Padina pavonica* and *Halimeda tuna*) were more abundant at mean pH 8.1 (both in 2007 and 2008), although the thin calcitic crust *Hydrolithon cruciatum* and the lightly calcified aragonitic crust *Peyssonnelia squamaria* were more abundant at mean pH 7.8 (S2).

Table 4: ANOVA results for carbonate saturation states ($\Omega_{calcite}$ and $\Omega_{aragonite}$) and ~~the~~ species richness of aragonitic and calcitic calcareous species in 2007 and 2008. Both $\Omega_{calcite}$ and $\Omega_{aragonite}$ values had to be transformed to Log_{10} before the ANOVA analysis.

2007 South	**d.f.**	**F**	**P**	**Tukey's test**
Calcitic species	2	130.5	<0.0001	S1>S2>S3
$\Omega_{calcite}$	2	92.95	<0.0001	S1=S2>S3
Aragonitic species	2	38.65	<0.0001	S1=S2>S3
$\Omega_{aragonite}$	2	93.31	<0.0001	S1=S2>S3
2008 South				
Calcitic species	2	19.24	<0.001	S1=S2>S3
$\Omega_{calcite}$	2	92.95	<0.001	S1=S2>S3
Aragonitic species	2	13.46	<0.05	S1=S2>S3
$\Omega_{aragonite}$	2	93.31	<0.001	S1=S2>S3
2008 North				
Calcitic species	2	8.46	<0.05	N1=N2>N3
$\Omega_{calcite}$	2	51.42	<0.0001	N1=N2>N3
Aragonitic species	2	2.79	0.11	N1=N2=N3
$\Omega_{aragonite}$	2	51.27	<0.0001	N1=N2>N3

No differences were recorded among similar pH sectors (e.g. S1 of 2007, S1 of 2008, N1 and C3) between the two years for both calcite and aragonite species number.

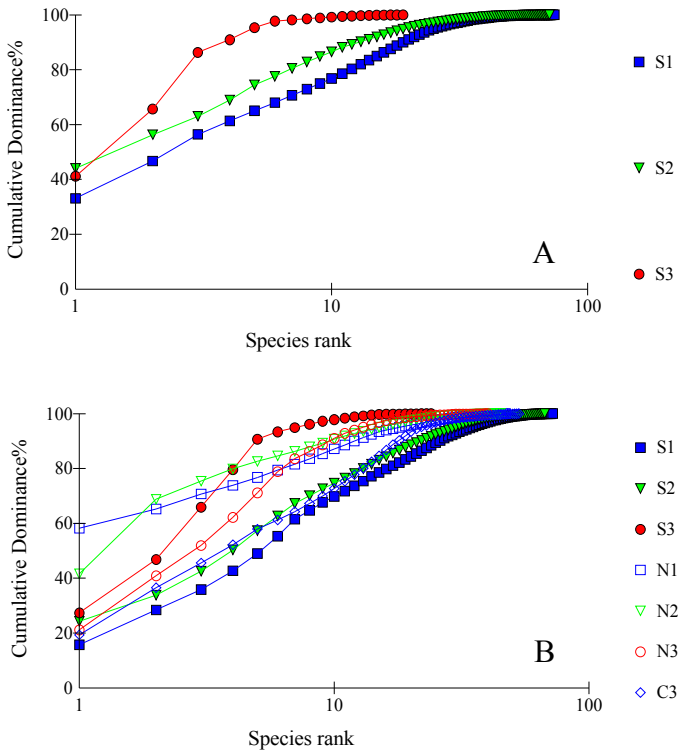


Figura 18: K-Dominance curves for macroalgal coverage along the pH gradients in the Southern sites in 2007 (A), and in the Southern, Northern and Control sites in 2008 (B).

The diversity and abundance of non-calcareous algae also shifted along the CO_2 gradient. For example, the Ochryophyte *Dictyota dichotoma* and the Rhodophyte *Hildenbrandia rubra* were present along the whole pH gradient with their highest percentage cover at mean pH 6.7 (S3). In contrast, *Sargassum vulgare*, *Cladostephus spongiosus*, *Osmundaea truncata* and *Chondracanthus acicularis* were confined to the most acidified zone in 2007 samplings while in 2008 the presence of *S. vulgare* specimens were also recorded at different pH values but with neglectful covers (Figure 15 and Figura 16).

These differences in species richness and abundance affected the structure of the community, as shown in Figura 18A (for the year 2007), where the cumulative percentage dominance of species is ranked on a logarithmic scale. The highest algal community complexity was evident at S1, whereas at S2 there was a shift towards greater dominance by fewer species and at S3 the simplified community was dominated by very few species. The same pattern was recorded the year later on both sides but with a less richness in species on the Northern side (Figura 18B).

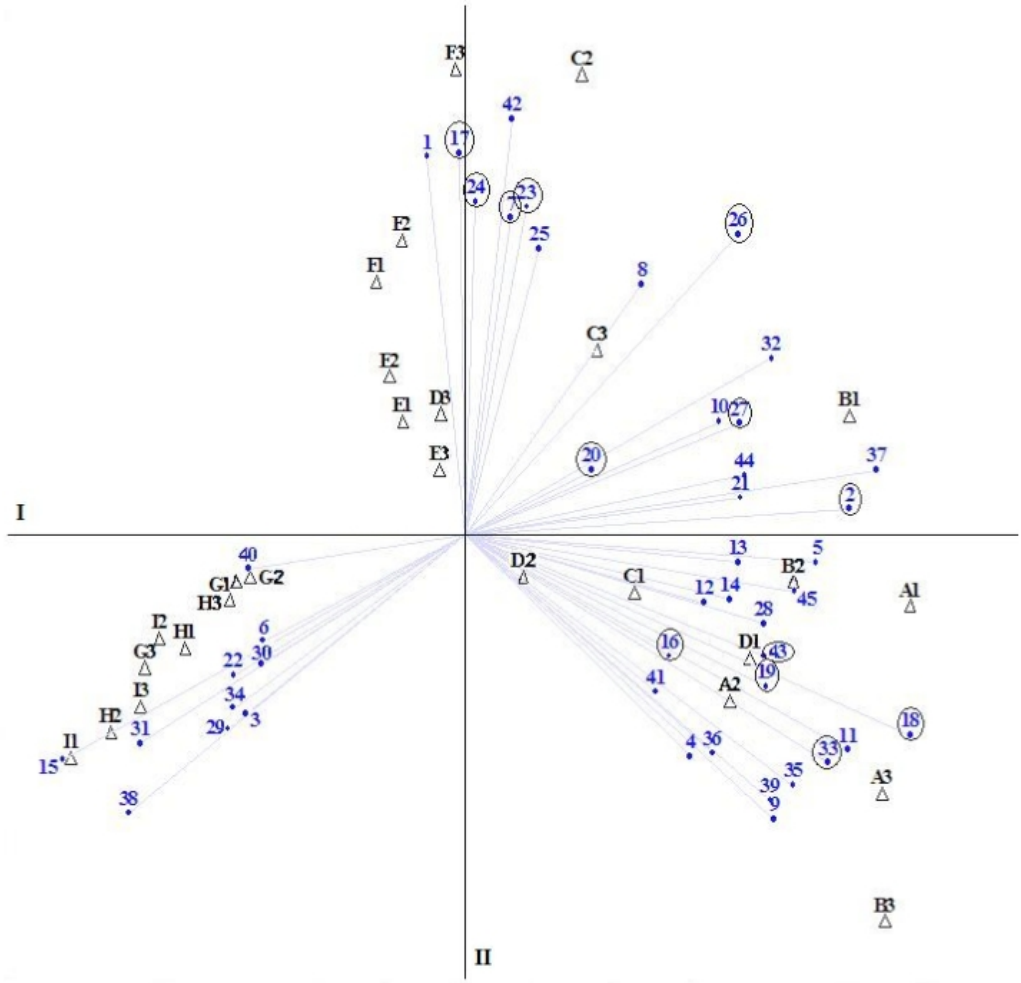


Figure 19: Principal component analysis (PCA) ordination for macroalgal cover data collected along a pH gradient in 2007. Triangles represent sampling locations, labelled with letters corresponding to Fig. 1.

Dots represent individual species (calcified species circled) and numbered correspond to species listed in Appendix I.

The PCA biplot (only performed for 2007 data set) (Figure 19) displays both sample (triangles) and species (numbers with vectors) distribution between the two principal components. The first component explains 26.8% of the species distribution with the second component it reaches 38.8%. The biplot displays a typical horseshoe shape because of non-linear relationships among species, for example due to competition for space and light.

Samples A-C group together on the right side of the diagram and were from mean pH 8.1 (S1) with many calcified species present (circled), samples D-F (from mean pH 7.8) group in the centre with fewer calcifiers and samples G-I from the most acidified area (S3) show a sharp discontinuity from the others. This differentiation is due both to species composition and their abundance differences among the three sites. Statistical differences in community composition among sectors are highlited by ANOSIM results (Table 5). SIMPER analysis of percentage species cover (Table 6 and Table 7) shows that averaged species abundances allow discrimination one sector from another.

Table 5: ANOSIM Global Test for species in 2007. Sample statistic (Global R): 0.886. Significance level of sample statistic: 0.1%. Number of permutations: 999 (Random sample from a large number). Number of permuted statistics greater than or equal to Global R: 0.

Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number ≥ Observed
S3 - S2	1	0.1	24310	999	0
S3 - S1	1	0.1	24310	999	0
S2 - S1	0.559	0.2	24310	999	1

This differentiation is quantified by dissimilarity percentage. SIMPER attributed the highest average similarity among quadrats of sector S3 dominated by *Dictyota dichotoma* and the highest dissimilarity (>89%) among S3 and the other two sectors (Table 6).

Table 6: SIMPER similarity analysis of macroalgal species coverage within sectors along the pH gradient in 2007.

Species	Average Abundance	Average Similitude	Similitude/ SD	Contribution %	Cumulative %
Sector S1					
Average similarity: 57.02					
Jania rubens	69.22	11.1	3.04	19.46	19.46
Valonia utricularis	28.5	6.69	6.3	11.73	31.19
Amphiroa rigida	10.28	4.53	2.96	7.95	39.14
Flabellia petiolata	20.33	2.67	0.7	4.68	43.82
Dictyota dichotoma	6.09	2.48	1.92	4.35	48.17

Sector S2					
Average similarity: 49.58					
Flabellia petiolata	66.22	12.73	1.54	25.68	25.68
Hydrolithon cruciatum	18.53	5.69	1.19	11.48	37.15
Jania rubens	10.09	3.83	1.16	7.73	44.89
Hildenbrandia rubra	4.44	3.78	2.42	7.63	52.52
Lobophora variegata	8.39	2.8	0.8	5.64	58.17
Sector S3					
Average similarity: 64.35					
Dictyota dichotoma	44.17	25.12	3.12	39.03	39.03
Hildenbrandia rubra	26.28	21.07	6.37	32.75	71.78
Osmundea truncata	4.7	5.11	1.8	7.94	79.71
Chondracanthus acicularis	4.96	4.05	0.83	6.29	86.01
Sargassum vulgare	22.17	3.85	0.54	5.99	91.99

The highest percentage contributions to the dissimilarities were due to the erect calcareous Rhodophyta *Jania rubens,* which was dominant at mean pH 8.1 (S1), the erect Chlorophyta *Flabellia petiolata,* which was dominant at mean pH 7.8 (S2), and the erect Ochrophyta *Dictyota dichotoma,* which was dominant at mean pH 6.7 (S3). Of the turf forming species, the loss of *Valonia utricularis* and replacement by *Osmundea truncata* drove much of the community shift as pH decreased (Table 7).

Table 7: SIMPER dissimilarity analysis of macroalgal species coverage between pairs of sectors in 2007.

Species	Average Abundance	Average Abundance	Average Dissimilarity	Contribution %
Sectors S1 & S2				
Average dissimilarity: 58.22				
	Sector S1	**Sector S2**		
Jania rubens	69.22	10.09	5.39	9.25
Flabellia petiolata	20.33	66.22	5.05	8.67
Valonia utricularis	28.5	2.57	3.61	6.2
Hydrolithon cruciatum	2.53	18.53	2.85	4.9
Peyssonnelia squamaria	3.87	8.86	1.95	3.34
Sectors S1 & S3				
Average dissimilarity: 89.61				
	Sector S1	**Sector S3**		
Jania rubens	69.22	0	9.97	11.12
Valonia utricularis	28.5	0.12	5.97	6.66
Dictyota dichotoma	6.09	44.17	5.36	5.98
Hildenbrandia rubra	3.18	26.28	4.44	4.95
Flabellia petiolata	20.33	0	4.14	4.62
Sectors S2 & S3				
Average dissimilarity: 89.37				
	Sector S2	**Sector S3**		
Flabellia petiolata	66.22	0	11.74	13.13
Dictyota dichotoma	1.34	44.17	8.94	10.01
Hydrolithon cruciatum	18.53	0	5.96	6.67
Hildenbrandia rubra	4.44	26.28	4.9	5.48
Sargassum vulgare	0	22.17	4.63	5.18

Significative differences among communities sampled in 2008 are highlighted by ANOSIM (Table 8).

Table 8: ANOSIM Global Test for species in 2008. Sample statistic (Global R): 0.765; significance level of sample statistic: 0.1%; Number of permutations: 999 (Random sample from a large number); Number of permuted statistics greater than or equal to Global R: 0

Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number Observed
S1 - C3	0.893	6.7	15	15	1
S1 - S2	0.333	8.6	35	35	3
S1 - S3	1.	2.9	35	35	1
S1 - N1	0.938	2.9	35	35	1
S2 - S3	0.958	2.9	35	35	1
S2 - N2	0.646	5.7	35	35	2
S2 - N3	0.458	2.9	35	35	1
S3 - N3	0.74	2.9	35	35	1
N1 - N2	0.188	17.1	35	35	6
N1 - N3	0.74	2.9	35	35	1
N2 - N3	0.427	8.6	35	35	3

SIMPER analysis for 2008 data set showed the highest similarity among quadrats for S3 again, where *D. dichotoma* v. *intricata* was dominant (Table 9).

The highest dissimilarity was recorded between S3 and both S1 and S2 (>72%) where *D. dichotoma* v. *intricata*, *Sargassum vulgare* and *Cladophora prolifera* had the highest abundances in S3 whereas *Jania rubens* characterized S1.

Table 9: SIMPER similarity analysis of macroalgal species coverage within sectors along a pH gradient in 2008.

Species	Average Abundance	Average Similitude	Contribution %	Cumulative %
Sector C3				
Average similarity: 56.49				
Peyssonnelia squamaria	11.50	4.11	7.28	7.28
Titanoderma pustulatum	3.90	3.35	5.93	13.21
Dasya hutchinsiae	3.50	3.27	5.79	19.00
Peyssonnelia polymorpha	21.90	3.21	5.69	24.69
Titanoderma corallinae	2.75	3.13	5.53	30.22
Sector S1				
Average similarity: 55.40				
Jania rubens	19.38	4.86	8.78	8.78
Lobophora variegata	15.63	4.58	8.27	17.04
Valonia utricularis	7.75	3.41	6.15	23.19
Hildenbrandia crouaniorum	7.63	3.20	5.78	28.97
Amphiroa rigida	9.20	2.87	5.17	34.14

Sector S2				
Average similarity: 47.43				
Hildenbrandia crouaniorum	28.25	5.95	12.55	12.55
Dictyota dichotoma v. intricata	8.13	4.19	8.84	21.40
Peyssonnelia squamaria	2.95	2.93	6.17	27.57
Valonia utricularis	2.38	2.64	5.56	33.13
Flabellia petiolata	11.00	2.47	5.22	38.35
Sector S3				
Average similarity: 61.02				
Hildenbrandia crouaniorum	63.20	11.01	18.04	18.04
Dictyota dichotoma v. intricata	89.00	7.85	12.87	30.91
Sargassum vulgare	44.38	6.25	10.25	41.16
Dictyota dichotoma	8.80	6.24	10.22	51.38
Cladophora prolifera	62.03	5.46	8.95	60.33
Sector N1				
Average similarity: 47.02				
Flabellia petiolata	42.25	8.29	17.63	17.63
Peyssonnelia squamaria	5.08	4.88	10.38	28.01
Bryopsis plumosa	4.00	4.62	9.82	37.83
Hydrolithon cruciatum	1.93	3.93	8.35	46.18
Hildenbrandia crouaniorum	1.03	3.42	7.27	53.45
Sector N2				
Average similarity: 45.00				
Flabellia petiolata	33.50	8.83	19.62	19.62
Corallina elongata	51.25	6.66	14.80	34.43
Hildenbrandia crouaniorum	5.50	6.18	13.74	48.17
Falkenbergia sp.	0.98	3.65	8.10	56.28
Peyssonnelia rosa-marina	0.85	3.58	7.96	64.23
Sector N3				
Average similarity: 44.04				
Hildenbrandia crouaniorum	31.63	9.55	21.68	21.68
Cutleria multifida	29.38	5.62	12.77	34.45
Peyssonnelia squamaria	2.75	4.83	10.96	45.41
Sphacelaria tribuloides	0.18	2.61	5.92	51.33
Chaetomorpha linum	0.30	2.61	5.92	57.25

Table 10: SIMPER dissimilarity analysis of macroalgal species coverage between pairs of sectors in 2008.

Species	Average Abundance	Average Abundance	Average Dissimilarity	Contribution %
Sectors S1 & S2				
Average dissimilarity: 55.02				
	Sector S1	**Sector S2**		
Jania rubens	19.38	6.25	2.16	3.92
Flabellia petiolata	7.75	11.00	1.74	3.16
Dictyota dichotoma v. intricata	1.25	8.13	1.71	3.11
Lobophora variegata	15.63	0.60	1.67	3.03
Amphiroa rigida	9.20	10.25	1.47	2.68
Sectors S1 & S3				
Average dissimilarity: 82.24				
	Sector S1	**Sector S3**		

Dictyota dichotoma v. intricata	1.25	89.00	3.75	4.56
Jania rubens	19.38	0.00	3.36	4.08
Lobophora variegata	15.63	0.00	3.19	3.88
Cladophora prolifera	0.00	62.03	3.14	3.82
Sargassum vulgare	0.05	44.38	3.01	3.66
Sectors S2 & S3				
Average dissimilarity: 72.35				
	Sector S2	**Sector S3**		
Dictyota dichotoma v. intricata	8.13	89.00	2.95	4.08
Dictyota dichotoma	0.00	8.80	2.87	3.96
Sargassum vulgare	1.25	44.38	2.85	3.94
Cladophora prolifera	2.00	62.03	2.60	3.59
Flabellia petiolata	11.00	0.00	2.60	3.59
Sectors N1 & N2				
Average dissimilarity: 56.57				
	Sector N1	**Sector N2**		
Corallina elongata	0.78	51.25	3.60	6.37
Hydrolithon cruciatum	1.93	0.63	2.09	3.70
Titanoderma pustulatum	1.35	0.00	1.95	3.45
Lithophyllum incrustans	1.45	2.00	1.80	3.18
Peyssonnelia squamaria	5.08	8.13	1.66	2.93
Sectors N1 & N3				
Average dissimilarity: 72.80				
	Sector N1	**Sector N3**		
Cutleria multifida	0.13	29.38	3.97	5.46
Flabellia petiolata	42.25	11.50	3.76	5.16
Hildenbrandia crouaniorum	1.03	31.63	3.15	4.32
Spermothamnion repens	0.00	16.50	2.99	4.11
Antithamnion cruciatum	0.00	15.25	2.87	3.95
Sectors N2 & N3				
Average dissimilarity: 67.53				
	Sector N2	**Sector N3**		
Corallina elongata	51.25	1.75	4.43	6.56
Flabellia petiolata	33.50	11.50	3.53	5.23
Cutleria multifida	1.75	29.38	3.31	4.90
Spermothamnion repens	0.00	16.50	3.02	4.46
Antithamnion cruciatum	0.00	15.25	2.90	4.29
Sectors S1 & C3				
Average dissimilarity: 61.54				
	Sector S1	**Sector C3**		
Peyssonnelia bornetii	0.13	24.75	2.15	3.49
Peyssonnelia polymorpha	0.05	21.90	2.12	3.44
Lobophora variegata	15.63	0.15	2.03	3.30
Amphiroa rigida	9.20	0.00	1.92	3.12
Lithophyllum incrustans	1.38	7.25	1.56	2.54
Sectors S1 & N1				
Average dissimilarity: 72.07				
	Sector S1	**Sector N1**		
Jania rubens	19.38	0.00	3.32	4.60
Flabellia petiolata	7.75	42.25	3.06	4.25
Lobophora variegata	15.63	0.25	2.78	3.85
Amphiroa rigida	9.20	0.05	2.22	3.07
Valonia utricularis	7.75	0.23	1.91	2.65
Sectors S2 & N2				
Average dissimilarity: 66.85				
	Sector S2	**Sector N2**		
Corallina elongata	3.25	51.25	3.38	5.06
Dictyota dichotoma v. intricata	8.13	1.75	2.31	3.45
Flabellia petiolata	11.00	33.50	1.72	2.57

Amphiroa rigida	10.25	0.38	1.71	2.56
Caulerpa prolifera	9.00	0.00	1.60	2.40
Sectors S2 & N3				
Average dissimilarity: 65.16				
	Sector S2	**Sector N3**		
Cutleria multifida	0.00	29.38	3.36	5.16
Dictyota dichotoma v. intricata	8.13	0.50	2.40	3.69
Spermothamnion repens	0.00	16.50	2.25	3.45
Flabellia petiolata	11.00	11.50	2.09	3.21
Antithamnion cruciatum	0.88	15.25	2.04	3.13
Sectors S3 & N3				
Average dissimilarity: 69.50				
	Sector S3	**Sector N3**		
Dictyota dichotoma v. intricata	89.00	0.50	5.57	8.01
Sargassum vulgare	44.38	0.00	4.75	6.83
Cutleria multifida	0.00	29.38	4.48	6.45
Cladophora prolifera	62.03	0.13	4.04	5.82
Phyllophora crispa	35.90	4.00	3.39	4.87

In the Northern side N3 was significantly different from the others two sectors. Dissimilarities were attributed to the highest abundans of *Corallina elongata* in N2, to *Cutleria multifida* in N3, and *Flabellia petiolata* in N1 and N2 (Table 10).

Of the 101 macroalgal species recorded in 2007, 51% were turf-forming species, 25% crustose and 24% were erect forms. There was little change in the numbers of species in each category from pH 8.1 to pH 7.8 although whereas it was higher at pH 6.7 (Table 2).

The little change in the cover of erect category along the pH gradient is highlighted in the nMDS graph (Figure 20).

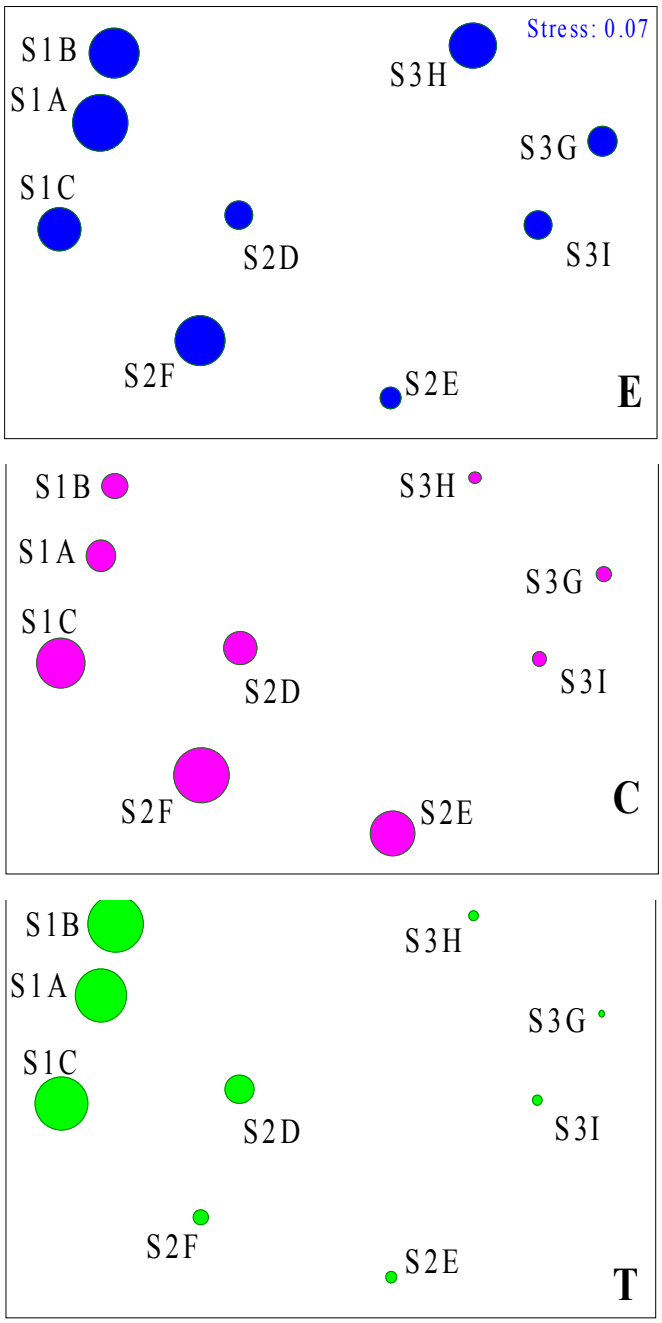


Figure 20: nMDS of coverage of E) erect, C) crustose, and T) turf macroalgal categories along the pH gradient from S1 (pH =8.1), S2 (pH =7.8) and S3 (pH =6.7) in 2007. Dot size corresponds to % coverage.

Crustose–algae remained abundant at mean pH 7.8 (S2) but were reduced in abundance in the area with highest CO_2 levels (S3). SIMPER analysis shows that turf algae face had a reduction and shift in species composition and area covered at mean pH 7.8 and they were particularly scarce in the most acidified sector as also shown by the high values of average dissimilarity between this sector and S2 and S1 (Table 11).

Table 11: SIMPER dissimilarity analysis of TURF algal group between pairs of sectors in 2007.

Species	Average Abundance	Average Abundance	Average Dissimilarity	Contribution %
Sectors S1 & S2				
Average dissimilarity: 49.34				
	Sector S1	***Sector S2***		
Valonia utricularis	17.5	2.57	3.11	6.3
Laurencia obtusa	1.74	0	3.04	6.15
Sphacelaria tribuloides	3.67	0.9	2.69	5.45
Herposiphonia tenella	0.6	0.06	1.93	3.91
Polysiphonia scopulorum	0.96	0.52	1.9	3.85
Sectors S1 & S3				
Average dissimilarity: 93.17				
	Sector S1	***Sector S3***		
Valonia utricularis	17.5	0.17	8.21	8.82
Sphacelaria tribuloides	3.67	0	5.79	6.21
Bryopsis plumosa	2.33	0	5.67	6.09
Osmundea truncata	0.22	2.7	5	5.37
Pseudochlorodesmis furcellata	1.01	0	4.44	4.76
Sectors S2 & S3				
Average dissimilarity: 91.76				
	Sector S2	***Sector S3***		
Osmundea truncata	0.12	2.7	6.25	6.81
Valonia utricularis	2.57	0.17	5.59	6.09
Griffithsia phyllamphora	1.23	0	5.07	5.52
Dasya corymbifera	0.054	0	5.05	5.51
Pseudochlorodesmis furcellata	1.14	0	4.66	5.07

In 2008 62 turf-forming species (T), 26 crustose-forming species (C) and 24 erect-forming species (E) were recorded (Table 3). As for 2007's data, in 2008 a similar number of species for each category was recorded into S1 and S2 while a drop was reported in S3. On the Northern side no differences were observed apart from the crustose category which number decreased in N3. Figura 21 shows a bubble plot of percent cover for each category.

Only on the South side, erect algae were more abundant at the lowest pH sector (S3); crustose forms had a similar distribution along the gradient on both sides. The highest differences were recorded by the turf category with an opposite trend on the two sides: lowest and highest in S3 and N3, respectively. SIMPER analysis (Table 12) confirmed these differences with high dissimilarity values and revealed shifts in species composition both between sides (exposition) and within sectors of the same side (pH gradient).

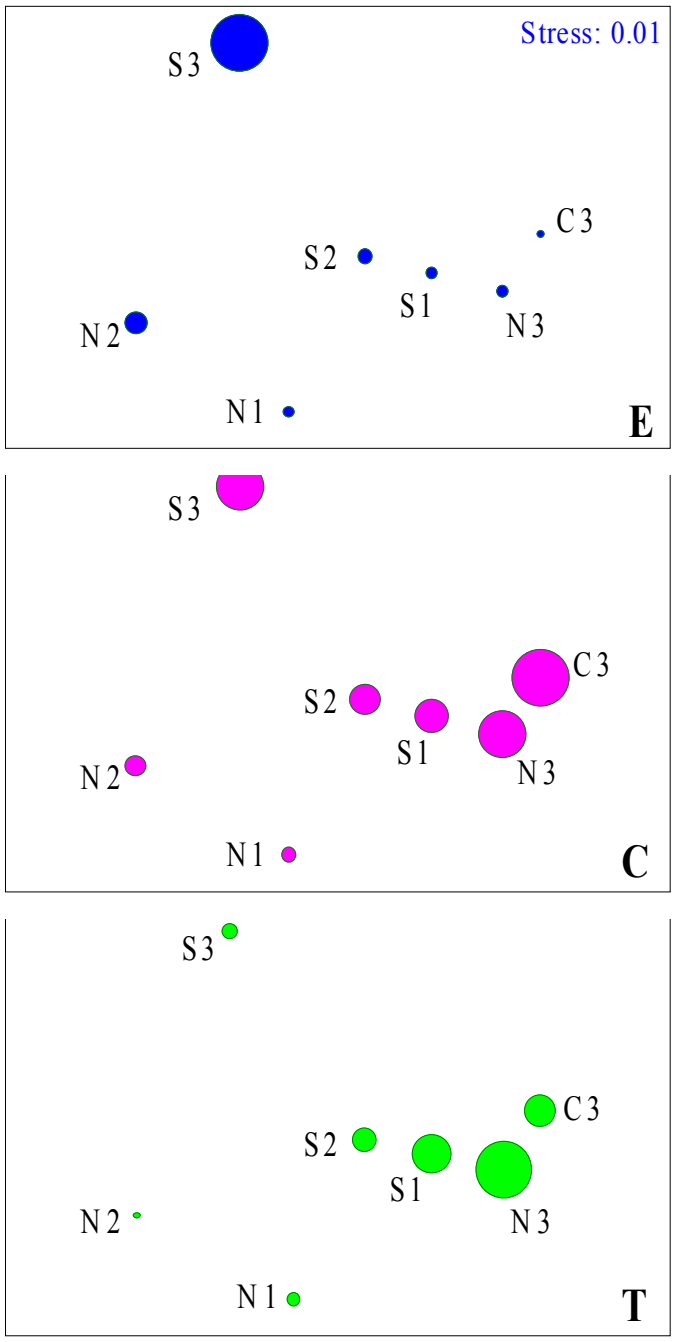


Figura 21: nMDS of coverage of E) erect, C) crustose and T) turf macroalgal categories on the North (N) and South (S) side of the Castello and in the Control site (C3) in 2008. Dot size corresponds to % coverage.

Table 12: SIMPER dissimilarity analysis of TURF algal group between pairs of sectors in 2008.

Species	Average Abundance	Average Abundance	Average Dissimilarity	Contribution %
Sectors S1 & S2				
Average dissimilarity: 67.74				
	Sector S1	**Sector S2**		
Valonia-utricularis	7.75	2.38	14.03	20.71
Bryopsis-plumosa	3.63	0.78	6.45	9.52
Sphacelaria-tribuloides	2.15	1.50	4.57	6.74

Lophosiphonia-cristata	1.25	1.70	4.12	6.08
Herposiphonia-secunda	0.00	1.48	3.82	5.64
Sectors S1 & S3				
Average dissimilarity: 86.32				
	Sector S1	**Sector S3**		
Valonia-utricularis	7.75	4.00	18.78	21.76
Bryopsis-plumosa	3.63	0.00	7.88	9.13
Antithamnion-cruciatum	0.03	1.73	5.60	6.49
Sphacelaria-tribuloides	2.15	0.00	5.29	6.13
Osmundea-truncata	0.10	2.00	4.72	5.47
Sectors S2 & S3				
Average dissimilarity: 85.26				
	Sector S2	**Sector S3**		
Valonia-utricularis	2.38	4.00	15.52	18.20
Antithamnion-cruciatum	0.88	1.73	7.49	8.78
Herposiphonia-secunda	1.48	0.00	6.68	7.83
Osmundea-truncata	0.10	2.00	6.33	7.43
Sphacelaria-tribuloides	1.50	0.00	5.10	5.98
Sectors N1 & N2				
Average dissimilarity: 77.59				
	Sector N1	**Sector N2**		
Bryopsis-plumosa	4.00	0.95	25.72	33.15
Pseudochlorodesmis-furcellata	1.55	0.13	9.60	12.37
Falkenbergia-sp.	0.28	0.98	8.67	11.18
Valonia-utricularis	0.23	0.60	5.82	7.50
Pterocladiella-capillacea	0.90	0.00	5.80	7.48
Sectors N1 & N3				
Average dissimilarity: 90.10				
	Sector N1	**Sector N3**		
Spermothamnion-repens	0.00	16.50	22.17	24.60
Antithamnion-cruciatum	0.00	15.25	18.72	20.77
Valonia-utricularis	0.23	3.58	11.17	12.40
Bryopsis-plumosa	4.00	0.98	9.93	11.02
Falkenbergia-sp.	0.28	1.60	6.15	6.83
Sectors N2 & N3				
Average dissimilarity: 87.06				
	Sector N2	**Sector N3**		
Spermothamnion-repens	0.00	16.50	24.15	27.74
Antithamnion-cruciatum	0.00	15.25	20.24	23.24
Valonia-utricularis	0.60	3.58	13.20	15.16
Falkenbergia-sp.	0.98	1.60	6.30	7.24
Bryopsis-plumosa	0.95	0.98	4.81	5.53
Sectors S1 & C3				
Average dissimilarity: 76.98				
	Sector S1	**Sector C3**		
Valonia-utricularis	7.75	3.10	13.11	17.04
Dasya-hutchinsiae	0.03	3.50	8.23	10.69
Bryopsis-plumosa	3.63	3.00	6.97	9.06
Cladophora-coelothrix	0.65	3.75	6.25	8.12
Sphacelaria-cirrosa	0.63	2.50	4.62	6.01
Sectors S1 & N1				
Average dissimilarity: 83.86				
	Sector S1	**Sector N1**		
Valonia-utricularis	7.75	0.23	20.54	24.49
Bryopsis-plumosa	3.63	4.00	10.94	13.05
Sphacelaria-tribuloides	2.15	0.00	5.50	6.56
Pseudochlorodesmis-furcellata	1.25	1.55	4.56	5.44
Dasya-corymbifera	1.40	0.00	4.52	5.39

Sectors S2 & N2				
Average dissimilarity: 83.30				
	Sector S2	**Sector N2**		
Valonia-utricularis	2.38	0.60	9.52	11.43
Herposiphonia-secunda	1.48	0.00	8.71	10.46
Sphacelaria-tribuloides	1.50	0.00	6.42	7.70
Antithamnion-cruciatum	0.88	0.00	5.84	7.01
Lophosiphonia-cristata	1.70	0.00	5.71	6.85
Sectors S2 & N3				
Average dissimilarity: 86.32				
	Sector S2	**Sector N3**		
Spermothamnion-repens	0.00	16.50	19.56	22.66
Antithamnion-cruciatum	0.88	15.25	17.82	20.65
Valonia-utricularis	2.38	3.58	11.20	12.98
Falkenbergia-sp.	0.60	1.60	4.12	4.77
Herposiphonia-secunda	1.48	0.00	4.06	4.70
Sectors S3 & N3				
Average dissimilarity: 87.12				
	Sector S3	**Sector N3**		
Antithamnion-cruciatum	1.73	15.25	21.82	25.04
Spermothamnion-repens	0.00	16.50	21.61	24.80
Valonia-utricularis	4.00	3.58	15.27	17.53
Falkenbergia-sp.	0.00	1.60	6.06	6.96
Osmundea-truncata	2.00	0.08	4.95	5.68

The effect of water acidification on the algal reproductive pattern has been analyzed only on 2007's data. Only seven genera were contemporaneously found with reproductive structures at different pH values (Figura 22) in 2007; among them, *Polysiphonia scopulorum*, *Osmundea truncata* and *Dictyota dichotoma* were able to reproduce at very high levels of CO_2. There was even an increase in reproductive frequency recorded in the high CO_2 areas for *D. dichotoma* and *O. truncata*, whereas reproduction in calcareous species appeared to be negatively affected (Figura 22).

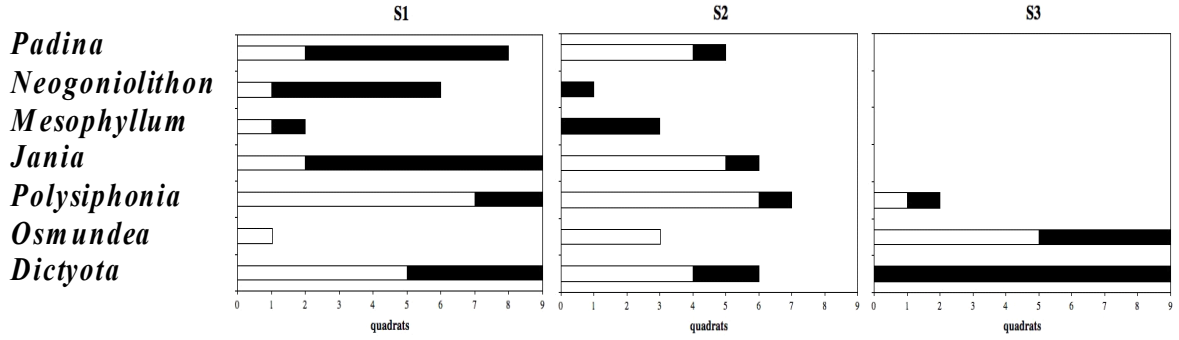


Figura 22: Occurrence (black bars) and absence (white bars) of reproductive structures in species present in at least two sectors with different pH (S1: pH= 8.1; S2: pH=7.8; S3: pH= 6.7).

All the algal species identified during the two sampling periods are listed in Table12. Each of them was assigned to a morpho-functional group (see Table 1).

Results for 2007 community analysis both at taxonomic and morpho-functional group levels are showed in Figura 23. n-MDS analysis gives back the same differences among sectors –by using distinct approaches: sector with the lowest pH has a significant different algal composition in comparison with the other two sectors.

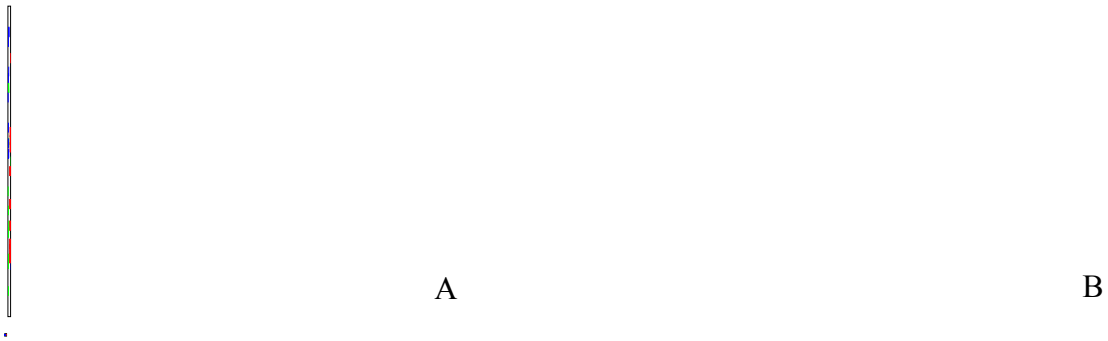


Figura 23: n-MDS of taxonomic species (A) and Algal Groups (B) in 2007.

ANOSIM confirmed the difference among sectors (Table 14).

Table 14: Global Test for Algal Groups in 2007. Sample statistic (Global R): 0.808. Significance level of sample statistic: 0.1%. Number of permutations: 999 (Random sample from a large number). Number of permuted statistics greater than or equal to Global R: 0.

Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number Observed
S3 - S2	0.99	0.1	24310	999	0
S3 - S1	1.	0.1	24310	999	0
S2 - S1	0.409	0.2	24310	999	1

The relative dominances (*% DR*) along the pH gradient of the ten different Algal Groups are showed in Figure 12, Normal (S1) and medium (S2) pH sites are characterized by diverse calcareous species (AG9, AG11, AG12,) (i.e. *Flabellia petiolata, Phymatolithon lenormandi*i, and *Iania rubens*, respectively) and by uniseriate and polisiphonous filamentous species (AG1 and AG2). These groups disappear in the lowest pH sector (S3), where more structural complex species, corticated, laminar and terete algae (AG6 and AG7) (i.e *Dictyota dichotoma, Chondracanthus acicularis*, respectively), leathery algae (AG8)(i.e *Sargassum vulgare*) and non-calcareous encrusting algae (AG5) (i.e *Hildebrandia rubra*)), replace them and are dominant.

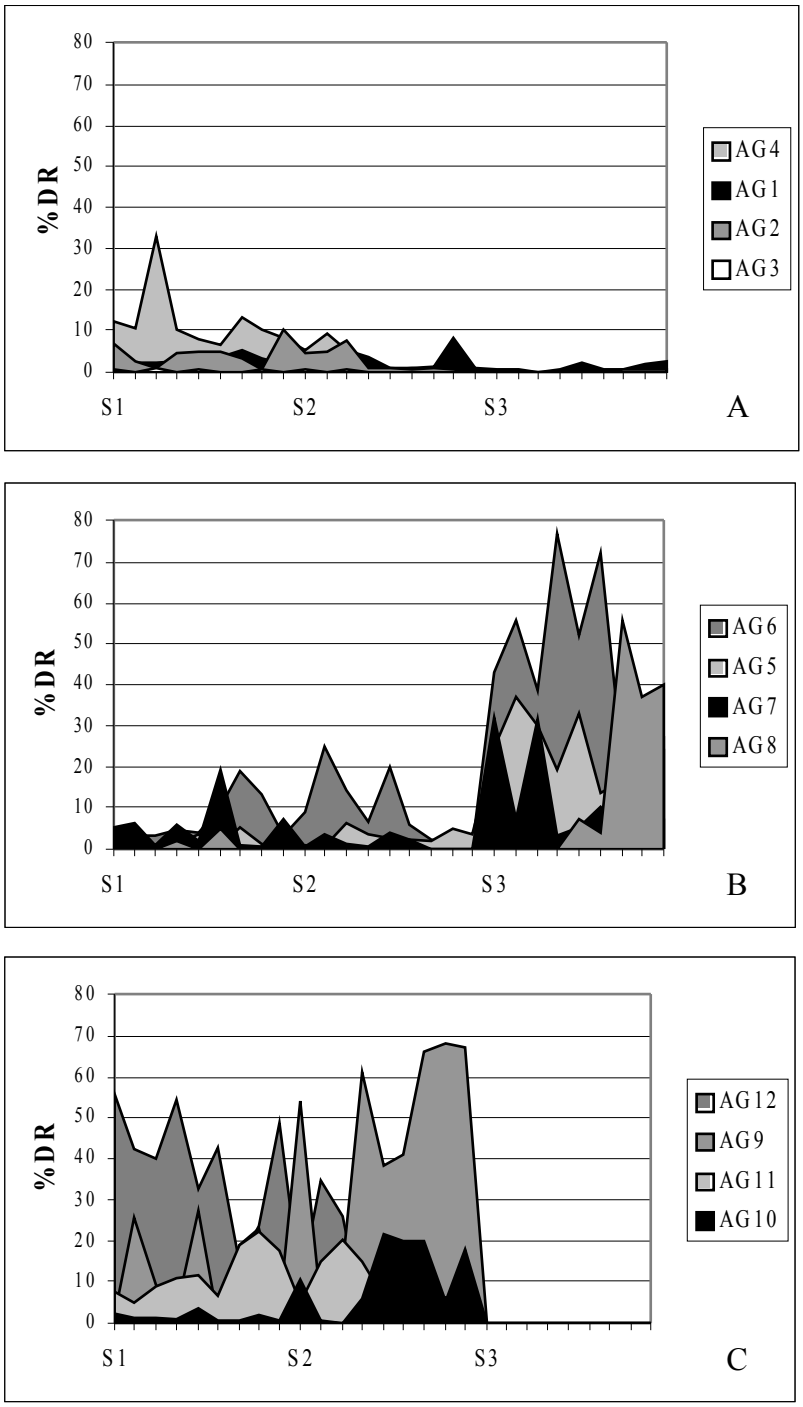


Figura 24: Percentage of Relative Dominance of Algal Groups along the pH gradient in 2007. A: from AG1 to AG4; B: from AG5 to AG8; C: from AG9 to AG 12 (for AG legend, see Table 1)

The same nMDS analysis, performed on 2008's data, underlines the relevance of different hydrodynamic regimes with different water chemistry in structuring the algal communities. In fact, differences among exposition are responsible of a less evident distinction in algal occurrence between sectors with different pH values (Figure 25A and B); however normal and medium pH sites are always different from the lowest pH site, for both taxa and morpho-functional groups.

A B

Figure 25: n-MDS of both species (A) and Algal Group (B) in 2008.

ANOSIM analysis for 2008's data set according to AG classification confirm a significant difference among pH and the geographic exposition (Table 13).

Table 13: Global Test for Algal Groups in 2008. Sample statistic (Global R): 0.616; significance level of sample statistic: 0.1%; number of permutations: 999 (Random sample from a large number); number of permuted statistics greater than or equal to Global R: 0

Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number ρ Observed
S1 - C3	0.786	6.7	15	15	1
S1 - S2	0.333	5.7	35	35	2
S1 - S3	1.	2.9	35	35	1
S1 - N1	0.781	2.9	35	35	1
S2 - S3	0.979	2.9	35	35	1
S2 - N2	0.563	2.9	35	35	1
S2 - N3	0.438	5.7	35	35	2
S3 - N3	0.729	2.9	35	35	1
N1 - N2	0.188	14.3	35	35	5
N1 - N3	0.813	2.9	35	35	1
N2 - N3	0.708	2.9	35	35	1

Figura 26 shows the Algal Groups composition along the pH gradient on both Southern and Northern side. The simplest AGs (fromAG2 to AG4) showed a higher dominance at higher pH, except for AG1 which was prevalent in the acidified area. In particular *Antithamnion cruciatum* and *Spermothamnion repens* were dominant in N3 while *Cladophora prolifera* in S3.

More complex thalli (from AG6 to AG8), once again, recorded a higher relative dominance at lowest pH sites: *Hildenbrandia crouaniorum* and *Cutleria multifida* were the dominant species in the Northern side, while *Dictyota dichotoma* v. *intricata*, *Sargassum vulgare* and *Hildenbrandia crouaniorum* in the South.

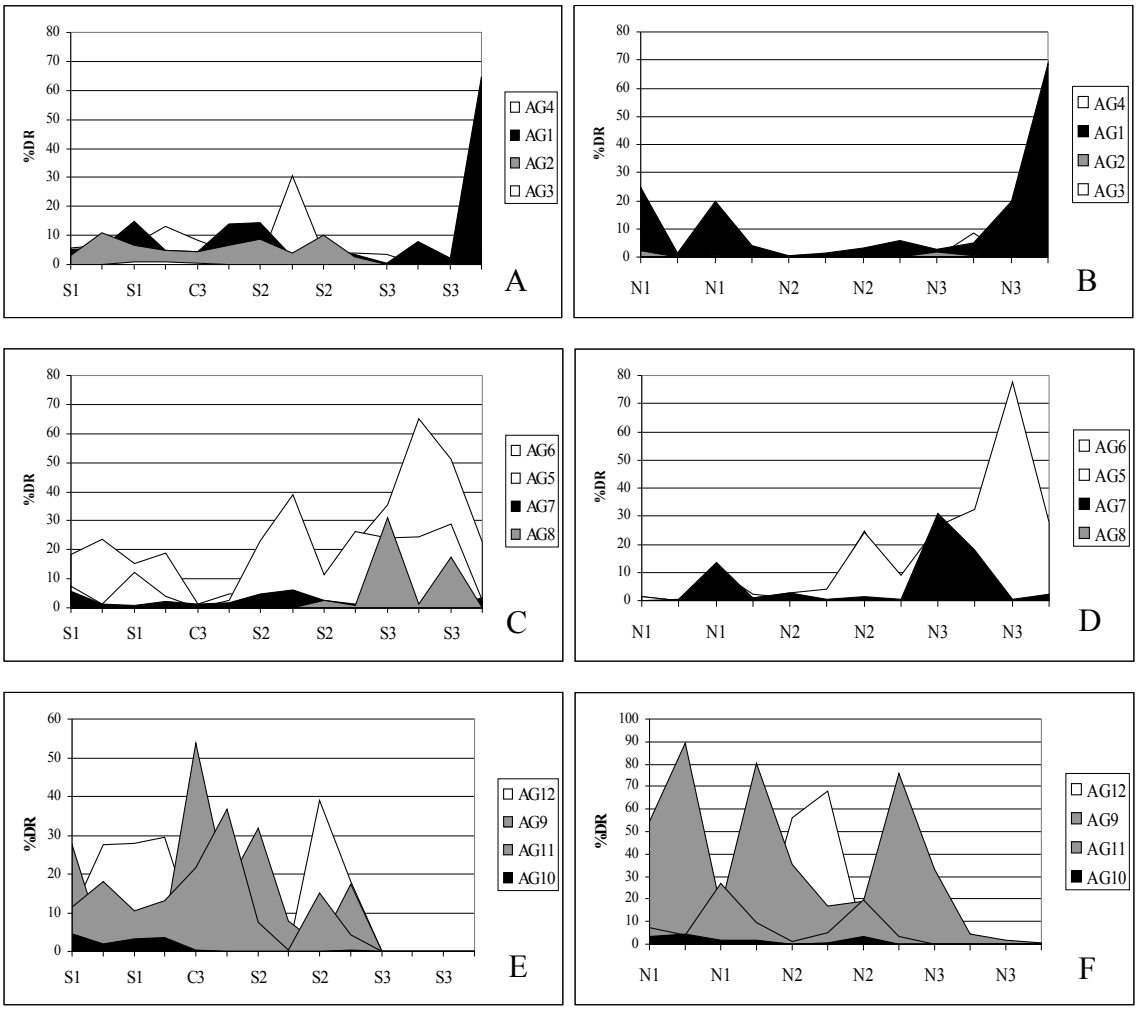


Figura 26: DR% of the Algal Groups along the pH gradient in 2008 on both Suothern (A, C, E) and Northern (B, D, F). A-B: from AG1 to AG4; C-D: from AG5 to AG8; E-F: from AG9 to AG 12.

On the opposite, calcareous AGs (from AG9 to AG11) showed a drop moving to more acidified areas in both Southern and Northern side, except for the occurrence of *Peyssonnelia squamaria* (AG9) in N3.

Algal communities on artificial substrata

A total of 49 macroalgal species, in different sectors and at different time intervals, were recorded. Among them, 18 Rhodophyta, 23 Ochrophyta and 8 Chlorophyta were identified (Appendix III).

Figure 27: nMDS for communities among sectors after two (A), threee (B) and four (C). months

C B

Figure 27 shows nMDS graphs for macroalgal communities' composition during time among different sectors. Significant separation (Stress value ≤ 0.04) was observed among sectors along pH gradient and exposition. In particular, the "acidification effect" (horizontal axis) was already evident after two months (Figure 27A) and it went on more and more in the time, separating the most acidified site from the others (Figure 27C). The effect of different hydrodynamic regimes (vertical axis) between the two sides was always evident.

Changes in community structure at different time intervals among sectors with different pH and exposition are represented in Figure 28.

Figure 28: nMDS plot for communities in different sectors during different times. Samples are evidenced according to pH (Normal, Mid and Low pH).

An increase in the community complexity at different time scale, both in the Southern (Fig. 29A) and Northern (Fig. 29B) sides of the Castello was evident. This pattern was confirmed by the high similarity inside each sector at the same time interval obtained by the SIMPER analysis (Appendix IV). The highest differences between the two different expositions occured during the initial phase of the colonizing process (two months - May), when the south communities are very poorly structured. The *Mirionema sp.* and *Feldmannia sp.* high covers are responsible of these differences.

The K-dominance curves according to different pH values (Fig. 30) showed the effect of water acidification in the colonizing the artificial substrates. After 4 months, differences among sectors with different water acidification were due to the presence of calcareous species at normal pH, such as *Titanoderma mediterraneum* (S1 and N1), and *Hidrolithon farinosum* (C3) whereas *Chaetomorpha linum* and *Feldmannia* sp dominated the sites at the lowest pH (S3 and N3, respectively) (Appendix IV).

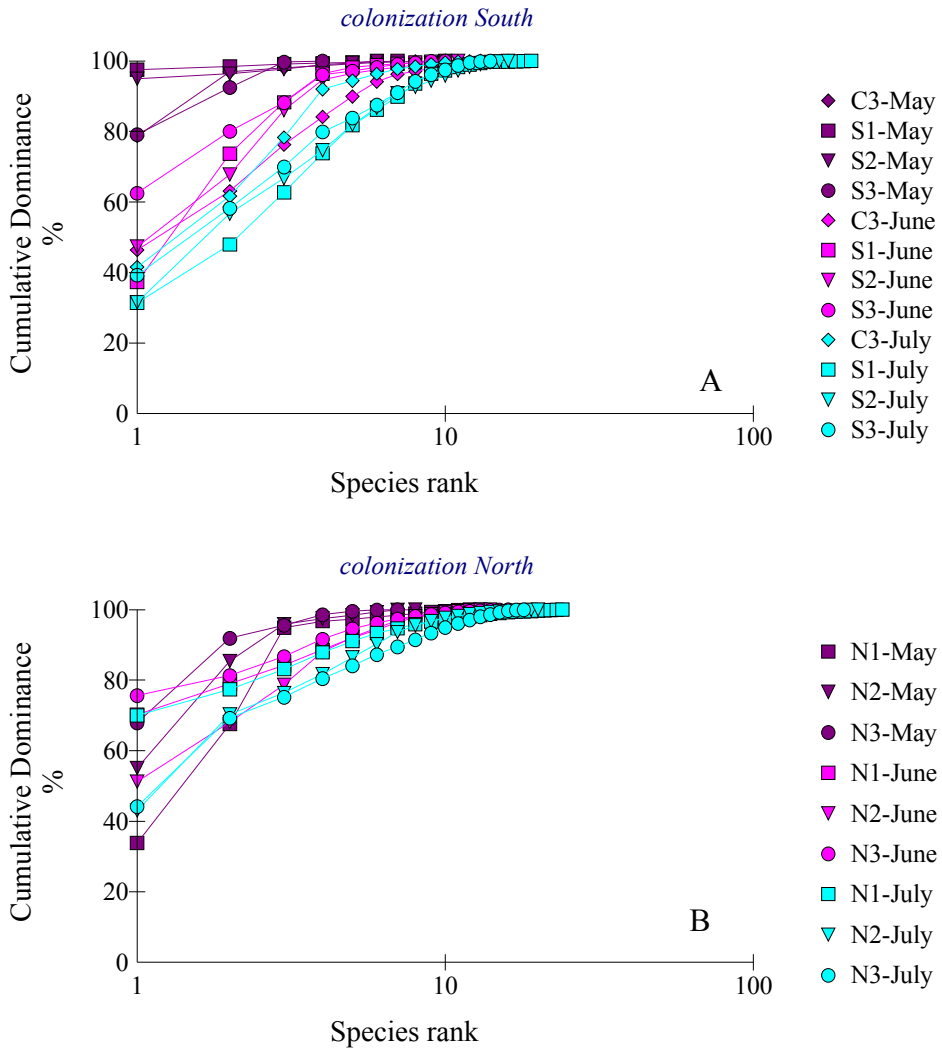


Figura 29: K-Dominance curves for all months in the Southern (A) and the Northern side (B).

Figura 30: K-Dominance curves for all sectors during May (A), June (B), July (C). Normal pH (C3), Normal pH South (S1), Mid pH South (S2), Low pH South (S3), Normal pH North (N1), Mid pH North (N2), Low pH North (N3).

C

B

3. Different responses in ecophysiological traits in target species along a pH gradient

3.1. Introduction

At present, the phenomenon of ocean acidification excites more interest because of its effects on marine organisms.

Coastal system is one of the most vulnerable ecosystems because of anthropic perturbation (Airoldi *et al*., 2007). In this environment macroalgae play an important structural and functional role and every change in their abundance and composition may cause reliable modifications not only to the associated communities but also to the entire food web.

Photosynthesis represents a key process for macroalgae as well as for all plant species. This process responds quickly to changes of environmental factors in the habitat and for this reason can be considered a good indicator in monitoring the health status of vegetal organisms and the occurring of stress conditions.

At the moment, a few number of studies have been focused on the mid and long term effects of pH changes on macroalgal community in their natural environment. The most part of experiments have been performed in laboratory, on a reduced number of species, in order to assess only short term responses (Kübler *et al.*, 1999; Beer *et al.*, 1996; Menéndez *et al.*, 2001; Israel & Hophy, 2002; Semesi *et al*., 2009).

Some macroalgal species have shown a significant rise in photosynthetic rates in response to CO_2 increase (Giordano *et al*., 2005). Photosynthetic activity has been studied almost at higher pH compared to present.

Only some species may increase their photosynthetic rate at higher pH value likely due to their capacity to convert bicarbonate (the most abundant form of inorganic carbon at higher pH) in CO_2 (Beer *et al.*, 1996; Johnston *et al*., 1992; Raven, 2003; Menéndez *et al.*, 2001). Different studies suggest that many species will be greatly benefited by the air CO_2 enrichment. Langdon *et al*. (2003) reported that "laboratory studies have found that the photosynthesis of many macroalgae is limited by inorganic carbon supply in natural seawater," citing also the studies of Borowitzka and Larkum (1976), Borowitzka (1981), Gao *et al*. (1993), Beer & Koch (1996).

On the other hand, Middelboe *et al*., (2007) have been observed that at higher pH values photosynthetic rates decreased with comparable inorganic carbon availability, suggesting the direct role of pH effect through physiological mechanisms such as membrane transport or internal pH regulation.

Natural CO_2 vents at Castello Aragonese, in Ischia, have been created a natural pH gradient likely for million of years (Hall-Spencer *et al.*, 2008, see Chapter 2) which make it suitable to study long term responses of macroalgae to different pH variation.

In this chapter pH effect on photosynthetic efficiency of some macroalgae living at Castello at different pH values, has been investigated both in laboratory and *in situ* studies.

The principal goal of this research was to assess through transplanting experiments, the adaptation capability of some macroalgae species to live in low pH environment as well as the possibility that the species present in not-acidified environment could activate acclimation mechanisms in their photosynthetic apparatus to overcome pH changes. In addition, molecular analysis was carried out to investigate cellular stress induction by acidification by using a marker of DNA damage. The marker was the enzyme poly-ADPR-polymerase (PARP) that is involved in the regulation of several cellular functions related to the maintenance of cellular integrity.

3.1.1. Photosynthesis in aquatic systems and its plasticity

Photosynthesis is the biological conversion of light energy into chemical bond energy that is stored in the form of organic carbon compounds. Approximately 40% of the photosynthesis on Earth occurs in aquatic environments each year (Falkowski, 1994).

A multitude of evolutionary adaptations and physiological acclimation of the photosynthetic apparatus have occurred in aquatic organisms. In nature photosynthetic processes are constantly modified. There are a great number of temporal variations in photosynthetic response that occur in both short and long time scales. The “short time scale” regards the physical, biochemical and physiological responses within the life span of an organism. These responses are collectively called *acclimation*. On the contrary, the “long time scale” regards the ecological and evolutionary *adaptation* processes through selection of phenotypic traits (Falkowski, 1994).

To better understand the photosynthetic process in aquatic environments, it is important to consider the underwater light distribution in terms of quantity and quality. In the water the scattering leads to spectral bias toward the blue, absorption by water itself is superimposed on the scattering process. Water absorbs strongly in the red and infrared. The net effect of both scattering and absorption enriches the penetrating down welling spectrum in the blue and blue-green wavelengths, progressively prevailing in deep waters.

Photosynthesis responds quickly to the irradiance daily variation. Circadian rhythms in photosynthetic response are not simply due to changes in stechiometry of reaction centres

or component of the Calvin-Benson cycle; they may arise from time-dependent changes in the activity of specific components of the photosynthetic apparatus (Falkowski, 1994).

Pigment content in macroalgae depends on irradiance values and is governed by diurnal rhythm. A different pigment complement is present in sun and shade adapted algae. The shade adapted algae have usually a higher content of photosynthetic pigments. The daily pattern represents for algae an indication of pigments rapid turnover in the natural environment (Hader *et al.*, 2002).

All photosynthetic pigments harvest light that is the driving force for photosynthesis. The photosynthetic-irradiance curve (P-E) describes the light utilization capacity by photosynthetic organisms and can be typically divided into three distinct regions: the light limited region, the light saturated region, and the photoinhibition region. In the darkness there is a net consumption of O_2 and evolution of CO_2 due to respiratory activity. At low irradiances photosynthetic rate are linearly proportional to irradiance; a doubling of intensity produces almost a doubling of photosynthetic rate. The light intensity at which photosynthesis balances respiration is called the compensation light intensity (E_c). The initial slope of the photosynthesis irradiances curve is proportional to the maximum quantum yield of photosynthesis (Kok, 1984). In the literature related to the acquatic organisms, the initial slope of the P *vs* E curve is often indicated by the symbol. As the irradiance increases, photosynthetic rate rises to a saturation level, P_{max}. At light saturation level, the rate of photon absorbed exceeds the rate of steady-state electron transport from water to CO_2. The intersection of and P_{max}, is often called the light-saturating parameter, and is indicated by E_k (Figure 31).

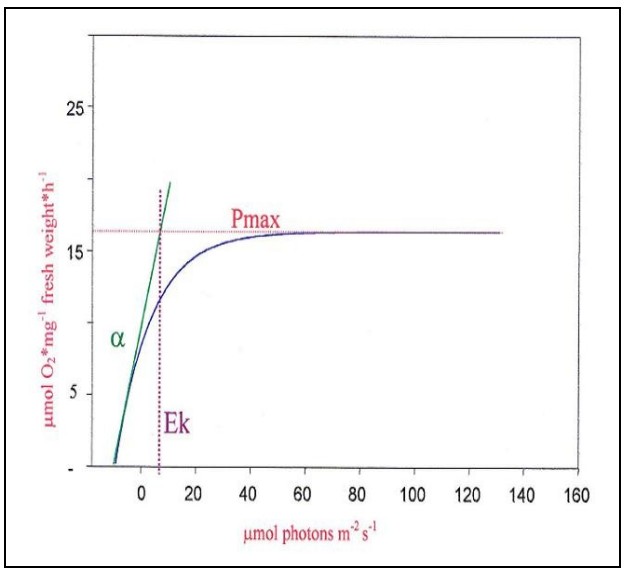


Figure 31: A photosynthetic light-response curve in aquatic environment

Further increases in irradiance beyond light saturation can lead to a reduction in photosynthetic rate (Baker & Bowyer, 1994). This reduction, which is dependent on both light intensity and the exposure duration, is often called *photoinhibition*. This phenomenon leads to a reduction in the photochemical efficiency of photosystems. The reduction in photochemistry may be due to photodamages at photochemical apparatus or to establishment of photoprotective mechanisms (Hader & Figueroa, 1997).

Because of the strong relationship between photosynthetic rates and irradiances, P*vs*E curves constantly change. The optimum position for a photoautotroph organism with respect to photosynthesis-irradiances curve is at the saturating irradiance E_k.

The benthic organisms cannot control the irradiance field, but are able to maintain photosynthetic rate at or near E_k by the adjustment of size and composition of the light harvesting system.

Most of the short-term adjustments are achieved via changes in the light harvesting system. These modificatons include alterations in non-photochemical quenching of excitation energy and photoacclimation via changes in the rates of synthesis of light harvesting complexes. Additional adjustments in the number of functional photosynthetic reaction centres and in the maximum electron transport rates arise as a consequence of metabolic feedback from the Calvin-Benson cycle into the photosynthetic electron transport chain.

Also temperature affects photosynthetic activity. It is well known that photoinhibition occurs when the light energy absorption by reaction centres exceeds the rate of photochemistry (Powels, 1984). However photoinhibition can occur also at moderate light intensity when carbon metabolism is limited by low temperature. Because of the relationship between irradiance and temperature on the maximum rate of photosynthetic electron transport and cells ability to repair photodamage, the effect of temperature on the maximum rate does not follow a simple Arrhenius function, but a more complex relationship may be found (Falkowski & Raven, 1997).

3.1.2. Photosynthetic performance measurements: chlorophyll fluorescence

Light energy that is absorbed by chlorophyll in a leaf can undergo three fates: a) it can be used to drive photosynthesis (photochemistry), b) it can be dissipated as heat or c) it can be re-emitted as fluorescence.

These three processes occur in the plant cell simultaneously. Any increase in the efficiency of one process will result in a decrease in the yield of the other two. Thus, measuring the yield of chlorophyll fluorescence we can obtain information about changes in the efficiency of photochemistry and heat dissipation. As heat dissipation of energy is low and remains constant in an order of time of few seconds, it follows that the fluorescence emission decreases proportionally with increasing of photochemical efficiency. This phenomenon is known as "quenching" of fluorescence by photosynthetic electron transfer through the photosystems and can be assessed by mean of fluorescence measurements. The total amount of chlorophyll fluorescence is very small (only 1-2% of total light absorbed) and the spectrum of fluorescence is different from that of absorbed light. The fluorescence yield can be quantified by exposing photosynthetic tissues to light of defined wavelengths and by measuring the amount of light re-emitted at longer wavelenghts (Maxwell & Johnson, 2000).

Changes in the yield of chlorophyll fluorescence were observed for the first time by Kautsky *et al.* in the 1960. They found an increase in the chlorophyll fluorescence over a period of around 1s when photosynthetic material was transferred from the dark into the light. Once PSII absorbs light and the plastoquinone Q_A has accepted one electron, it is not able to accept another until it has passed the first onto the subsequent electron carrier (Q_B). During this period, the reaction centres becomes "closed". So when a leaf is transferred from darkness into the light, PSII reaction centres are progressively closed and, as a consequence of reduction electron acceptors in the photosynthetic pathway, an increase in the yield of chlorophyll fluorescence occurs. However, the fluorescence level typically starts to fall again over a time-scale of a few minutes. In fact there is an increase in the rate at which electrons are transported away from PSII as a consequence of light-induced activation of enzymes involved in carbon metabolism; such quenching is referred to as "photochemical quenching". At the same time, an increase occurs in the efficiency with which energy is converted to heat (non-photochemical quenching). Generally, changes in these two processes will be complete within about 15-20 min, depending on plant species (Johnson *et al.*, 1990).

In order to obtain useful information by measurements of chlorophyll *a* fluorescence, it is necessary to distinguish between the photochemical and non-photochemical quenching.

At present the most utilized instruments for fluorescence investigations are a new class of fluorometers called PAM (pulse amplitude modulated fluorometers). In particular in this PhD thesis we have used a particular kind of PAM fluorometer: the diving PAM (Walz, Germany) able to measure the fluorescence indexes underwater.

By means of PAM fluorometers is possible to distinguish between photochemical and non-photochemical processes using the *saturation pulse* method (Schreiber *et al*., 1986, 1994). The "light doubling" technique allows to transiently reduce to zero the photochemical quenching (Bradbury & Baker, 1981; Quick & Horton, 1984). In this approach, a short duration, high intensity flash of light is given so that PSII reaction centres are transiently closed. The saturating flash is emitted by halogen bulb at >2000 μmol photons $m^{-2}s^{-1}$. During the flash, the chlorophyll fluorescence reaches the highest value and the photochemical quenching is zero. In this moment all absorbed energy has to be re-emitted as thermal dissipation (unchanged) and by fluorescence. This maximum fluorescence is indicated as F_m', if measured for light adapted plants. Comparison of this maximum value with the steady state yield of fluorescence in the light (F') gives information about the efficiency of photochemical quenching and PSII performance.

As well as changes occurring in the efficiency of photochemistry, the efficiency of heat dissipation (i.e. non-photochemical quenching) can change depending on various internal and external factors. Such changes are reflected as changes in the level of maximum fluorescence. Unlike photochemistry, it is not possible to inhibit heat dissipation totally, so it is not possible to measure the yield of chlorophyll fluorescence in the absence of non-photochemical quenching. Hence, all estimation of non-photochemical quenching are strictly relative to some dark-adapted point (termed F_m). For this reason, it is necessary to determine this reference point by measuring F_m and F_0 on dark-adapted plants. F_0 is the lowest fluorescence value because it is measured when all reaction centres are open (dark-adapted status) and in the absence of an actinic (photosynthetic) light. From F_m and F_0 the quantum yield of electron transfer in PSII (Y) is derived:

$$Y= (F_m-F_0)/F_m= F_v/F_m$$

This maximum or potential quantum yield is measured in dark-adapted plants where all reaction centres are open. The F_v/F_m measurement has to be performed after at least 10-15 min of leaf dark adaptation in order to allow the complete relaxing of photosystems. The F_v/F_m value for terrestrial plants in healthy status is about 0.83 (Bjorkman and Demming, 1987), whereas for seagrasses as *Halophila ovalis* it is about 0.73-0.75 (Ralph and Burchett, 1995; Ralph, 1999). A decrease from these values indicates the occurring of stress and the presence of a quenching mechanism.

A decline in F_v/F_m can be interpreted as photoinibition or photodamage or both; generally the former is dependent on processes which are easily reversible (e.g. photoprotective non-photochemical quenching), whereas the latter depends on very slowly reversible non-photochemical quenching processes (Cavender-Bares & Bazzaz, 2004).

The measurement of quantum yield performed on light adapted plants, gives another parameter, termed effective quantum yield of PSII (Y or Δ_{PSII}). The fluorescence variables F_t and F_m' measured in the light correspond to F_0 and F_m measured in the dark:

$$\Delta_{PSII} = (F_m'-F')/F_m'$$

The effective quantum yield of PSII is calculated following Genty *et al*., (1989). This parameter has been often used as stress indicator for photosynthetic apparatus exposed to low temperature (Cavender-Bare & Bazzaz, 2004).

The effective quantum yield of PSII multiplied by the number of photons absorbed by PSII, represents a measure of the linear electron transport rate through photosystem II.

The electron transport rate (ETR) can be calculated as:

$$ETR= \Delta_{PSII} *PAR*0.5*ETR\ factor$$

Where PAR (Photosynthetically Active Radiation) is the incident irradiance as measured with a light sensor at the leaf surface; 0.5 is the result of an assumed equal distribution of photons absorbed by the two photosystems; ETR factor describes the proportion of incident photons absorbed by photosynthetic pigments; for a wide range of terrestrial leaves it is 0.84 but underwater as the reflection of thallus is negligible, the ETR factor can be approximate to 1 and can be ignored in the previous equation. This is called relative ETR (rETR) and is calculated as:

$$rETR= \Delta_{PSII} *PAR*0.5$$

In addition to measuring ETR *in situ*, the fluorometer used for our investigations, the diving PAM, can provide a range of artificial irradiances (by the internal halogen lamp) useful to investigate the response of photosynthetic apparatus at different light intensities. If the time of exposure to different step of light are short (10s each), no significant change on photochemistry will be obtained. These measurements generate a so-called *rapid light curve*

(RLC) (Beer *et al*., 2001); a RLC provides information on the capacity of photosynthetic apparatus to utilize the light (Longstaff *et al*., 2002; Ralph & Gademann, 2005) and can provide information on stress-inducing events (Runcie & Riddle, 2004). Some authors have found linear correlations between ETRs curves and oxygen evolution (photosynthetic rate) at lower irradiances (Longstaff *et al*., 2002; Carr & Bjork, 2003) in particular when $\Phi_{PSII} > 0.1$ (Beer & Axelsson, 2004). A RLC is similar to P-E curve; but, differently from P-E curves, a RLC does not reach the steady state during each light step; for this reason Φ_{PSII} and rETR indicate the actual state of photosynthesis, not the optimal state. As described for P-E curves, RLC have three distinct regions: the light-limited, the light-saturated and the photoinibition region. At low irradiance, photosynthesis is limited by light. The rise of the curve in the light-limiting region (α) is proportional to efficiency of light capture (effective quantum yield). Minimum saturating irradiance (E_k) is determined by the interception of α with the maximum photosynthetic rate. E_k is related to quenching; photochemical quenching dominates the region below E_k, non photochemical quenching the region above E_k.

Under moderate irradiance, the capacity of the electron transport chain limits photosynthesis and the curve reach a plateau, where maximum photosynthetic capacity occurs ($rETR_{max}$). At higher irradiance values (supra-saturating) the curve often leans to decline. In a traditional P-E curve, the decline is usually associated to photoinibition processess; on the contrary, in RLCs the decline could be linked to a dynamic down-regulation of PSII, because time is not insufficient for the induction of photodamage (Ralph & Gademann, 2005).

To find a good relationship between photosynthetic rate and ETR (Beer & Bjork, 2000), the fluorescence data must be corrected for the approximate proportion of incident quanta absorbed by the pigments of PSII. Thus, the previous formula must be adjusted by the absorption factor (AF):

$$ETR = \Phi_{PSII} * PAR * 0.5 * AF$$

AF can be approximated by placing the leaf sample between the actinic light source and quantum light sensor and measuring the light transmitted through the leaf according to:

$$AF = (\text{incident PAR} - \text{transmitted PAR})/\text{incident PAR}$$

AF (absorbance factor) is determined according to Beer *et al.* (2000). The ETR describes the capacity of photosystems to use the incident light and can be used to compare the photosynthetic efficiency of different portions of the same individual, of different species or of the same species in different conditions (Beer *et al*., 2001).

In absence of stomatal limitation, ETR is well correlated with the CO_2 assimilation rate, although it includes also all electron transport to alternative CO_2 sinks such as electron flow to oxygen and detoxification of oxygen radicals as well as nitrogen assimilation. A decrease in electron transport rate indicates a reduction in CO_2 fixation and may be associated, among different stress factors, to low temperatures (Cavender-Bares & Bazzaz, 2004).

Other parameters can be derived by fluorescence measurements; the first one is the photochemical quenching, qP, it reflects the PSII efficiency and is due by energy transformation at PSII reaction centres. Photochemical quenching give information on the oxidation status of PSII acceptors: if the acceptor pool is completely reduced (closed), qP=0; if is entirely oxidized (open), qP=1. qP is calculated as follows:

$$qP = (F_m' - F')/(F_m' - F_0')$$

The second parameter is represented by non-photochemical quenching, qN. and depend on pH gradient (Horton & Ruban, 1994, Demmig-Adams, 1990, Krause & Weis, 1991). It represents non-radiative pathways of energy de-excitation, occurring mainly through heat and redistribution of excitation energy from PSII to PSI; qN involves photoprotective mechanisms such as the xanthophylls cycle and is calculated as:

$$qN = 1-(F_m'-F_0')/(F_m-F_0)$$

Another coefficient for non-photochemical quenching is the Stern-Volmer quencing (NPQ) calculates as:

$$NPQ = (F_m-F'_m)/F'_m$$

NPQ is more sensitive to energy dissipation within the antennae matrix (Schreiber, 2004).

During photoinibition, photosynthetic quantum yield and the photochemical quencing (qP) decrease while the non-photochemical quenching rises (Hader & Figueroa, 1997; Hader *et al.*, 1998). Although photoinibition mechanisms are still unclear, they can be interpreted as active regulatory processes able to protect the photosynthetic apparatus from excessive radiation. In fact during intensive solar radiation, active oxygen species can be produced by transfer of excessive excitation energy from excited chlorophyll molecules to ground state (triplet) oxygen molecules. The reactive oxygen species (ROS) damage the structure of photosynthetic apparatus. In particular, one of main target of ROS is the D1 protein of reaction centre of PSII that plays a key role in energy transfer (Sundby *et al*., 1993). Any modification in D1 limits the linear photosynthetic electron flow. Algae growing in surface water are well adapted to high solar radiation and less predisposed to photoinhibition than those growing in deeper habitat (Hader et al., 1998)

The qN is generally defined as the decrease of fluorescence not due to photochemical process. It is induced by the built-up of a pH-gradient across the thylacoid membrane, by state transition (Raven & Geider, 2003), and by photoinhibition (Karukstis 1991; Krause & Weis, 1991; Buschmann, 1999; Hader *et al*., 2001). Also F and Fm' curves provide information on the development of the trans-thylacoid proton gradient and on thermal energy dissipation (Ralph & Gademann, 2005) in particular a value of qP <0.4 indicating an energization of thylacoid membrane (Schreiber, 2004). When the fluorescence yield (F) increases rapidly, it means that a greater proportion of PSII reaction centres become inactivated. An high fluorescence yield is normally linked with the build up of a proton gradient across the thylacoid membrane (ΔpH). The ΔpH is driving force for ATP production, while Calvin cycle activity is the main sink for ATP. When F increases and remains elevated, there is a limitation of CO_2 fixation (Ralph & Gademann, 2005).

Moreover the photosynthetic apparatus is protected by down-regulation of the electron transport chain (ETR) and involvement of the violaxanthin cycle (Hader *et al*., 2001).

However the differences between high-light adaptation and low-light adaptation are clear: algae grown at high-light intensities have lower chorophyll content, high photosynthetic capacity and active photoprotective mechanisms; on the contrary, algae grown in low-light environments show the opposite characteristics (Demming-Adams *et al*., 1999; Ralph & Gademann, 2005). These features can influence photosynthetic activity according to the prevailing light conditions, as well as the seasonal light regime. Pigment content usually takes several days to weeks for acclimation, while the xanthophyll cycle can

be regulated in minutes, and effective quantum yield can change in seconds (Raven & Geider, 2003; Ralph & Gademann, 2005).

3.1.3. Chlorophyll fluorescence for measuring stress and stress tolerance

Fluorescence measurements may provide an useful tool to assess plant photosynthetic performance. In particular, fluorescence can give information about the ability of plant to tolerate the environmental stresses and the occurring of damages at photosystems level.

Since F_v/F_m is very sensitive to plant stress, this parameter has been used as index to monitor the impact of environmental and anthropogenic stressors. Measurements made over a diurnal course can indicate the status of electron transport rate, the quantum PSII efficiency and the extent of photoinibition in response to light, temperature, pH and other factor (Bilger *et al.*, 1995). Generally the increase in basal fluorescence, F_0, may indicate the occurrence of photoinibitory damage in response to high temperature (Gamon & Pearcy, 1989), low temperature (Groom & Backer, 1992), excess of light (Ogren & Sjostrom, 1990) and water stress (Epron *et al*., 1992). At present, changes in F_v/F_m and F_0 are widely used as reliable diagnostic indicators of photoinibition (He et al., 1996; Valladares & Pearcy, 1997).

Up to now in marine environments the PAM fluorimetry was mainly used in the analysis of photosynthetic performance of seagrasses in order to investigate the photoinibitory damage risks (Ralph & Burchett, 1995; Dawson & Dennison, 1996; Longstaff *et al*., 1999; Ralph, 1999), temperature changes (Ralph, 1998), osmotic stress and desiccation (Ralph, 1998; Bjork *et al*., 2000), as well the contamination by heavy metals and petrochemicals (Ralph & Burchett, 1998) and herbicides (Ralph, 2000).

3.1.4. PARP as marker of cellular stress induction

Poly-ADPribosylation is a reversible post-translational modification of proteins catalyzed by the nuclear enzyme poly-ADPR polymerase (PARP) that uses NAD^+ as a substrate to synthesize polymers of adenosine diphosphoribose (poly-ADPR) (D'Amours *et al*., 1999; Chiarugi, 2002). Poly-ADPribosylation is involved in the regulation of several cellular functions related to the maintenance of genomic integrity (DNA repair, gene amplification, apoptosis) and to the expression and propagation of the genetic information (DNA transcription and replication, differentiation, neoplastic transformation) (Smith *et al*., 1998; Amé *et al*., 1999; Kickhoefer *et al*., 1999). The synthesis of poly-ADPR is an immediate response to DNA damage and is the first step in a cascade of events leading to either DNA

repair or apoptosis (Althaus *et al*., 1987; Jacobson *et al*., 1999, Chiarugi, 2002). The natural occurrence of a family of proteins with poly-ADPribosylating activity (PARP 1, PARP 2, V-PARP, tankyrase) underlines the important role of this modification in the regulation of various cellular functions (D'Amours *et al*., 1999; Jacobson *et al*., 1999; Smith *et al*., 1998; Amé *et al*., 1999; Kickhoefer *et al*., 1999; Chiarugi, 2002).

In plants, both PARP-1 and PARP-2 homologues were found with a very similar structure to animal polymerases (Babiychuk *et al*., 1998).

Both enzymes are localized in the nucleus and are equally induced by DNA breaks, while stresses such as cold, dehydration and heavy metals mainly induce PARP-2 (Doucet-Chabeaud et al., 2001).

When the stress causes too much ATP consumption, the plant suffers severe and permanent damages that lead to the cell death (Ying *et al*., 2005; Huang *et al*., 2009). Because in plants PARPs are major consumers of energy, several studies showed that both pharmacological and genetic PARPs inhibition reduce stress-induced energy consumption, protect plants against stress and prevent cell necrosis (Amor *et al*., 1998; De Block *et al.*, 2005). Recently, many researchers provided evidences for a role of plant PARP in energy homeostasis and stress tolerance (De Block *et al.*, 2005). Strong stresses induce poly(ADP-ribosyl)ation-activity, causing NAD^+ breakdown enhancing mitochondrial respiration. By reducing stress-induced poly(ADP-ribosyl)ation-activity NAD^+ breakdown is inhibited preventing high energy consumption (Vanderauwera *et al*., 2007). Under these conditions, plants preserve their energy homeostasis without an over-activation of the mitochondrial respiration, thus avoiding the production of reactive oxygen species (Amor *et al*., 1998).

A decrease of poly(ADP)ribosylation alleviates the NAD^+ and ATP, promoting an enhanced tolerance to drought, heat and high-light stresses.

3.2. Material and methods

3.2.1. Study site and target species

The Southern side of Castello Aragonese (40° 043.84' N; 13° 57.08' E), located at Island of Ischia (see Study Area), has been the sampling site during the summer season (July-August 2009) at about 1m depth.

In particular, two environments at different pH values have been considered to asses the effects of pH variation on physiology of some macroalgae.

S1 characterized by 8.1 units of pH was considered as "Normal pH" sector, S3 characterized by 6.78 units of pH was considered as "Low pH" sector.

For the eco-physiological analyses, three target species were selected according to their abundance and distribution along the pH gradient. The chosen species are: *Sargassum vulgare* (Phaeophyceae) present into the acidified environment; *Jania rubens* (Corallinaceae), mostly abundant at pH higher than 8 and completely absent into the acidified zone and *Dictyota dichotoma* v. *intricata* (Phaeophyceae) able to live all along the pH gradient even tough it is present with different abundances (see Chapter 2).

The measurements *in situ* and the sampling of thalli were performed always around noon (local time) in order to avoid possible effects related to the daily variation in the algal photoresponse.

Thalli collected for pigment analysis were free from epiphytes and immediately stored at -20°C.

3.2.2. Photosynthetic performance measurements: Photochemical parameters

In situ on intact thalli of the three target species (*Sargassum vulgare*, *Jania rubens* and *Dictyota dichotoma* v. *intricata*) were performed Rapid Light Curves (RLCs) in relation to the: effective PSII quantum yield (Δ_{PSII}), relative maximum PSII electron transport rate (rETRmax), photochemical (q_P) and non-photochemical quenching (q_N) by means of diving pulse amplitude modulated (PAM) fluorometer (Walz, Effeltrich, Germany). *In situ* on 10 min dark-adapted thalli, the maximum quantum yield of PSII (F_v/F_m) was also monitored.

RLC were derived through measurements of fluorescence (F_t) at 8 different light intensities (from 30 to 800 μmol photons m^{-2} s^{-1}). For each irradiance level, a saturating light pulse was applied and the maximal fluorescence (F_m') was determined. The difference

between F_m' and Ft is assumed to be the result of electron transport and, therefore, (F_m'-F_t)/ F_m' ($\Delta F/F_m$) can be used as an estimate quantum yield of PSII (Φ_{PSII}). Since *J. rubens* has not a laminar thallus did not determine the absorbance factor (AF) (Saroussi & Beer, 2007). For this reason relative ETR (rETR) were calculated for this species according to (Beer *et al*., 2001) as:

$$rETR = Y*PAR*0.5$$

The same parameter was calculated also for *D. dichotoma* and *S. vulgare*. Maximum photosynthetic rate (ETRmax), *alpha* (α) and saturating light intensity (E_k =rETR/α) (Ralph *et al*., 2002) of ETR *vs* light curves were estimated by the SigmaPlot software (SPSS Inc.) using the exponential function of Webb *et al.* (1974). When at the PPFDs used was observed an appreciable downturn in the curve, an additional term was introduced in the equation according to Platt *et al*. (1980). The photosynthetic parameters α and ETRmax *vs* E_k ratio were determined. α is the initial slope of the curve that represents the rate of photon conversion under low irradiances (Runcie & Riddle, 2004).

For the maximum quantum yield determination, thalli were darkened for 10 minutes to determine the ground basal, initial fluorescence (F_0) and then exposed to a single saturating flash in order to derive the maximum fluorescence (F_m). q_P and q_N were calculated according to van Kooten & Snel (1990).

3.2.3. Experimental design

For each target species 10 thalli of natural populations were considered to asses their photochemical performance. I*n situ* measurements were carried out on: *S. vulgare* in Low PH sector, *J. rubens* in Normal pH sector; *D. dichotoma* v. *intricata* in both Normal and Low pH environments. Thalli of natural populations were called *wild*.

In order to evaluate a possible stress induced by acidification on target species, transplanting experiments have been performed by moving algae from their native environment to other sites at pH different from the native one. In addition to assess the effect of transplanting practice, some thalli were transplanted also in their native environment.

In details, *S. vulgare* was transplanted from Low pH (S3) (i.e. native condition) to Normal pH environment (S1) and to S3. *J. rubens* was initially transplanted from Normal pH (S1) to to both Low pH (S3) and native Normal pH environment (S1). After only one week,

thalli transplanted in S3 were lost. For this reason S2 zone (pH = 7.68) characterized by pH values higher than S3, was used as. Low pH environment for the transplant of *J. rubens*.

D. dichotoma v. *intricata* was transplanted also from Normal pH to both Low pH and native Normal pH environment; *vice-versa* was made for thalli living in Low pH environment. Unfortunately all transplanted thalli of *D. dichotoma* v. *intricata* native of Low pH were lost and cannot be considered in the results. Transplanted thalli were called *transplant* followed by the name of the environment in which they were transplanted (e.g. *transplant_low* is a thallus transplanted in the low pH environment). A scheme of the experimental design is reported in Table 14.

Table 14: Species transplanted are showed. *New* indicate the non-native environment for the relative species.

Species and native environment	Transplanting environment	
	Low pH (S2 or S3)	Normal pH (S1)
S. vulgare Low pH (S3)	X (S3)	X (S1) *new*
J. rubens Normal pH (S1)	X (S2) *new*	X (S1)
D. dichotoma Low pH (S3)	-	-
D. dichotoma Normal pH (S1)	X (S3) *new*	X (S1)

Transplants were set up using plastic nets of 20*20 cm fixed to the rocky substrate by means of screws. On each net, 10 thalli of two target species were fixed on their meshes repeated for 3 times to prevent loss of thalli, for a total of 30 transplanted thalli for each species (Figure 32).

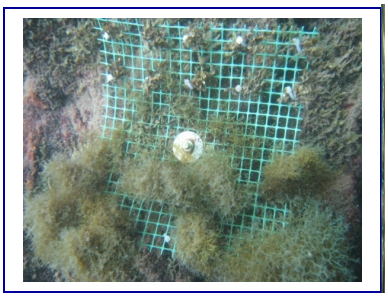


Figure 32: Transplanting nets with target species.

Transplanted thalli were let to acclimatize for three weeks, after this time a new set of fluorescence measurements was performed *in situ*. In order to assess possible seasonal changes during the transplanting period, fluorescence measurements were repeated also on natural populations simultaneously to transplanted thalli. Thalli of natural population on

which photochemical measurements were repeated after three weeks (as in transplanted thalli) were called *wild a*.

3.2.4. Laboratory experiments

In order to set the Diving-PAM fluorometer, first photochemical assessments were carried out in laboratory adjusting some parameter such as: intensity and time of the saturating pulse, the range of irradiance used for each species, the distance between the sample and the optical sensor and the assessment of the absorbing factor (AF).

In addition to the three target species, also the algae *Corallina elongata* and *Amphiroa rigida* (Corallinaceae) have been considered.

In all these species was analyzed the photochemical activity depending on the irradiance (Photosynthetic Photon Flux Density, PPFD, μmol photons m^{-2} s^{-1}). In particular the photochemical indexes: quantum yield of the linear electronic transport (Φ_{PSII}), electron transport activity (ETR), photochemical quenching (q_P) and non-photochemical quenching (q_N) were measured in response to increasing doses of PPFD ranging from 0 to 900 μmol photons $m^{-2}s^{-1}$. Φ_{PSII} was calculated according to Genty *et al.* (1989), ETR was measured according to Beer *et al*. (2001), q_P and q_N were determined following van Kooten & Snel (1990).

Maximum photochemical efficiency (F_v/F_m ratio) was measured on all 10 minutes dark adapted thalli.

3.2.5. Pigment analysis

Pigments were determined on *wild a* and *transplant* thalli of *Sargassum vulgare*, *Jania rubens* and *Dictyota dichotoma* v. *intricata*. Chlorophylls and carotenoids content of thalli were determined spectrophotometrically in 100% acetone according to Lichtenthaler (1987).

Pigments from 0.100 g of frozen tissue were extracted with a mortar in 100% acetone and centrifugated at 3000 rpm for 7'. Before centrifugation, samples were balanced and final volume was taken for each sample.

Total carotenoids (x+c), chlorophyll *a* and chlorophyll *b* were determined at 470 nm, 645 nm and 662 nm respectively. Then mg/g of fresh weigh of pigments was derived. In addition the ratios chl a/b and total chlorophyll/total carotenoids (a+b/x+c) were also determined.

3.2.6. PARP investigation

Isolation of nuclei

The isolation of nuclei was performed according to the method modified of Green *et al*. (1999). All operations were performed on ice or at 4°C. All algas were cut and resuspended in buffer A containing 10 mM TrisHCl pH 7.0, 1 mM EDTA, 1 mM EGTA, 1 mM $PhMeSO_2F$, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 0.5% Triton X-100 (1:4, w/v) and homogenized for 15-30 s at low speed using an Ultra Turrax T8 (IKAWERKE). The homogenates were filtered through three layers of cheesecloth, and the filtrates were centrifuged at 1500 x g for 30 minutes at 4°C. The pellets containing nuclei were suspended in buffer A and were centrifuged as above. This procedure was repeated four times to extract chloroplasts. Finally, the pellets were washed with buffer A without Triton X-100 (buffer B) and suspended in a small volume of buffer B containing 2% glycerol.

Assay of poly(ADPribose) polymerase

The enzymatic activity was routinely assayed in a reaction mixture (final volume 50 µL) containing 0.5 M Tris-HCl pH 8.0, 50 mM MgCl2, 10mM DTT, 0.4 mM [^{32}P]NAD^+ (10000 cpm/nmole) and a defined amount (20 µg protein) of whole nuclear fractions from algas. After incubation for 15 minutes at 25°C, the reaction was stopped by transfer onto ice and addition of 20% ((w/v) trichloroacetic acid (final concentration). The mixture was filtered through Millipore filters (HAWPP0001, 0.45 µm) and washed with 10% trichloroacetic acid. The activity was measured as acid-insoluble radioactivity by liquid scintillation in a Beckman counter (model LS 1701).

SDS-PAGE and Western Blot

Nuclear fractions from algae were electrophoresed onto 12% polyacrilammide mini-gel in 0.1% sodium dodecyl sulphate (SDS). Western Blot analysis was performed by electrotransferring proteins to a PVDF membrane using the Biorad apparatus at 200 mA for 2h at 4°C. Filter was incubated first with anti-poly(ADP-ribose)polymerase (H-250, Santa Cruz, CA, USA and after with the horseradish peroxidase-conjugated goat antirabbit secondary antibody (Pierce). Immunodetection by Enhanced Chemiluminescence was measured with a Quantity One Program in a Chemidoc apparatus (Bio-Rad).

3.2.7. Statistical analysis

The statistical analysis of data has been performed by analysis of variance (one way-ANOVA) with Tukey's *post hoc* test, for laboratory and *in situ* measurements for fluorescence parameters F_v/F_m, ETRmax, E_k, and *alpha*. Differences among curves of wild and transplanted thalli were analyzed by using two-way ANOVA (irradiances *vs* treatment) and Bonferroni *post hoc* test, for each photochemical parameter (Φ_{PSII}, qP and qN) and for each species.

Differences among chlorophylls and carotenoids content and their derived parameters (e.g a+b *vs* c+x ratio) were also analyzed through ANOVA and Tukey's *post hoc* test. All statistical analysis was performed by using statistical software package Prism 4 (GraphPad software Inc., 2005).

3.3. Results

3.3.1. Photochemical performance of target species

Results for effective PSII quantum yield (Φ_{PSII}), ETR, q_P and q_N are showed in Figura 33 for all considered species in laboratory measurements.

Highest values of (Φ_{PSII}), ETR and q_P were observed among different species, for *Dictyota dichotoma* and *Sargassum vulgare*. As regards thermal dissipation, the highest values of q_N were reported for *C. elongata* and *J. rubens* whereas *S. vulgare* showed the lowest ones.

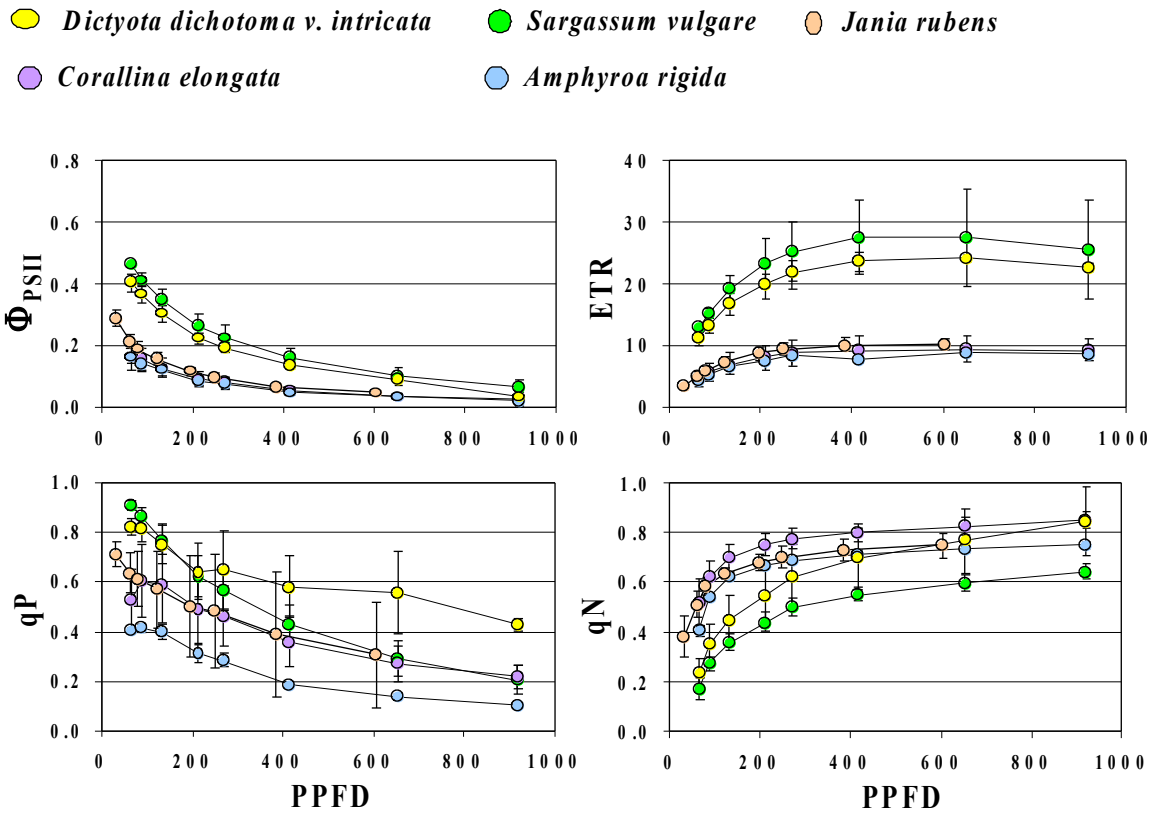


Figura 33: Quantum yield (Φ_{PSII}), electron transport rate (ETR), photochemical quencing (qP) and non-photochemical quencing (qN) in *D. dichotoma*, *S. vulgare, J. rubens*, *C. elongata* e *A. rigida,* to irradiance (PPFD, µmol of photons m^{-2} s^{-1}), measured in laboratory (means± standard error, n=4)

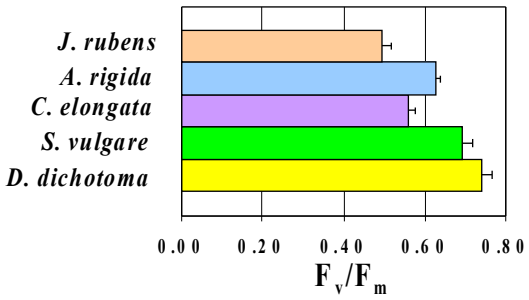


Figure 34: Maximum photochemical efficiency of PSII (F_v/F_m), measured in laboratory (pH>8) in *D. dichotoma*, *S. vulgare, J. rubens*, *C. elongata* e *A. rigida* (means ± standard error, n=4).

In is reported the maximum PSII photochemical efficiency for all species. *D. dichotoma* and *S. vulgare* showed higher values of F_v/F_m, compared to Corallinaceae. In particular, *J. rubens* showed the lowest value ($P<0.01$).

The same photochemical parameters measured in laboratory, were monitored also *in situ* (). The quantum yield of PSII electron transport (Φ_{PSII}) did not show significant differences among species at high irradiance. On the contrary in the range of 0-200 μmol photons $m^{-2}s^{-1}$, slightly difference was observed among *D. dichotoma* of lower pH environment (pH 6.72) and the other species.

The highest values of q_P and q_N were found in *J. rubens* whereas the lowest ones in *D. dichotoma* of both pH environments.

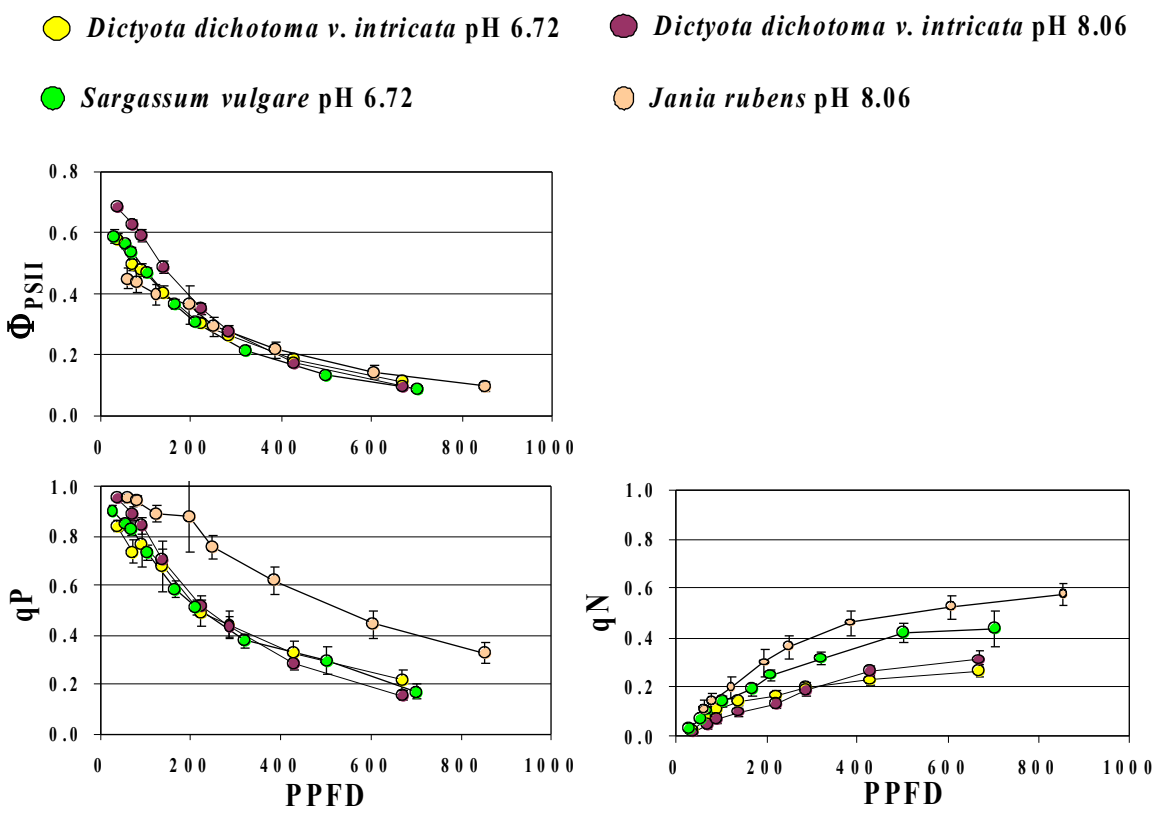


Figura 35: Quantum yield (Φ_{PSII}), electron transport rate (ETR), photochemical quencing (q_P) and non-photochemical quencing (q_N), to irradiance (PPFD, µmol of photons m^{-2} s^{-1}), measured in situ in *D. dichotoma* in both acidified (6.72) and normal (8.1) pH, *S. vulgare* in acidified environment and *J. rubens* in normal pH (means± standard error, n=9)

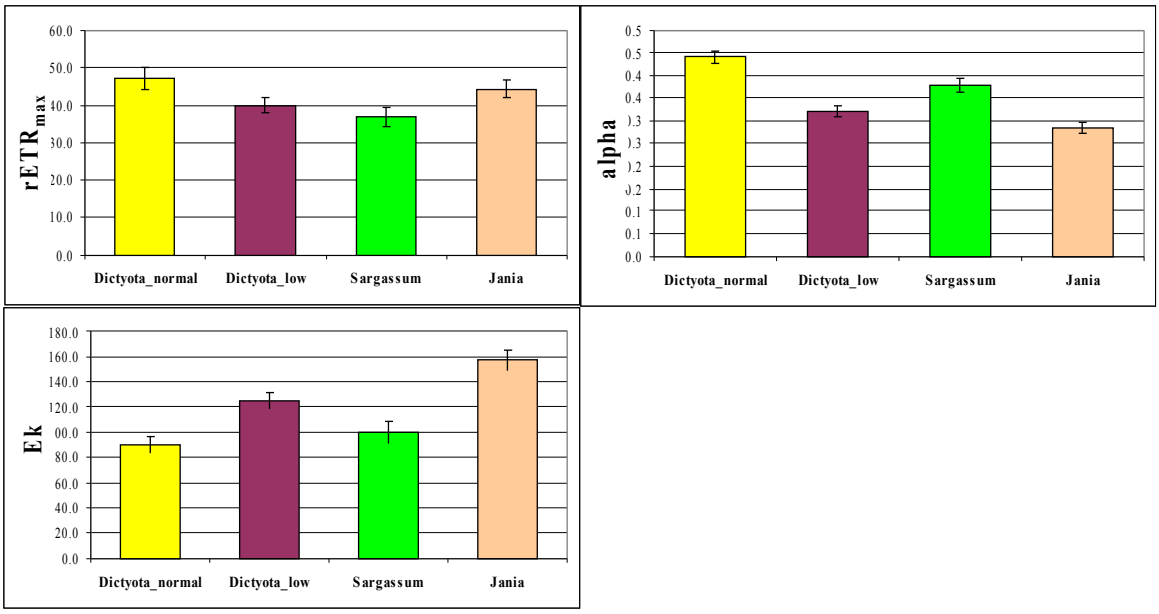


Figure 36: $rETR_{max}$, alpha and Ek for each target species (means± standard error, n=9).

shows the rETRmax, the alpha factor and Ek values measured *in situ* for the four target species. As regards rETRmax significant differences were observed between *D. dichotoma* of Normal pH, that presents the highest rETRmax, and *D. dichotoma* of Low pH ($p < 0.05$ according to Tukey's *post hoc* test) and between *D. dichotoma* of Normal pH and *S. vulgare* ($p < 0.01$) that exhibits the minimum value.

Among species, *D. dichotoma* of Low pH and *J. rubens* showed an alpha value significant lower ($p > 0.05$) compared to other algae. *D. dichotoma* from Normal pH showed the highest value for *alpha*. The highest Ek was observed for *J. rubens* while no difference was reported for *D. dichotoma* from both pH conditions and *S. vulgare*.

In Table 15 ANOVA results for rETRmax, alpha and Ek for each species are summarized.

Table 15: ANOVA results.

	Dictyota_Normal	Dictyota_Low	Sargassum_Low	Jania_Normal	F	P
rETRmax	47.1±2.89	39.8±2.13	36.9±2.43	44.3±2.33	6.06	<0.01
alpha	0.4±0.013	0.3±0.013	0.4±0.015	0.3±0.012	24.88	<0.01
Ek	89.9±6.30	124.9±6.90	99.6±8.52	157.5±8.05	15.59	<0.05

Figure 37 displays the PSII maximum photochemical efficiency for target species. No difference was observed among *D. dichotoma* of both pH environments and *S. vulgare* while the lowest F_v/F_m value was reported for *J. rubens* ($p < 0.0001$, Kruskal-Wallis test).

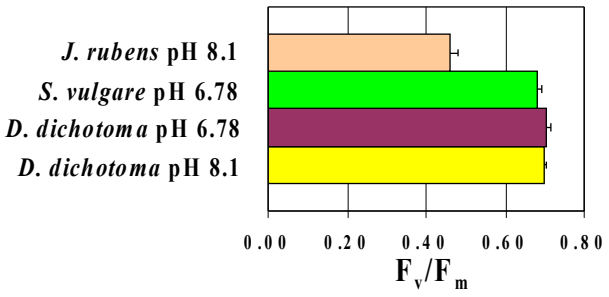


Figure 37: Maximum photochemical efficiency of PSII (F_v/F_m) in situ on target species (means± standard error, n=9).

3.3.2. Ecophysiological response to pH variation

Sargassum vulgare

Photochemical PSII efficiency (Φ_{PSII}), photochemical (q_P) and non-photochemical quenching (q_N), have been reported for *Sargassum vulgare* (Figure 38).

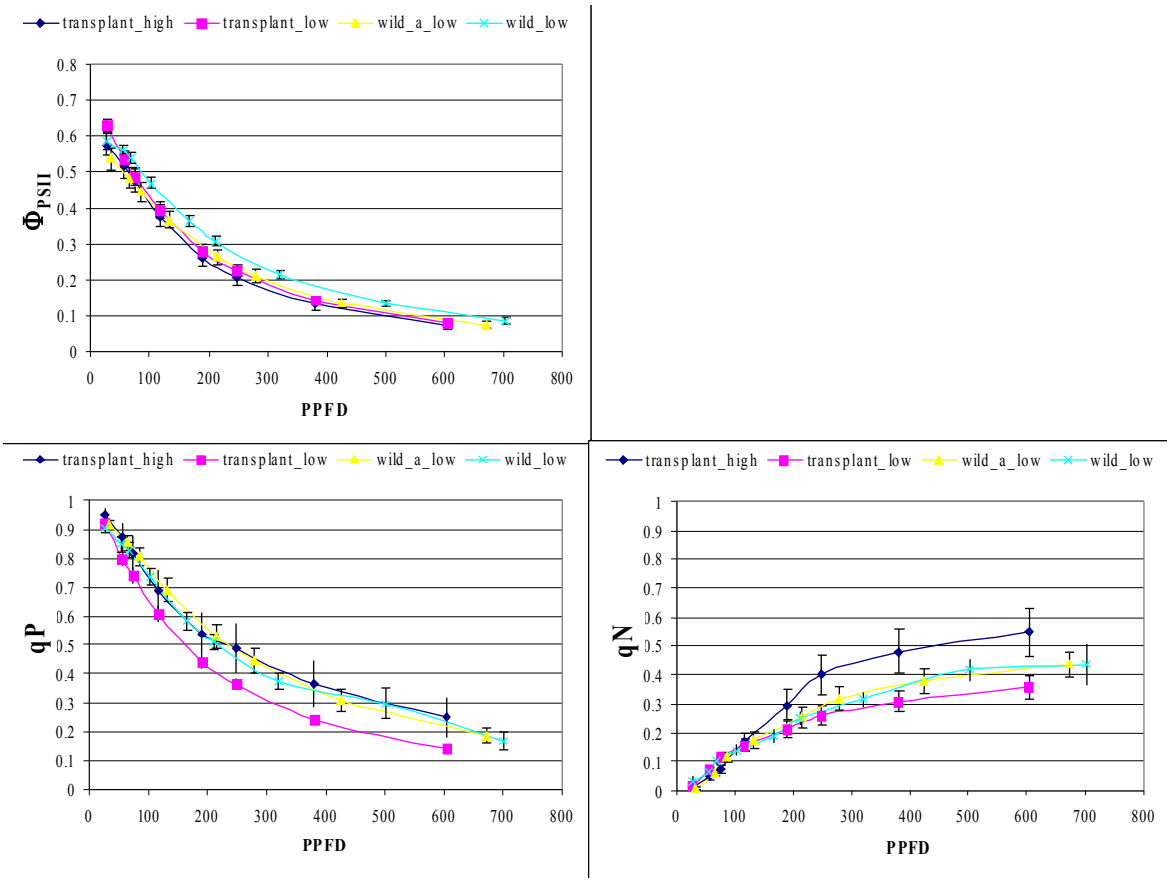


Figure 38: Photochemical PSII efficiency (Φ_{PSII}), photochemical (q_P) and non-photochemical quenching (q_N) in both transplanted and wild thalli of *S.vulgare* (means± standard error, n>9)

As regard transplanting experiment, there is no difference for the Φ_{PSII} among wild and transplanted thalli. Lowest values of photochemical quenching (q_P) were observed for thalli transplanted in native area ($p<0.001$), i.e. low pH condition, while little difference ($p>0.05$) was observed between wild and transplanted thalli in higher pH conditions. The last ones are very similar to *wild a* thalli in which measures were carried out simultaneously to transplanted thalli. On the other hand, highest values of q_N ($p<0.01$) were observed in thalli transplanted in higher pH conditions while similar values were recorded for both wild and *wild a* thalli and transplanted thalli in native conditions.

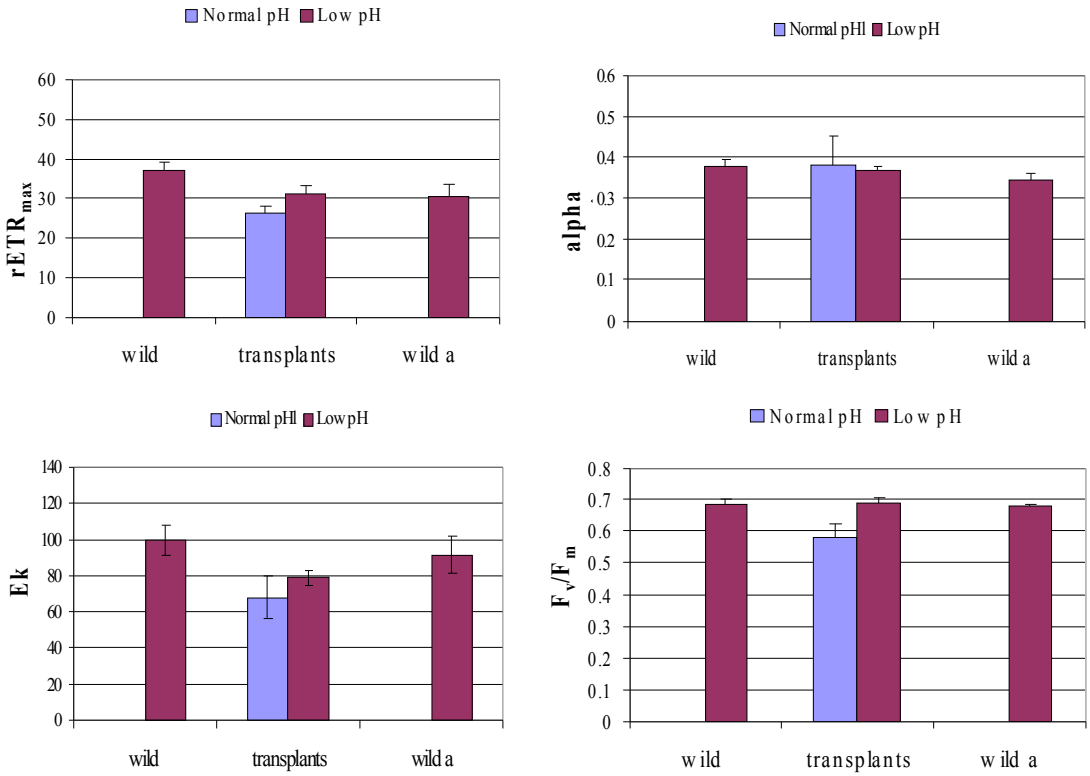


Figure 39: $rETR_{max}$, alpha, Ek and F_v/F_m for *S.vulgare* in transplanted and wild thalli (means± standard error, n>9).

In Figure 39 are reported the rETRmax, the alpha factor, the E_k and the maximum PSII photochemical efficiency (F_v/F_m) for the species *Sargassum vulgare*. $rETR_{max}$ evidences no significant differences among *wild*, *wild a* thalli and transplanted thalli in both pH environments ($F = 1.94$, $p = 0.3315$); *alpha* values did not present any differences among treatments ($F = 1.12$, $p = 0.0867$) whereas Ek decreases significantly in thalli transplanted in both higher pH and native environment compared to wild thalli ($p<0.05$). The highest value of E_k was found in wild thalli ($p<0.001$).

As regards the maximum PSII photochemical efficiency, the highest values of F_v/F_m were observed in low pH conditions while a significant decrease was detected in transplanted thalli in higher pH ($F = 4.77$, $p < 0.01$). No difference was recorded in wild thalli and transplanted thalli in native area.

In Table 16 ANOVA results for $rETR_{max}$, alpha and E_k in *Sargassum vulgare* are summarized.

Table 16: ANOVA results for *S. vulgare*.

	Wild_Low	Trans_Normal	Trans_Low	Wild a_Low	F	P
rETRmax	36.9±2.43	26±2.22	31.2±1.67	30.4±3.16	1.94	>0.05
alpha	0.4±0.015	0.4±0.026	0.4±0.01	0.3±0.021	1.12	>0.05
Ek	100.2±8.52	68±11.78	78.9±4.53	91.4±10.34	45.48	<0.05

Jania rubens

In Figure 40 are reported the photochemical indexes Φ_{PSII}, ETR, q_P and q_N for *Jania rubens*.

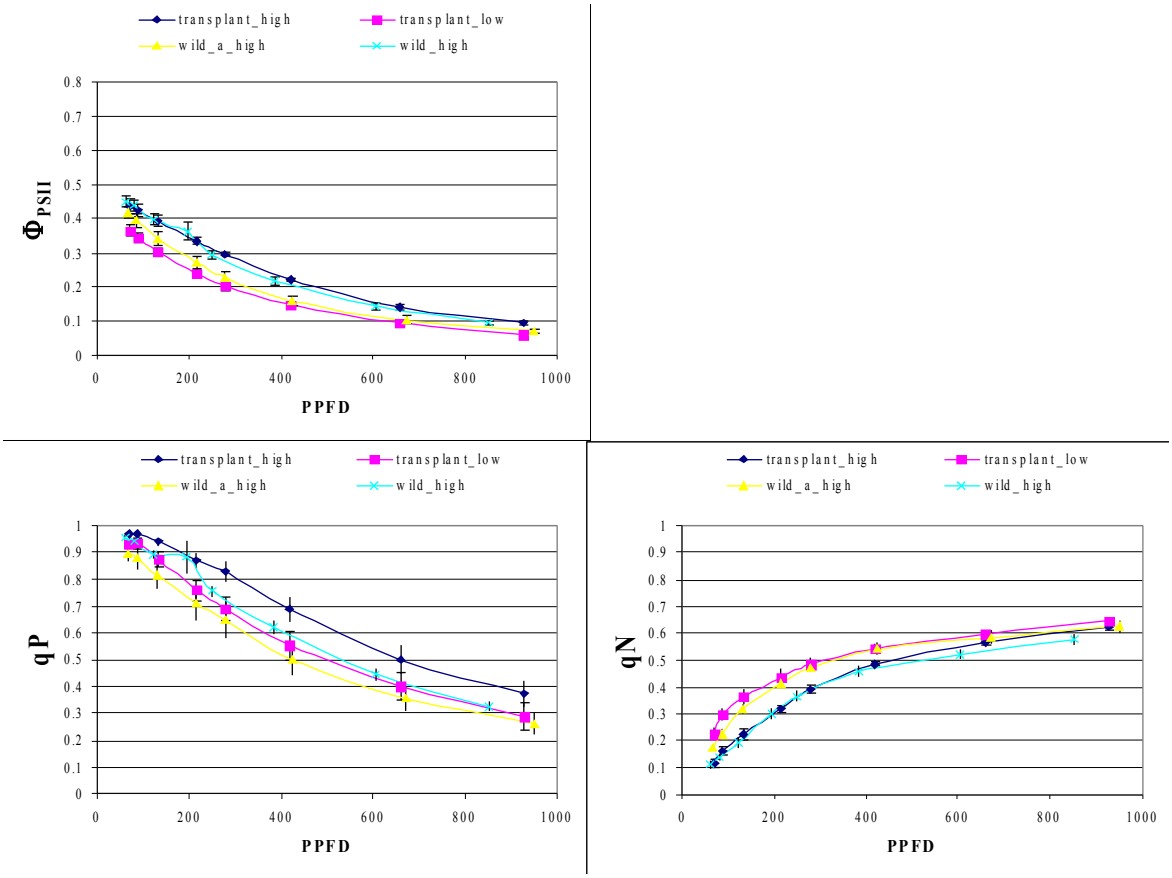


Figure 40: Photochemical PSII efficiency (Φ_{PSII}), photochemical (qP) and non-photochemical quenching (qN) in both transplanted and wild thalli of *J. rubens* (means± standard error, n>9).

The quantum yield of PSII electron transport (Φ_{PSII}) did not show any difference among wild and transplanted thalli at higher pH. In thalli transplanted at lower pH Φ_{PSII} strong decrease showing the lowest values. A similar trend was observed for *wild a* thalli.

Higher values of q_P were observed for transplanted thalli in higher pH sector. No differences ($p>0.05$) among transplanted thalli in lower pH, wild thalli and wild *a* thalli were

found. As regard q_N, the highest values were reported for transplanted thalli in lower pH condition principally at lower irradiances ($p<0.01$). Similar ($p>0.05$) values were observed for both transplanted and wild thalli at higher pH.

In Table 17 are summarized the ANOVA results for $rETR_{max}$, alpha and E_k in *Jania rubens*.

Table 17: ANOVA results for J. rubens.

	Wild_Normal	Trans_Normal	Trans_Low	Wild a_Low	F	P
rETRmax	44.3±2.3	48.3±1.8	32.5±2.6	35.6±4	7.09	<0.01
alpha	0.3±0.012	0.3±0.017	0.2±0.013	0.3±0.0075	3.59	<0.05
Ek	157.5±8.05	185.8±17.08	145.3±14.9	122±13.8	2.48	>0.05

Figure 41 shows $rETR_{max}$, the alpha factor, the E_k and the maximum PSII photochemical efficiency (F_v/F_m) for the species *Jania rubens*. As concerns $rETR_{max}$, significant differences were detected between wild thalli and transplanted thalli in lower pH environment ($p < 0.01$). Statistical significant differences ($p < 0.01$) were also evidenced between transplanted thalli in the different pH conditions, with lowest value for thalli transplanted in low pH sector, and between wild a and transplanted thalli in native environment ($p< 0.05$).

The minimum value of *alpha* was observed for transplanted thalli in low pH environment ($p < 0.05$) whereas no differences were reported for wild thalli and transplanted thalli in native condition. No difference was observed among wild thalli and transplanted thalli as regard as E_k values.

The highest F_v/F_m ratio was observed at higher pH conditions for both transplanted and wild thalli. Maximum photochemical efficiency decreases significantly ($p < 0.01$) in thalli transplanted in lower pH conditions.

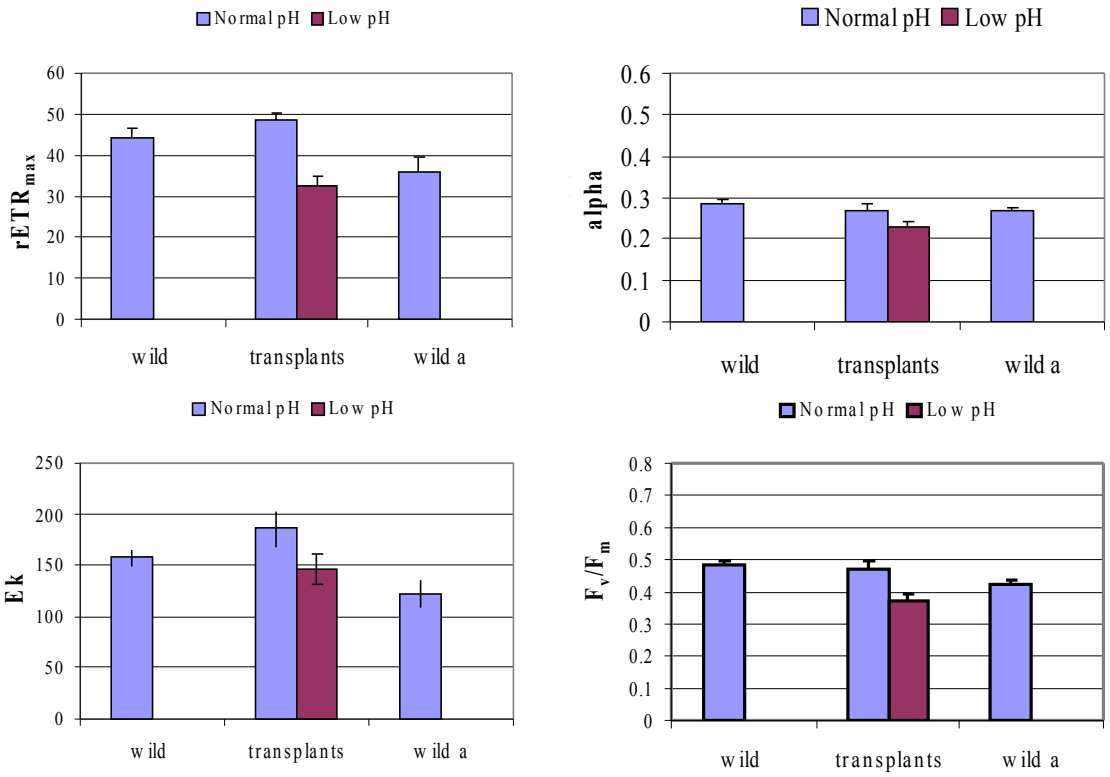


Figure 41: $rETR_{max}$, alpha, Ek and F_v/F_m for *J. rubens* in transplanted and wild thalli (means± standard error, n>9).

Dictyota dichotoma

D. dichotoma showed lower values of quantum yield of PSII electron transport (Φ_{PSII}) in transplanted thalli in low pH, compared to transplanted thalli in native area (normal pH) ($p < 0.01$). The highest values were observed for wild thalli in higher pH area principally at lower irradiances ($p > 0.01$) (Figure 42).

The highest values of q_P (Figure 42) were observed for transplanted thalli in native pH environment (normal pH), whereas the lowest were observed for *wild a* thalli of lower pH environment. However at lower irradiances (< 100 PPFD) q_P values are very similar among different treatments and control thalli.

Highest values of q_N (Figure 42) have been showed by *wild a* thalli of higher pH environment. *Wild a* thalli from lower pH environment together with *wild* thalli from both lower and higher pH showed lowest values of q_N being similar among them ($p > 0.05$). Transplanted thalli of higher pH, lower pH and native environment showed differences at higher irradiances for qN ($p < 0.01$).

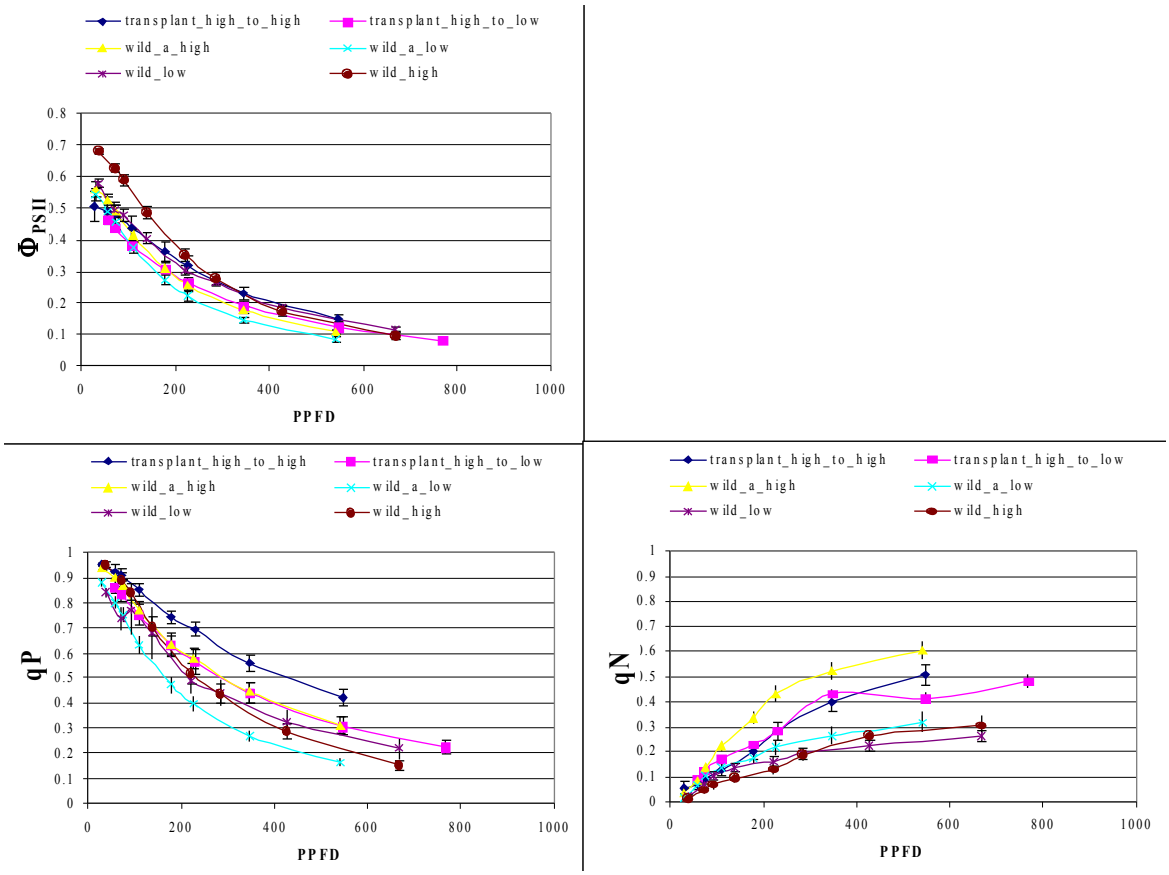


Figure 42: Photochemical PSII efficiency (Φ_{PSII}), photochemical (q_P) and non-photochemical quenching (q_N) in both transplanted and wild thalli of *D. dichotoma* (means± standard error, n>9).

Figure 43 showed $rETR_{max}$ mean values with SE for *Dictyota dichotoma*. Significant differences were observed between wild thalli from Normal pH environment and transplanted thalli in Low pH ($p < 0.001$) and between *wild* and *wild a* thalli from Normal pH ($p < 0.001$). Significant differences were observed for *alpha* between wild thalli of different pH condition ($p < 0.01$). *alpha* decreases in thalli transplanted in both pH environments ($p < 0.01$) and in *wild a* thalli from Normal pH environment ($p < 0.05$).

As regards E_k, higher values were observed in transplanted thalli in native environment, compared to wild thalli of Normal pH ($p < 0.001$, according to *post hoc* Tukey's test). No difference was observed in transplanted thalli of both pH conditions neither in wild and *wild a* thalli of Normal pH. The maximum PSII photochemical efficiency (F_v/F_m) showed no statistical difference among *wild* and *wild a* thalli of both pH conditions. Lowest values ($p < 0.01$) of F_v/F_m were recorded for transplanted thalli in both higher and lower pH environment. In Table 18 are summararized the ANOVA results for $rETR_{max}$, alpha and E_k in *D. dichotoma*.

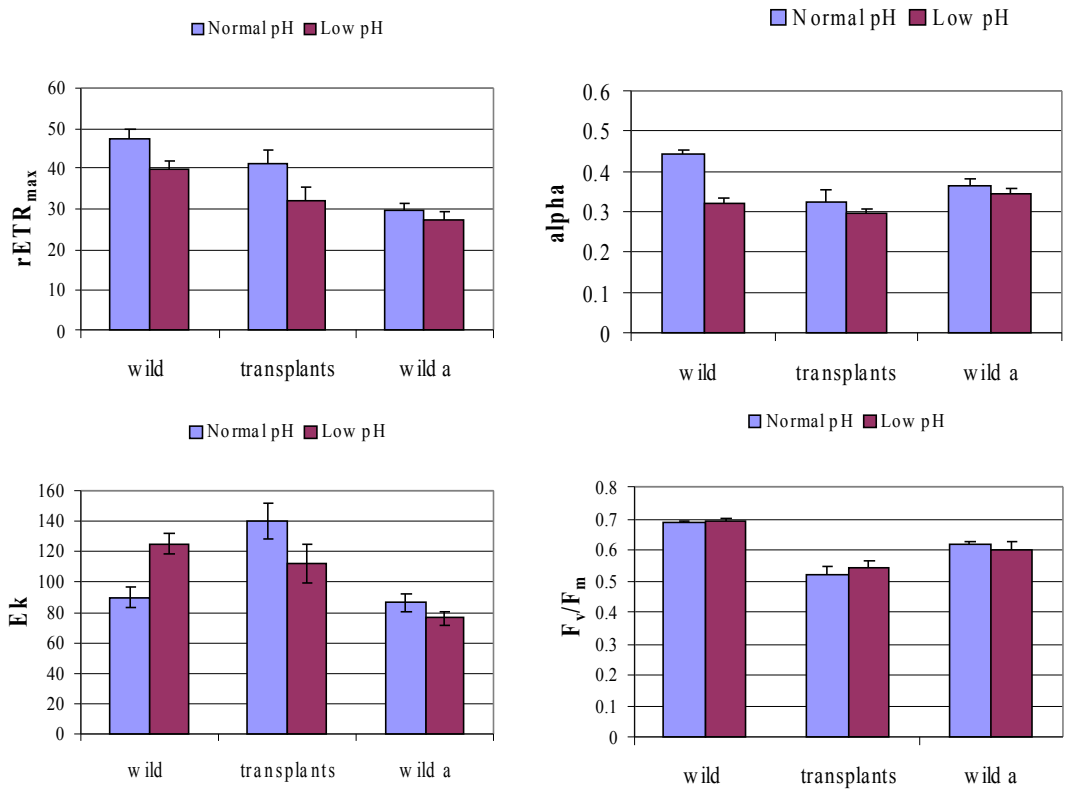


Figure 43: $rETR_{max}$, alpha, Ek and F_v/F_m for *D. dichotoma* in transplanted and wild thalli (means± standard error, n>9).

Table 18: ANOVA results for *D. dichotoma*.

	Wild Normal	Wild Low	Trans Normal	Trans Low	Wild a Normal	Wild a Low	F	P
rETRmax	47.1±2.89	39.8±2.13	41.1±3.42	32.1±3.69	29.7±1.64	27.4±1.90	11.73	<0.001
alpha	0.4±0.013	0.3±0.013	0.3±0.033	0.3±0.013	0.4±0.017	0.3±0.014	10.02	<0.001
Ek	89.9±6.30	124.9±6.90	140±11.59	112.4±13.22	86.5±5.79	76.4±4.57	8.38	<0.001

3.3.3. Pigments analysis

Pigments content and derived parameters (e.g. Chl a/b, a+b/x+c) have been analysed for each target species. All species show a similar pigments ratio even among treatments (Figure 44).

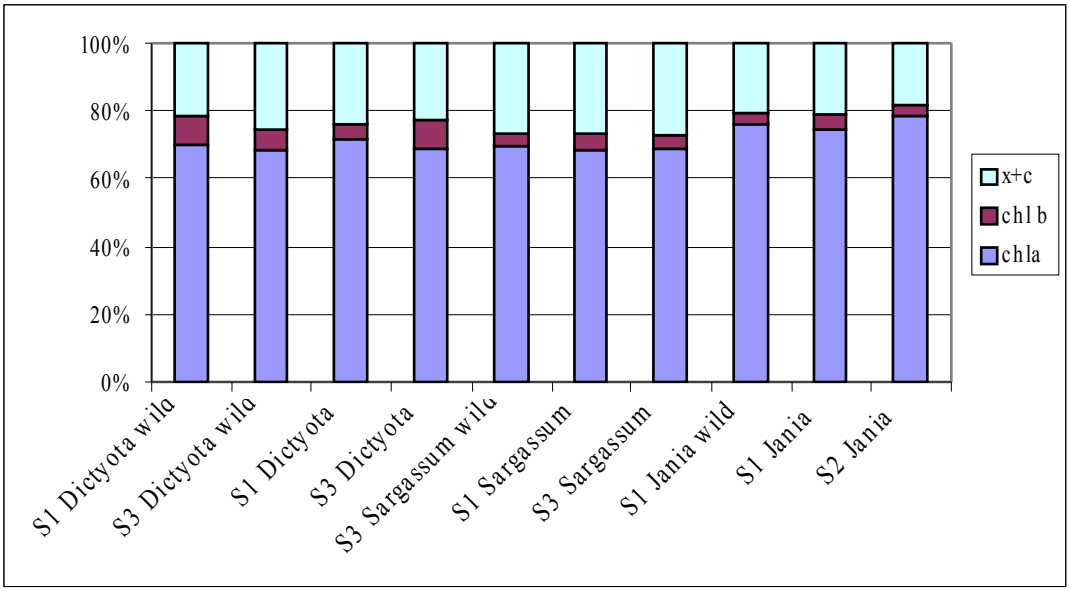


Figure 44: Pigment ratios in transplanted and wild thalli of target species.

Sargassum vulgare

In Figure 45 is showed the Chlorophyll *a* and b content, the total chlorophyll (a+b) and the total carotenoid (x+c) content, ratio of chlorophyll a/b and the ratio of total chlorophyll/total carotenoid (a+b)/(x+c) at different pH for the species *S. vulgare*. The maximum value of Chl *a* (Figure 45) was found in thalli of *S. vulgare* transplanted in native environment ($P < 0.001$), i.e. at lower pH conditions; on the contrary, wild and transplanted thalli in normal pH showed comparable content of chlorophyll a ($P > 0.05$).

Chlorophyll *b* (Figure 45) showed no statistical difference in *wild a* thalli and transplanted thalli in both high and low pH environments ($P > 0.05$). Total Chlorophyll content (*a*+*b*) showed the same pattern of Chlorophyll *a*, as it was expected, with a maximum value for thalli transplanted in native environment (Figure 45).

Total carotenoid content (*x*+*c*) was the highest in thalli transplanted in native condition, on the contrary, thalli transplanted in normal pH conditions exhibited comparable content (Figure 45).

The ratio *a/b* showed no difference in thalli transplanted in non-native environment, i.e. in normal pH condition (Figure 45). At low pH, (*a*+*b*/*x*+*c*) ratio decrease in thalli transplanted compared to thalli *wild a*. No significant difference was fount between *wild a* thalli and thalli transplanted in normal pH (Figure 45).

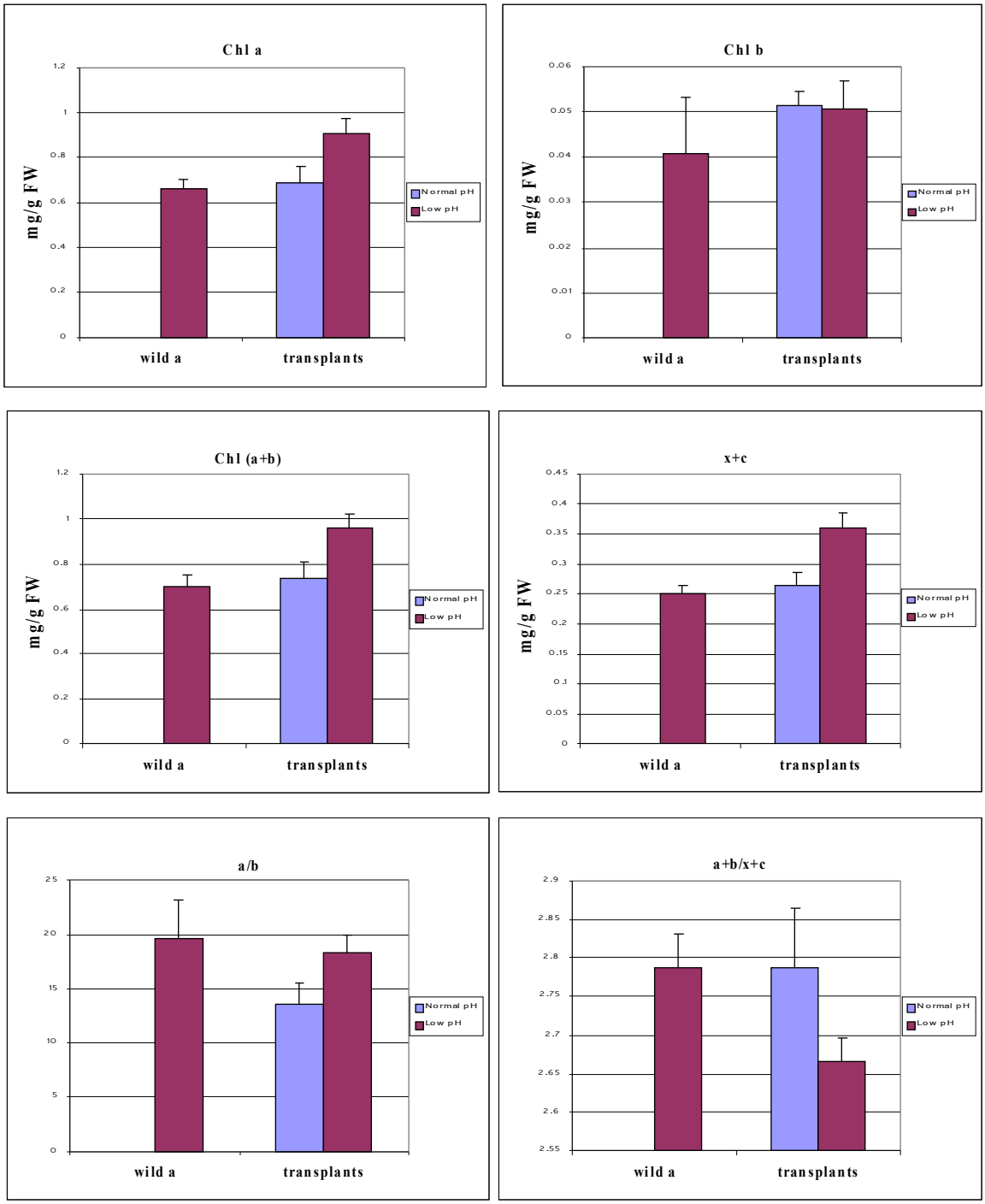


Figure 45: Pigment analyses in *S. vulgare* for *wild a* and transplanted thalli (means± standard error, n=4).

<u>*Jania rubens*</u>

In Figure 46 is showed the Chlorophyll *a* and b content, the total chlorophyll (a+b) and the total carotenoid (x+c) content, ratio of chlorophyll a/b and the ratio of total chlorophyll/total carotenoid (a+b)/(x+c) at different pH for the species *J. rubens*. Chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoids showed lower value in thalli transplanted in low pH environments compared to thalli *wild a* in normal pH (Figure

46). The ratios *a/b* and (*a+b/x+c*) exhibited comparable value between *wild a* thalli and thalli transplanted in lower pH environment without statistical differences (P> 0.05) (Figure 46).

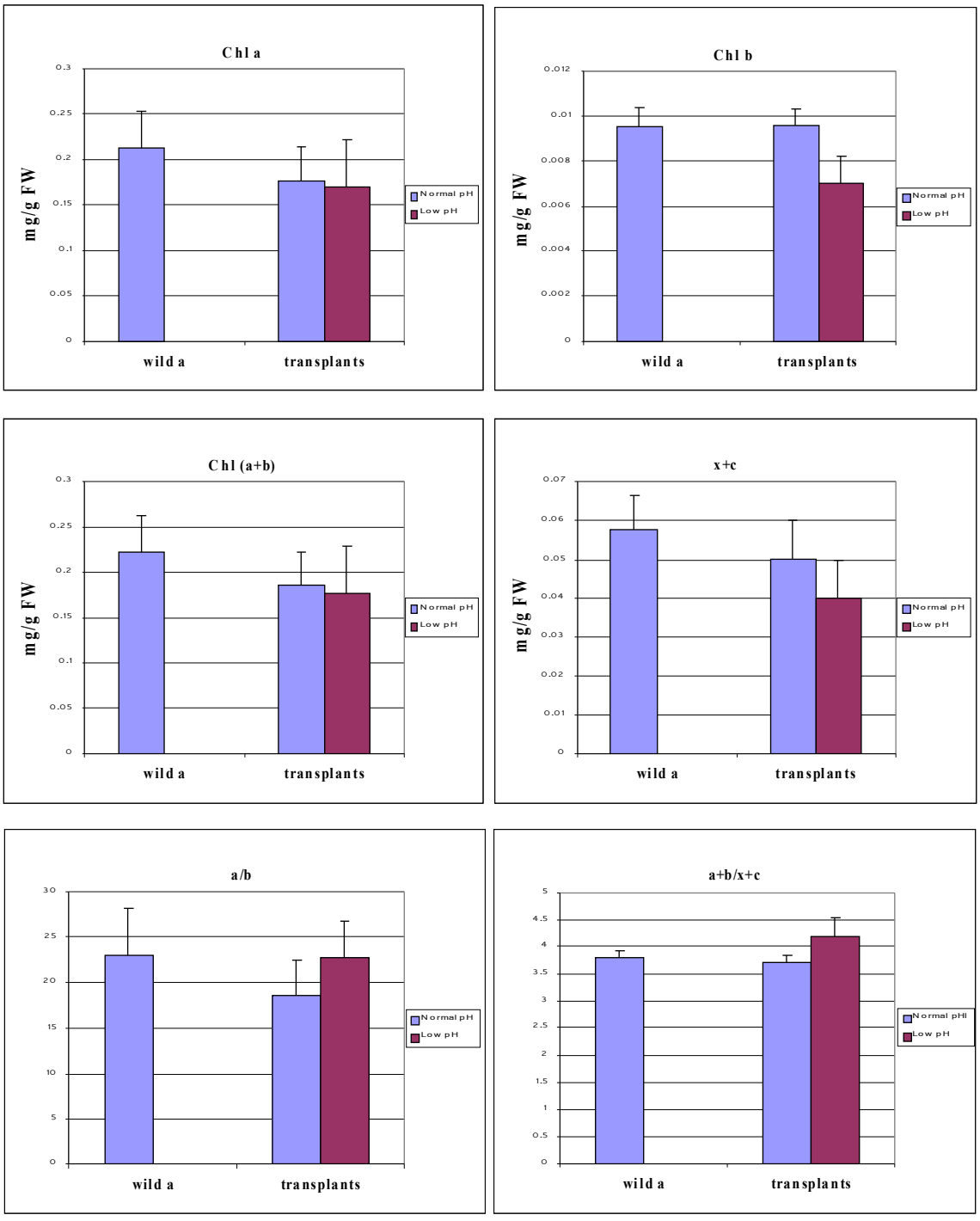


Figure 46: Pigment analyses in *J. rubens* for *wild a* and transplanted thalli (means± standard error, n=4)..

<u>*Dictyota dichotoma*</u>

In Figure 47 is showed the Chlorophyll *a* and b content, the total chlorophyll (a+b) and the total carotenoid (x+c) content, ratio of chlorophyll a/b and the ratio of total chlorophyll/total carotenoid (a+b)/(x+c) at different pH for the species *D. dichotoma*.

Chlorophyll *a* showed comparable values in both wild a and transplanted thalli ($P > 0.05$). *Wild a* thalli of lower pH environment showed the highest ($P < 0.01$) content of Chlorophyll *a* (Figure 47).

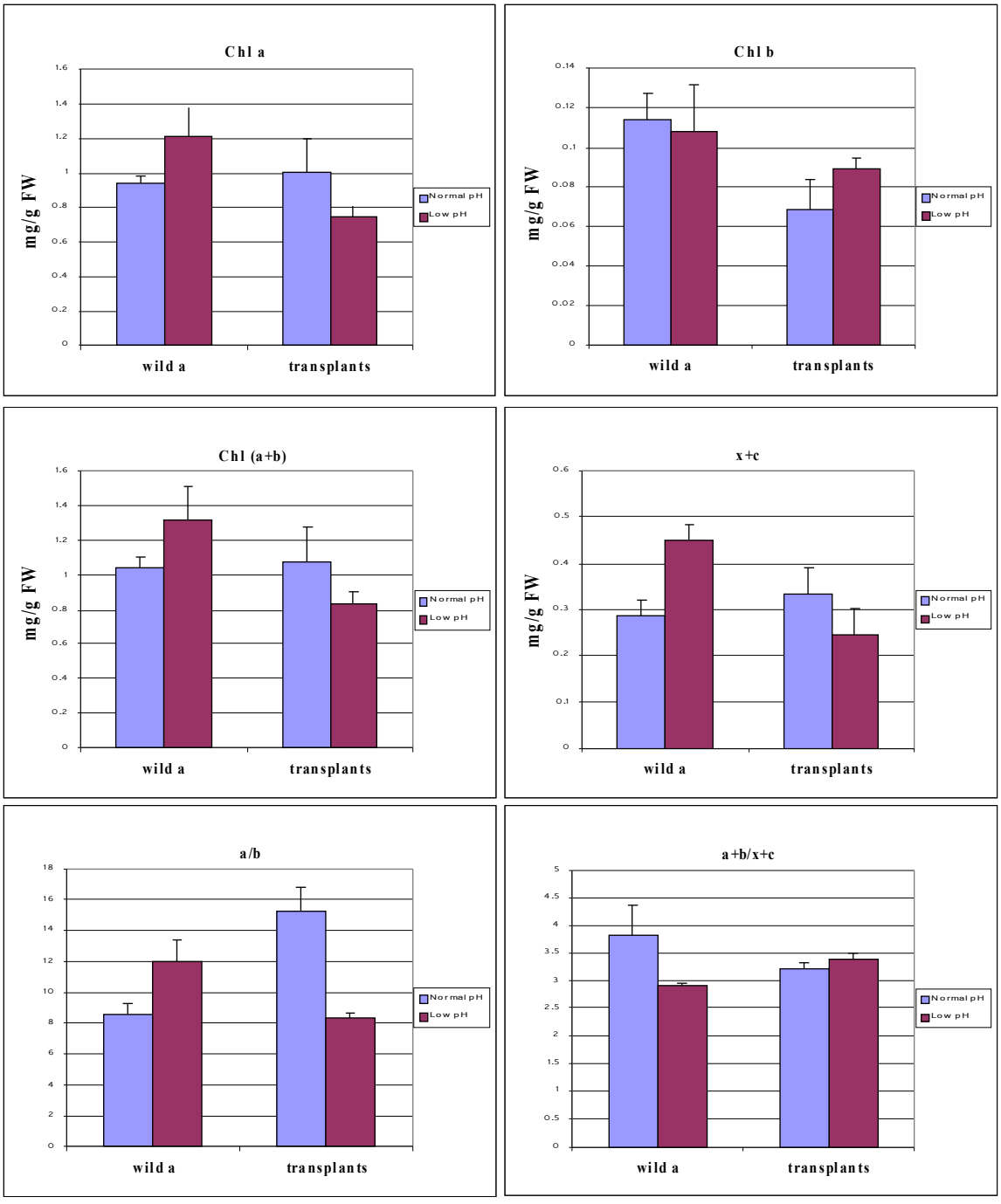


Figure 47: Pigment analyses in *D. dichotoma* for *wild a* and transplanted thalli (means± SE, n=4).

Chlorophyll *b* of transplanted thalli was lower ($P < 0.05$) than wild a thalli at both pH conditions. Total chlorophylls content mirrored the same pattern of chlorophyll *a* (Figure

47). Total carotenoid showed the highest amount in *wild* thalli of lower pH environment (P< 0.05), whereas thalli transplanted from higher pH to lower pH environment showed a comparable carotenoid content (P> 0.05) (Figure 47). The highest *a/b* ratio was found at low pH in *wild a* thalli compared to transplanted ones (P< 0.05). At normal pH the situation is reversed: transplanted thalli shoed higher a/b ratio than *wild a* ones (P< 0.001). Total chlorophyll *vs* total carotenoids ratio showed a higher value in *wild a* thalli of normal pH compared to thalli low pH, no difference between transplanted thalli in both pH conditions was found.

3.3.4. PARP activity

The nuclear fractions obtained from all transplanted and wild population thalli of *Sargassum vulgare*, *Jania rubens* and *Dictyota dichotoma* v. *intricata*, were assayed for ADP-ribosylating activity at 25°C and pH 8.0 (Figure 48).

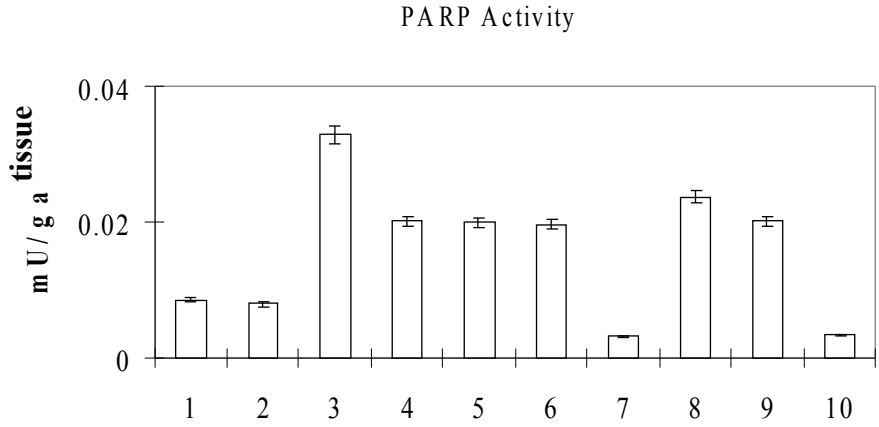


Figure 48: PARP activity in 1= Wild thalli of *S. vulgare* (S3); 2=Transplanted thalli of *S. vulgare* in S3; 3= Transplanted thalli of *S. vulgare* in S1; 4= Wild thalli of *J. rubens* (S1); 5=Transplanted thalli of *J. rubens* in S1; 6=Transplanted thalli of *J. rubens* in S2; 7= Wild thalli of *D. dichotoma* (S3); 8= Wild thalli of *D. dichotoma* (S1); 9= Transplanted thalli of *D. dichotoma* from S1 to S1; 10= Transplanted thalli of *D. dichotoma* from S1 to S3.

In *S. vulgare* samples, higher levels of activity were found in the transplanted thalli in S1 (pH 8.1) compared to wild population and transplanted thalli in S3 (pH 6.57), in which a comparable activity was measured. As regard *D. dichotoma*, the lowest activity was found in transplanted thalli in S3 (pH 6.57) and in wild thalli in S3. Transplanted thalli in S1 (pH 8.1) showed about 50% of poly(ADPribosyl)ating activity measured in wild S1 pH 8.1. The PARP activity in *J. rubens* samples seemed to confirm the previous hypothesis: a comparable (ADPribosyl)ation was found in all *Jania* samples growth in similar pH conditions.

Immunochemical analyses

Nuclear fractions from all examined algae thalli were subjected to Western Blot with anti-PARP1 polyclonal primary antibodies, to confirm the presence of an endogenous poly(ADPribosyl)ating enzyme. In all samples a band at lower molecular weight (about 70 kDa) and a second signal of about 140 kDa were observed (Figure 49).

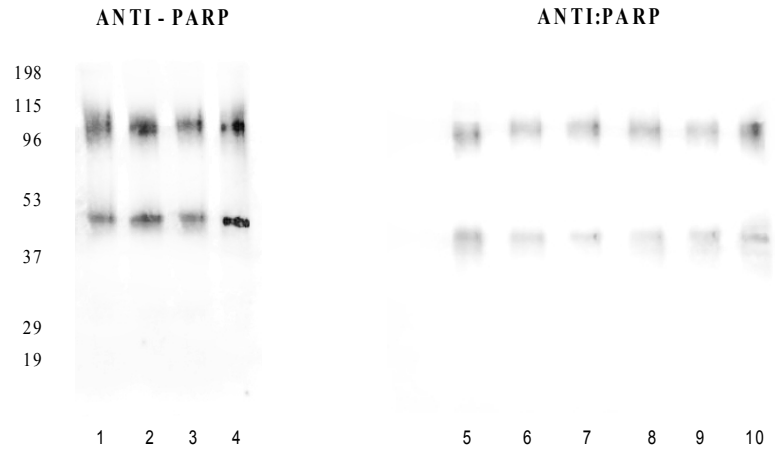


Figure 49: Western blot for 1= Transplanted thalli of *D. dichotoma* from S1 to S1;; 2= Transplanted thalli of *D. dichotoma* from S1 to S3; 3= Wild thalli of *D. dichotoma* (S1); 4= Wild thalli of *D. dichotoma* (S3); 5= Wild thalli of *J. rubens* (S1); 6= Transplanted thalli of *J. rubens* in S2; 7= Transplanted thalli of *J. rubens* in S1; 8= Transplanted thalli of *S. vulgare* in S1; 9= Wild thalli of *S. vulgare* (S3); 10= Transplanted thalli of *S. vulgare* in S3.

4. Changes in genetic diversity along a natural pH gradient

4.1. Introduction

4.1.1. The species problem

Species are considered the essential level of biological organization, and as such they are fundamental to several fields of investigation as well as to practical applications of evolutionary and biology. There is great controversy among biologists about the definition of species, to the extent that nobody has ever not heard anything about the issue known as "the species problem".

Recently, two of the major journals of evolutionary biology have devoted several papers in special issues to the question of: "what are species?" (Journal of Evolutionary Biology, volume 14, 2001, pp. 889 ff.; and Trends in Ecology and Evolution volume 16, 2002, pp. 326 ff.), there have been several books (Otte and Endler, 1989; Hey, 2001; Claridge, Dawah and Wilson, 1997; Howard and Berlocher, 1998) and of course numerous articles.

In research manuscripts, where the species is involved (species descriptions, taxonomic revisions, phylogeographic hypothesis, etc.), it is very rarely evident which species concept the researcher adheres to.

Mayden (2002) reviews the reasons for the disagreement surrounding the species issue. Besides the main one (the failure of scientists in appreciating the nature of species as individuals), he highlights some epistemological reasons for the disagreement surrounding the species issue. First, the perception of reality and judgements of different scientists is reflective of their academic background. "If one is a morphological taxonomist or systematist, then there will likely be more emphasis placed on these types of traits validating the existence of a species, even in the face of dramatic genetic differences between morphologically similar forms". Furthermore, the potential of resolving issues regarding biological diversity using molecular tools is currently particularly advertised. "Although the methods being employed in molecular studies can be more informative in some cases, it is equally likely that they will either not provide information in other cases or the information generated is of questionable validity to ask evolutionary and biodiversity questions because of questions of homology (Stauffer and McKaye, 2001).

4.1.2. Speciation and sister, cryptic and sibling marine species

The knowledge of what species are, how they originate and how they can be distinguished is of paramount importance in order to understand taxonomic relationships existing among them. This topic has practical applications to several fields, as biodiversity conservation, public health and human diet. Papers dealing with it are published at increasing rate and, today, terms as "sister", "sibling" and "cryptic" species dominate their titles and abstracts. Even removing the misunderstanding coming from the fact that camouflaged or secretive species are referred to as "cryptic" by some authors, there seems not to be a general agreement on the real definition of these terms. Most authors consider cryptic species to be synonymous with sibling species (Saez and Lozano, 2005), others regard sibling species as "cryptic sister species" (Knowlton, 1986).

Before continuing, it is essential to make clear this point. Sister species are two species derived from the same immediate common ancestor; cryptic species two or more distinct species that are erroneously classified as a single nominal species because they are, at least superficially, morphologically indistinguishable. Sibling species are the closest relative of each other and have not been distinguished from one another taxonomically. Finally, a pair or a group of cryptic (or sibling) species are considered as a species complex. Bickford et al. (2007) provide the most recent survey on literature on cryptic and sibling species. Their work confirms the extreme actuality of the issue (over 3500 published papers in the last 50 years), often occurring as a byproduct of investigation on other biological issues and rather biased towards animal taxonomy. Despite the increased present-day attention on it, the problem of identifying cryptic species is not exactly a novelty and has probably appeared immediately after the origin of the idea of species. Obviously, this problem has been affected by the debate on species concepts and researchers have based the recognition of cryptic on their favourite species concept. For the reasons reported above, an attempt to deal with the issue of cryptic species identification will be made in this thesis, remembering the differences among species definition, speciation processes and species delimitation, thus disentangling this issue from the above discussed "species problem". Until now, most taxonomists described species observing the dictates of morphological (or "typological") species concept. Each description corresponds to a "lot" of type specimens, known as "type series" housed in museums or private collections. After its formulation, Biological Species Concept became the most popular species concept, and tests for interbreeding were commonly accepted as method to identify cryptic species. Indeed, as referred before, speciation is not always accompanied by morphological change; the true number of biological species is likely to be greater than

species described on the basis of purely morphological grounds. Unfortunately, mechanisms of reproductive isolation differ among taxa and this method has soon been proven not being universally appropriate. Today, the production and the analysis of molecular data require no specialized knowledge of the anatomy, ecology, behaviour or biogeography of the taxa involved. For this reason, DNA sequencing is becoming the most largely employed method in taxonomical research on cryptic species at the extent that it has been suggested it "should be incorporated in the research of alpha taxonomists (i.e. biologists that discover, describe, and name new species) as a matter of routine and/or that genetic material should be preserved so that subsequent molecular analysis is possible" (Bickford et al., 2007). Methods based on neutral molecular markers make up a new powerful tool to obtain information on hierarchical relatedness and relatives rates of evolution (i.e. molecular clocks). They are in agreement with all Phylogenetic Species Concepts and succeed in overcoming the constraints imposed by the strict application of reproductive isolation tests.

Starting from the awareness that the multidisciplinary approach is the unique tool making possible the most realistic "vision" of the taxonomic status of a species, before to focus on the single cases analysed in this thesis, we'll make a rapid excursus on the problems met by the researchers, to explain the mechanisms leading to speciation and to forming sibling species, ubiquitous and so common in the sea (Knowlton, 1993).

These difficulties are also linked to the distinction made among sympatric, parapatric and allopatric species. Their peculiarities are not even so clear (Futuyma and Meyer, 1980), and become more complicated in the sea, where it is really hard both to delineate the characteristics of larvae and/or adults marine individuals, and to document their dispersal power (Burton, 1983). Besides, the hypothesis on speciation have been made considering principally divergence in terrestrial or freshwater animals, grouped in small populations and capable of low spreading, making so impossible to deal with populations characterised by high dispersal ability and high fecundity like the major part of marine organisms (Otte & Endler, 1989). Referring to the biogeographic characteristics of species, to the sympatric ones belong groups who, even if do not share the same habitat (for example differences in depth, salinity), come in contact, even for a brief time, during their larval or adult stages; the parapatric species are identifiable with taxa who do not overlap their distributions along a continuous coastline; the allopatric species correspond to groups able to disperse across and among the oceans (Knowlton, 1993). The latters, thanks to planktonic larvae and free-swimming adults, are able to move for thousands of kilometres, leading to believe that the rapid gene flow slows the speciation process. These groups, instead, can provide the

exception to the rule “slow species divergence in allopatry” (Palumbi, 1992). What are the mechanisms thanks to which allopatric populations in the sea can show at least a slight genetic structure? The sea heated differently at the poles and at the equator, provides complex circulations and makes gene flow neither continuous nor random (Schopf, 1979). Looking at large geographical scale, gyres surely constrain larval dispersion in the ocean; focusing on smaller geographical features, instead, near-shore currents, steep temperature gradient (Schopf, 1979) and processes as “plate effects” can represent valid barriers to gene flow (Scheltema & Williams, 1983, Kay, 1983). Also the isolation by distance (considered principally for large geographical scales) (Palumbi, 1994) and the capacity of some species to control their dispersal power (Burton & Fieldman, 1982), have been catalogued as possible explanations to the presence of genetic structure in allopatric populations. Much consideration is also given to the recent history of the species analysed: the changes of the sea level of the tropical Pacific Ocean, during the Pleistocene (Paulay, 1990) or the rise of the Isthmus of Panama (Knowlton et al., 1993) help to explain the genetic structure found in populations with high dispersal power. Particularly, Pleistocene may have been a period of rapid speciation (Palumbi, 1994). However, reproductive isolation is always an essential request for the speciation process (Mayr, 1942, Coyne, 1992, Knowlton, 1993): obstacles to the reproduction, studied principally in sympatric species and mainly due to pre-zygotic barriers, are consequences of mate preference or habitat specialization choose by different taxa of marine invertebrates (Snell & Hawkinson, 1983, Grassle & Maciolek, 1992, Knowlton *et al*., 1993). What, still today, appears really difficult to establish is what are the genetic mechanisms controlling the reproductive isolation (Palumbi, 1992, Palumbi, 1994).

Much problematic is to analyse groups living in habitat like deep sea (Gage & Tyler, 1991), or extremely simple taxa like sponges, where it results hard to find diagnostic characters capable to distinguish sibling species (Boury-Esnault *et al*., 1992, Solè-Cava *et al*., 1992).

Knowlton (1993) provided an extensive review on marine species taxa “whose distinctiveness has been the source of substantial taxonomic debate or whose discovery was based on non morphological characters is included under the rubric of sibling species”. Clearly, under this criterion, the examples provide range “from species that are readily distinguished morphologically once the appropriate characters are considered ("pseudosibling species") to species that are only imperfectly isolated from each other ("semispecies")”. Despite that her work constitutes the first review of literature on marine cryptic species, (even if she referred to them as “sibling”).

Among brown algae, three species of the genus Macrocystis: *M. angustifolia*, *M. integrifolia* and *M. pyrifera*, are biologically compatible. However these entities are still conserved as "morphologic species" since it is possible differentiate them at first sight (de Reviers, 2006). On the other hand, cases of hybridization are well known in the same genus (i.e. Fucus) and between different genera of Laminariales (Nereocystis and Macrocystis) as well as undistinguishable species cannot interbreed. In general it is very difficult to apply the biological species concept and for this reason today's systematists generally make extensive use of molecular biology to avoid to describe individuals as new species based on a different morphology (de Reviers, 2006).

4.1.3. The genus *Dictyota*: life cycle, distribution and ecology

Genus *Dictyota* belong to the class Phaeophyceae (brown algae), order of *Dictyota*les, family *Dictyota*ceae, is very common in intertidal and subtidal habitats along rocky coasts worldwide (Guiry & Guiry, 2010)

Species of the genus *Dictyota* have thallus flattened, ribbon-like, erect or prostrate, with smooth, dentate, crenulate or ciliate margins; attachment by basal rhizoids or marginal rhizoidal processes scattered along the edges of the thallus or restricted to the base, stoloniferous holdfasts present or absent; branching dichotomous, anisotomous or alternate, rarely falcate; apices obtuse, rounded, apiculate or acute; phaeophycean hairs and superficial proliferations present or absent (Guiry & Guiry, 2010); each dichotomy originate from the division of the lenticular apical cell (Figure 50, c).

Cross sectioning in whatever position shows two kind of cells forming the parenchymatous thalli of the genus: the inner one is constituted by colourless medullary big cells with a single nucleus, the cortical ones by much more smaller cells, with a single nucleus and numerous discoid cromatophores. The relative number of medullar layers is variable while the cortical layer is generally one.

Reproductive structures, identified in tetrasporanges, antherids and oogones, develop on three different kinds of individuals (sporophytes, male gametophyte and female gametophyte, respectively) but they are always dependent on the swelling of some cortical cells which split up in two cells by means a division which is always parallel to the surface thallus. The lower cell remains small and sterile, forming the stalk cell, whereas the top one develops in a reproductive structure.

Tetrasporanges can develop alone or togheter forming small groups. They develop through the condensation of their content, becoming spherical and splitting up in four spores.

Oogonia differ from tetrasporangia because they are grouped in elongated sores of 20-40 adjacent cells, much smaller than sporanges, closed and deformed by their mutual pressure.

Antherids are also grouped in elongated sores. After the division from the basal sterile cell, the mother cell develops topwards and the content become lighter. During antheridial development, many divisions occur both crosswise and lengthwise. Once mature, the antherid is composed by very numerous greyish cubical niches; each of them develops in an antherozoid, a male gamete, which has just one flagellum, different from other Phaeophyceae; the second flagellum is reduced to the basilar corpuscule. Although some cells have developed towards as antherid, remain sterile surrounding the sore which is retained even after antherozoids release giving a particular appearance to empty sores.

Tufts of hairs are very frequent in younger parts of the thallus; they are rapidly transient and once lost they leave scairs, often confused with those of the antherids (Hamel, 1939).

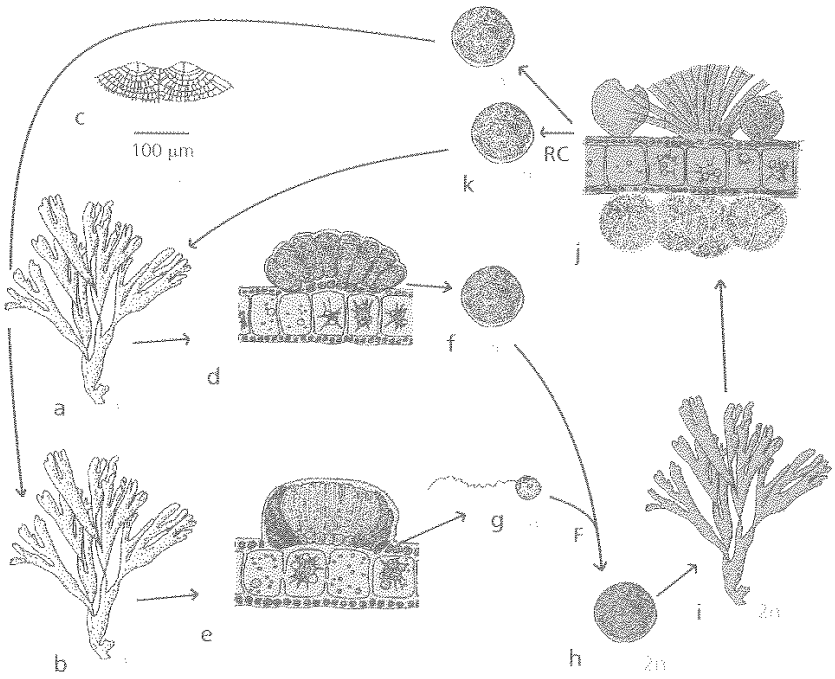


Figure 50: a. Female thallus; b. Male thallus; c. lenticular apical cells; d. Oogone; e. Antherid; f. Oosphere; g. Antherozoid; h. zygote; i. Sporophyte; j. Sporangia with phaeophycean hairs; k. spores.

The life cycle (Figure 50) is a digenetic isomorphic haplodiplontic one, i.e. gametophytes (a and b) and sporophytes (two generation = digenetic), similar morphologically (isomophic), alternate (haplodiplontic) during the cycle.

Every oogone (d) releases one oosphere (f), the female gamete, whereas each antherids (e) releases many antherozoids (g). When one antherozoid fertilizes an oosphere, the zygote (h) grows in a diploid sporophyte (i). Once mature the sporophyte will develop

unilocular sporanges (j) in which meiosis originates four unflagellated haploid spores (k). Each spore germinates in a haploid gametophyte (de Reviers, 2002).

Dictyota is constituted by annual species which disappear in less favourable period but surviving as microscopic resting stages (microthalli). Natural populations are a mix of both sporophytes and gamametophytes even though the sporophytes results always more abundant all the year (van den Hoek, 1984; Tronholm *et al*., 2008).

Genus *Dictyota* is composed by 75 species, currently accepted taxonomically and it is considered as a cosmopolitan genus (Guiry & Guiry, 2010).

Until now five species and two varieties are accepted along the Italian coasts (Furnari et al., 2010); they are listed below with their phytogeographic element, according to Cormaci et al., 1982:

- *Dictyota dich*otoma (Hudson) J.V. Lamouroux is the type species of the genus (Cosmopolitan),
- *Dictyota dichotoma* v. *intricata* (C. Agardh) Greville (Sub-cosmopolitan),
- *Dictyota fasciola* (Roth) J.V. Lamouroux (Sub-cosmopolitan),
- *Dictyota fasciola* v. *repens* (J. Agardh) Ardissone (Indo-Atlantic),
- *Dictyota linearis* (C. Agardh) Greville (Sub-cosmopolitan),
- *Dictyota mediterranea* (Schiffner) G. Furnari (Mediterranean),
- *Dictyota spiralis* Montagne (Atlantic).

In particular *D. mediterranea* was never recorded along the Campania's coast (South Italy, Thyrrenian Sea) (Furnari *et al*., 1999; Serio *et al*., 2006).

4.1.4. Aim

In order to understand if long term acidification may or not favour any local adaptation, the genus *Dictyota* has been chosen as model; in fact it can live all along the natural pH gradient present at Castello, Ischia (Gulf of Naples) (see Study Area).

Moreover, it could be interesting which are the physiological mechanisms which allow these species to cope with acidification compared to the other species, analyzing the expression of functional genes. However I had to begin identifying species by a genetic point of view for two reason: the first one was the lack of genomic information on most of brown algal species.

The second reason is that the genus *Dictyota* has recently been the target of a molecular taxonomical study (Tronholm *et al*., 2010). In fact a combination of high morphological variability (Scnetter *et al*., 1987; Hwang *et al*., 2005; both in Tronholm et al.,

2008) and a lack of distinctive characters makes an accurate description difficult and have driven a high number of species description or wider geographical range as in *D. dichotoma* (De Clerck, 2003).

In this chapter the attention has been focused on molecular taxonomy of the genus *Dictyota* along the pH gradient and the assessment of a genetic adaptation to the acidification.

4.2. Material and Methods

4.2.1. Plant material

Three target species of the genus *Dictyota* have been chosen along the pH gradient. Five thalli of them have been sampled in the Southern side of Castello Aragonese, both in S1 (pH = 8.1) and S3 (pH = 6.57) at 0.5- 1 meter depth, in September 2010 (Appendix VI).

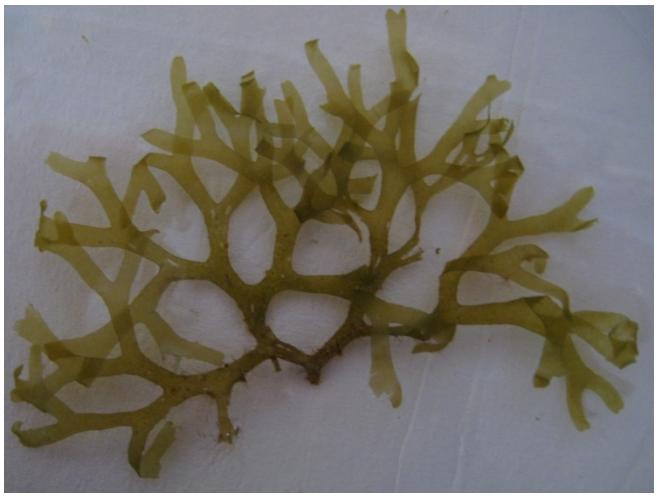
The first one has been identified as *D. dichotoma*. Morphology indicates that the thallus is completely erect, attached by basal rhizoid forming a discoid holdfast. The width of the axes is homogeneous throughout the thallus, apices rounded; branching is dichotomous. This species is considered distributed worldwide (Guiry & Guiry, 2010) inhabiting the shallow vertical shaded rocky shores until 2m depth (Cabioc'h *et al*., 2006).

The second one was a variety, identified as *D. dichotoma* v. *intricata*, very similar to *D. dichotoma* but differentiated by narrow axes especially in the distal portion of the thallus with acute apices, with branching angle of 50° (Afonso Carillo & Sanson, 2000) and distributed worldwide (Guiry & Guiry, 2010). *D. dichotoma* v. *intricata* live in both photophilous and shaded biotopes on shallow rocky shores until 10m depth (Cabioc'h *et al*., 2006).

Both of them have been identified along the vertical subtidal rock of Castello during the community characterization (see Chapter 2).

The last one was not identified on the vertical rock but living with high abundance on the bottom of the most acidified zone. It was in earlier times identified in the field, during the frequent activities in the site, as *D. linearis* showing a narrow morphology all along the thallus and forming larger balls. But taking some sample to confirm their identity with *D. linearis* they have been finally identified as *Dictyota pulchella* Hörnig & Schnetter, morphologically similar to *D. dichotoma* v. *intricata*, since constituted by narrow axes especially in the apical part of the thallus but branching angle of 90° or more and acute apices (Afonso Carillo & Sanson, 2000). They commonly occur as an entangled turf or as unattached benthic drift (tumbleweeds) (Beach *et al*., 2003) in shallow areas with calm water down to the lower limit of the infralittoral (Littler *et al*., 2000, Bernecker, 2008; Samper-Villareal *et al*., 2008). This species was segregated from the *D. dichotoma* complex by Hörnig & Schnetter (1988). It resulted reproductively isolated from *D. dichotoma* v. *intricata* despite the same chromosome number (De Clerck, 2003). It was never recorded in the

Mediterranean Sea, living mostly in the Caribbean Sea, the central Western Atlantic Ocean and in Canary Island (Guiry & Guiry, 2010).



Dictyota dichotoma

Dictyota dichotoma v. *intricata*

Dictyota pulchella

Thallus size has been observed, although no quantitative data are available, for the different species in the two different pH environments. *D. dichotoma* did not vary in the size along the gradient whereas both *D. dichotoma* v. *intricata* and *D. pulchella* become much smaller in the normal pH environment.

4.2.2. Molecular phylogeny

Molecular phylogeny, also known as molecular systematics, is the use of the sequence of molecules to gain information on an organism's evolutionary relationships. The result of a molecular phylogenetic analysis is graphically represented in a so-called phylogenetic tree.

The methodological steps are summarised below:

- choice of the molecular marker
- genomic DNA extraction
- DNA target amplification by PCR
- PCR products separation and purification
- PCR products sequencing
- sequence alignment
- phylogenetic reconstruction and analysis of data

Choice of molecular markers

According to Avise *et al*. (1987) organelles DNA is an ideal molecular system for phylogenetic analysis because it is distinctive but enormously distributed to a wide range of organisms; it can be easily isolated and analysed and it has a genetic structure not complicated by repeating DNA, pseudogenes, transposable elements and introns. Furthermore, it is linearly transferred, without recombination or other genetic rearrangements; it generates qualitative information which can be phylogenetically compared and, finally, its evolutionary rate makes it appropriate to investigations at a species level. Mitochondrial genome (mtDNA) in most taxa consists in a relatively small circular molecule with a length ranging from 15 to 20 kb. It contains the information for 13 mRNA (codifying for proteins involved in electron transport chain and phosphorylative oxidation), two rRNA (12S for the small ribosomal subunit and 16S for the big one) and 22 tRNA, plus a so-called "D-loop" region which is the most variable and it is involved in replication and transcription. MtDNA shows a fast evolutionary change whose rate can be even ten times that observed for nuclear DNA (Birky, 1995). Although insertions and deletions are not rare, most of the modifications consist in single-base mutations with a clear predominance of transitions. Variability increases proceeding from more conserved tRNA and rRNA genes, through mitochondrial protein genes (averagely conserved), to the control region or D-loop, the less conserved region, on which mtDNA length depends.

However, plastidial genome (cpDNA) is mostly used in algae compared to mtDNA for phylogenetic purposes (De Clerck *et al*., 2006; Tronholm *et al*., 2010). An explanation could be the higher information derived from cpDNA. This is likely due to the presence of more then one symbiotic event for some algal groups with subsequent indipendent lost and/or replacing in several eukaryote lineages, compared to mitochondria which were interested by just one symbiotic event with a single kind of ancestor. However heterokont (brown algae) plastids are demonstrated to be monophyletic (Le Corguillé *et al*., 2009). cpDNA also is a circular molecule of 130 kb in brown algae containing 140 genes encoding essential plastid proteins, involved in transcription, protein synthesis and transport, and photosynthetic metabolism, such as component of ATP synthase, cytochrome, photosystem I and II complexes, 30 tRNA genes and 2 ribosomal operons encoding 16S, 23S and 5S rRNA (Le Corguillé *et al*., 2009).

The high rate of substitution of mtDNA and cpDNA sequences seems to be attributable to a high mutational frequency as a consequence of a low efficiency in DNA repair systems and to the lack of DNA polymerase proofreading.

The other fundamental difference between organelles DNA and nuclear DNA is the modality of hereditary transfer. Genetic material of mitochondria and chloroplasts is transferred by extranuclear non-mendelian heredity, because it is passed clonally and independently to the offspring through the citoplasm being also uniparental inherited for some oogamous algal groups, as it is the case of *Dictyota* (Tronholm *et al*., 2010a). In a phylogenetic perspective, the entire mtDNA molecule represents a non-recombining genealogic unit with an enormous number of alleles (Avise, 1994). Studies on mitochondrial sequences demonstrated that transversions accumulate progressively and transitions/transversion rate can be very informative for population genetics and systematics (Moritz *et al*., 1987).

For the reasons reported above, plastidial DNA has been considered appropriate for this investigation and two chloroplast genes, rbcL and psbA have been chosen for the molecular approach. The rbcL gene codes for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (McIntosh *et al*., 1980). On the other hand psbA gene codes for the D1 protein photosystem II (PSII) reaction centre; it is fundamental for the electrons crossing from the PSII to the electron transport chain. Because rbcL and psbA are both coding regions, molecular evolution of this marker is relatively constrained or 'slow', and it has been used in several studies focusing on species level (De Clerck *et al*., 2006; Tronholm *et al*., 2010a, 2010b; Sattarian, 2006).

Genomic DNA extraction

An optimal extraction should allow to isolate high molecular weight DNA molecules, that is entire molecules as less deteriorated as possible; to eliminate every chance of contamination from genetic material coming from different organisms than those under study; and remove from DNA most of cellular components. Researchers employ several methodologies and techniques and protocols vary according to the kind of tissue chosen as a DNA source.

In order to clean individuals from the presence of microscopic epiphytes as well as possible, thalli were washed shaking them in 45 μm filtered seawater before being weighted. Extraction genomic DNA is reported in Appendix VII.

Every tool used in the described operation has been accurately washed with 90% ethanol and sterilized by flame before a new use on another specimen. Using of sterile and disposable tools combined with cleaning and sterilizing devices used on more than one specimen, are of basic importance to prevent contamination among different samples. DNA extraction (see protocol in the Appendix VII) begins with physical degradation of cells by means of tissue homogenization, performed with mortar and pestle, combined with the action of lytic enzymes (Proteinase K). This causes the dissolution of cellular contain in a buffer which role is to limit DNA degradation. The isolation from other fractions (RNA, proteins, carbohydrates, pigments, etc.) released by the cells in the solution occurs by mean of chemical isolation and repeated extraction and washing steps in microcentrifuges (see Palumbi, 1996). In the case of algae, the extraction of sufficiently pure DNA is particularly difficult for the marked presence of complex polysaccharides and pigments in their tissues. These compounds, indeed, tend to be purified and isolated together with DNA and may inhibit the activity of several enzymes as ligase, polymerase and restriction enzymes (Winnepenninckx *et al*., 1993; Rumpho *et al*., 1994; Palumbi, 1996; Sokolov, 2000), which are essential in most of the commonly employed analysis. Several method have been devised in order to remove polysaccharides and other tainting compounds and, probably, the most employed one includes the CTAB detergent (Cetyltrimethylammonium bromide) (Doyle & Doyle, 1987; Levitan & Grosberg, 1993), which creates complexes with carbohydrates molecules and can be subsequently extracted in phenol. In this work, this method has been optimised for the genus *Dictyota* and employed in the extraction.

DNA target amplification

The method of polymerase chain reaction, universally known as PCR, allow to replicate a specific DNA sequence in a very large number of copies in a few hours starting from a solution containing even a very small quantity of DNA (Mullis and Faloona, 1987)

PsbA - The primers for psbA are constructed on the basis of genbank sequences: PsbA 20F and PsbA 20R (forward: 5'-ATGACTGCTACTTTAGAAAGACG-3') (reverse: 5'- GCTAAATCTARWGGGAAGTTGTG -3'). They amplify a 900 bp region. For the amplification of this region in this study the PCR was set at the following conditions: 94°C for 3 min, 30 cycles of 94°C for 1 min, 52°C for 40 sec and 72°C for 1 min and a final elongation step of 10 min at 72°C.

RbcL - Universal primers for rbcL (Fig. 51) are known as RbcL 68F (5'-TGCCWAAATGGGRWAYTGGGATGC-3') combined with RbcL 1380R (5'-TATCTTWCCATAAATCTAAHGC-3') and RbcL 496F (5'-AGATTAGAYAHATTTGGWCGT-3') combined with S3r (5'-AAACATCCTTGTGTAACTCTC-3') (Draisma *et al*., 2001; De Clerck *et al*., 2006):

Figura 51: rbcL primers

For the amplification of this region in this study the PCR was set at the following conditions: 94°C for 3 min, 28 cycles of 94°C for 1 min, 46°C for 1 min and 72°C for 2 min and a final elongation step of 10 min at 72°C.

PCR products separation and purification

In this work, the separation of PCR products was carried out by gel electrophoresis. This technique is based on differential migration of molecules, according to their charge, conformation or molecular weight, through a gel matrix to which is applied an electric field. The gel can be made of starch, polyacrylamide, agarose or cellulose acetate. This method has been largely employed, from the 60s, to study genetic variability of natural population at the level of proteins. If two individuals have sufficiently different genotypes at one locus which codify for a protein, they will produce two slightly different molecular forms of that protein which will be identifiable and separable by mean of electrophoresis (Russel, 1998). This technique have been applied mainly to population genetics, but also to phylogenetic analysis: isozymes have been used as diagnostic markers for a priori species identification, even also there have been cases in which they have failed in discriminating genetically taxa morphologically distinct (Murphy *et al*., 1996) and these cases can be considered a limitation in the application of proteins in molecular systematics. The causes of this failure could be explained by the fact that a considerable portion of genetic variability does not generate differences in the mobility of proteins in a gel (Bullini, 1983; Murphy *et al*., 1996). In this work purified BioRad ® agarose has been used in order to separate DNA molecules produced by PCR, but also to verify the efficiency of DNA extractions and to test the result of DNA purification from gel (see below). A system for horizontal electrophoresis consists in a Plexiglas tray containing a buffer and equipped with two electrodes (platinum threads placed at the extremity of anodic and cathodic chambers) connected to a power generator. The samples are loaded in wells obtained in a thin gel layer which is placed inside the tray. The agarose, extracted and purified from marine algae, is a linear polymer with a low melting point which, changing into gel at room temperature creates a colloidal grid in which submicroscopic holes are formed. The dimensions of this matrix, which affect the migration of samples, depend on agarose concentration: the higher is the concentration, the narrower will be the molecular net and the slower will run the DNA molecules.The linear relationship between the electrophoretic mobility of DNA (μ) and gel concentration (τ):

$$\log \mu = \log \mu 0 - Kr\, \tau$$

where $\mu 0$ is the electrophoretic mobility of free DNA and Kr the coefficient of slowing, depending on gel properties and on the size and the conformation of migrant molecules (Sambrook *et al*., 1989). In order to separate DNA fragments differing in length by 100 or more base pairs it is necessary to use a concentration of agarose of 1.5 – 2.0 % (weight/volume) (Sambrook *et al*., 1989). In this study a solution of agarose at a

concentration of 1% has been used to check genomic DNA extraction whereas for the amplification products a solution of agarose at 1.5 % has been shown sufficiently high to separate the bands of interest and low enough to not giving problems in the purification step.

To allow the visualization of bands, to photograph or to excise, the agarose solution has been marked with the fluorescent dye ethidium bromide (Et-Br) (5 µl in 100 ml of solution). Et-Br is an interposing substance which inserts between DNA bases; fragment so marked, if placed on a UV transilluminator, becomes fluorescent and can be easily identified. Because of the gel transparency, in order to load the DNA sample in the wells, it is necessary to stain the sample with the so called Blue dye (6x loading dye), which contains 0.09% bromophenol blue, 0.09% xilene cyanide FF, 60mM EDTA and 60% glycerol. The role of glycerol is to make the solution more viscosity and making it to deposit in the wells bottom. The two dyes give good information about DNA migration, with no need too remove the gel from the tray, in that they differ in colour and in migration speed. In order to purify desired PCR products obtained, the bands corresponding to them in the gel have been excited on a UV transilluminator with a sterile scalpel, trying to take as little agarose exceeding the band as possible. The small gel portions obtained have been treated with gel extraction kits QIAquick ® (Qiagen, GmbH, Hilden, Germany). The kit consists of a silica-gel membrane, placed in removable columns on 2ml tubes, which has the property to adsorb DNA molecules. Nucleic acids can bound to silica-gel only at high concentrations of caotropic salts and the adsorption has an efficiency of 95% at pH $\leq$ 7.5 and decreases abruptly at higher pH values. Polysaccharids and proteins, conversely, have a low o null affinity for the silica-gel matrix at these concentations. This allows to remove, by repeated washing cycles in centrifuge, the residues of proteins, salts, non incorporated nucleotides, primers and all contaminants as ethidium bromide, agarose, low molecular weight DNA and RNA molecules, recovering more than 90% of high molecular weigth DNA (see Hillis *et al*., 1996). By lowering the concentration of salts, using for example bidistilled water or a buffer, DNA is eluted from silica-gel matrix and passes into solution.

PCR products sequencing

DNA has been sequenced directly from PCR product by an automatic sequencer with the"Dye terminator" method of Beckman Instruments ® (Molecular biology service, Stazione Zoologica "A. Dohrn", Napoli). The solution for the sequencing reaction (20 µl) has been prepared in an eppendorf ® tube mixing 4.5 µl of one of the two primers used during the fragment amplification and 15.5 µl of non diluted purified PCR product. For each amplified fragment, two mixes have been prepared, one with "forward" primer and the other with the "reverse" primer, in order to obtain a "double strand" reading of two obtained chromatograms. The chromatogram files (Fig. 52) obtained were opened and read with Chromas Lite v. 2.01 software (© 2005 Technelysium Pty Ltd.).

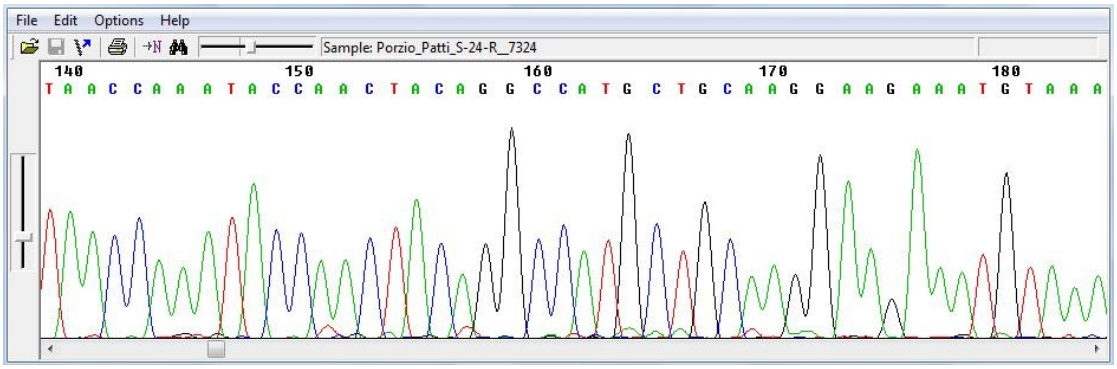


Figura 52: Image of the chromatogram files.

Once the sequences were obtained, they were subject to identification by comparison with biosequences online databases. The numerous projects and researches aiming to the identification of nucleotidic sequences of complete genomes or specific regions of several organisms have produced (and continue to produce exponentially) an enormous amount of sequences, whose biological function is, at the beginning, often unknown (anonymous sequences). The realization of database in which sequences already known and characterised are deposited, provide a precious support to researchers which can compare newly obtained sequences with those of the database gaining information on their functional features or simply verifying if they belong to a precise genome region or a particular taxon. This search provides the evidence that the fragment amplified and sequenced is the correct one and, indirectly, to exclude the presence of contamination by exogenous DNA. Among the most useful tools of this huge "biosequences library" there is a powerful search engine (http://www.ncbi.nlm.nih.gov/ BLAST/Blast.cgi), which is based on MegaBLAST algorithm, derived from BLAST (Basic Local Alignment Search Tool). These algorithms have been implemented to make a very quick comparison between an aminoacidic or a nucleotidic

query sequence with other sequences already present in the database, optimising velocity, sensibility and process selectivity (Fig. 53). BLAST algorithm employs the method implemented by Altschul *et al*. (1990) for searching regions of local similarity between the query sequence and the sequences of database collection and offers, with respect to analogous algorithms, as, for example, FASTA, higher speed for the same level of sensibility and not only. The application of BLAST is lead by statistical properties of the query sequence and those of database sequences, providing a significance value which allows evaluating, on statistical grounds, the reliability of homology relations among sequences (Attimonelli *et al*., 1997).

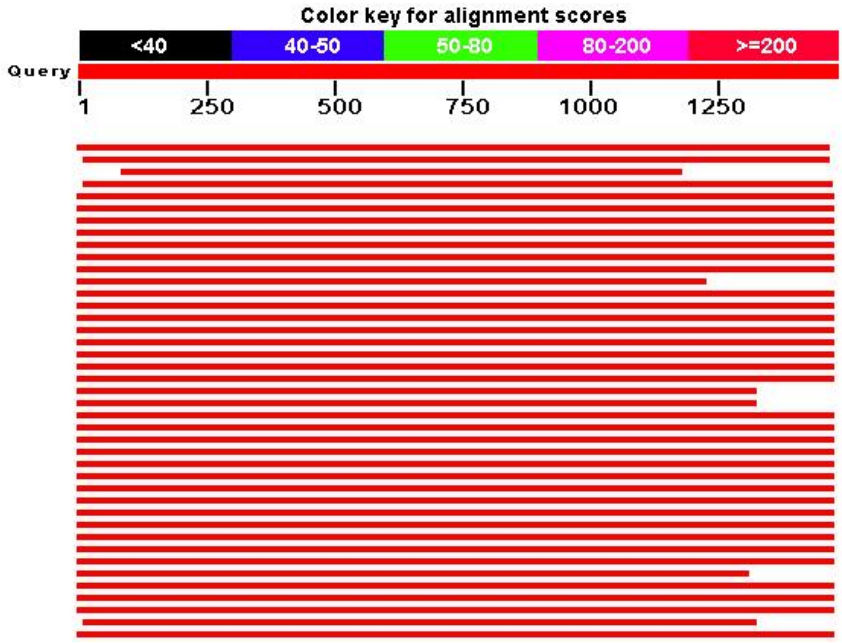


Figura 53: BLAST output using Megablast (highly similar sequences). Distribution of blast hits on the query sequence.

4.2.3. Data analysis

Sequences alignment

In bioinformatics, a sequence alignment is a way of arranging the primary sequences of DNA, RNA, or protein to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the sequences. Aligned sequences of nucleotide or aminoacid residues are typically represented as rows within a matrix. Gaps are inserted between the residues so that residues with identical or similar characters are aligned in successive columns. The process of alignment is basic for a correct phylogenetic interpretation and, in general, also for an attempt to minimise incoherencies among different sequences by mean of gap insertion. A gap should not correspond necessarily to a real insertion o deletion in the DNA, but is exclusively aimed to place all the bases which have the same relative position, with respect to adjacent bases of the same sequence, in the same absolute position, with respect to all analysed sequences. In other words, the goal is to position each base in the correct column of a matrix, constituted by the array of aligned sequences, on which molecular alignment will be performed (Caputo, 1998).

The process is executed by the existing algorithms with a twofold approach: a first alignment step (pair-wise alignment), which pairs the most similar sequences among all possible pairs, and a second step (multiple alignment) in which a multiple alignment is evaluated comparing and assembling the pairs obtained during the first step. In each step some gaps are inserted to maximise the similarity among sequences, according to user settings. The results of computer elaborations must be analysed and evaluated "manually" in a phyletic perspective and weighing the added gaps (which do not represent real biological entities) in terms of number and efficiency. With this process are identified the homologies among sequences, which are those similarities due to heredity of the same character from a common ancestor. Estimating homologies among nucleotidic sequences is a basic requirement of phylogenetic analysis, but it should be taken into account that the "character nucleotide" does not have a real ontogenesis (but only in terms of biosynthesis) and can only exist in 4 states (A, G, C, T) identical for all the characters!

The homology has been often used as synonym of similarity. When two sequences are said homologous by a certain percentage it means that they have the same percentage of equal nucleotides at the same position: the mistake comes from the fact that homology is a non quantifiable qualitative property, (Moritz & Hillis, 1996). A similarity in base position does not necessarily depend on the origin from a common ancestor; it is necessary, indeed, to

distinguish among orthology (origin from a speciation process from a common ancestor), paralogy (which depends on a gene duplication event), xenology (in which horizontal gene transfer is implied), paraxenology (a combination of paralogy and xenology) and plerology (due to genetic conversion) (Patterson, 1988).

In this study, multiple alignments have been obtained by means of several steps, starting from CodonCode Aligner software v. 1.5.1 (CodonCode Corp., 2006) which allows to verify the reading of multiple sequences automatically generated from chromatograms and to obtain groups of aligned and contiguous sequences. This has been performed with the assembling tool, carried out leaving unchanged the default settings (70% of minimum percent identity and 25 bp of minimum overlap length). Sequences groups thus generated have been then manually edited with Bioedit v. 5.0.6 (Hall, 1997; http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) in order to remove useless sequences extremities and to fix a uniform length. The last step has been performed with ClustalX v. 1.7 (Thompson, 1997; ftp://ftp.ebi.ac.uk/pub/software/clustalw2), which has generated the definitive multiple alignment (see Alignments Figure 57 and 58) on which the following analysis have been executed.

Mean Evolutionary Diversity for the entire population was estimated for psbA and rbcL. Evolutionary analyses were conducted in MEGA v5b (Tamura *et al*., 2007). All ambiguous positions were removed for each sequence pair. The number of base substitutions per site from mean diversity calculations within the entire Population is shown (see eq. 12.73 in Nei & Kumar, 2000). Standard error estimate (SE) was obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Kimura 2-parameter model (Kimura, 1980).

Phylogenetic reconstruction

The goal of this step is to assemble a phylogenetic tree representing a hypothesis about the evolutionary ancestry of a set of genes, species, or other taxa. The topics discussed in this introduction are treated in detail in Felsenstein (2003). The phylogenetic trees are constructed by computational software by means of algorithms. Just because they represent hypotheses, these trees are unlikely to perfectly reproduce the evolutionary tree that represents the historical relationships between the species being analyzed. For example, the real historical tree representing the evolution of some species may differ from the historical tree of a single homologous gene shared by those species. Phylogenetic trees generated by computational phylogenetics can be either rooted or unrooted depending on the input data

and the algorithm used. A rooted tree is a directed diagram that explicitly displays a most recent common ancestor, usually a hypothetical sequence that is not represented in the input. On the contrary, unrooted trees plot the distances and relationships between input sequences without making assumptions regarding their descent. An unrooted tree can always be produced from a rooted tree, but a root cannot usually be placed on an unrooted tree without additional data on divergence rates, such as the assumption of the molecular clock hypothesis. Independent information about the relationship between sequences or groups can be used to simplify the tree search range and to provide a root for unrooted trees. For this purpose, tree generating methods involve the inclusion of at least one outgroup sequence known to be only distantly related to the sequences of interest in the query set. This usage can be seen as a type of experimental control. If the outgroup choice has been appropriate, the outgroup will have a much greater genetic distance and thus a longer branch length than any other sequence, and it will appear near the root of a rooted tree. Choosing an appropriate outgroup requires the selection of a sequence that is moderately related to the sequences of interest; too close a relationship defeats the purpose of the outgroup and too distant adds noise to the analysis. Care should also be taken to avoid situations in which the species from which the sequences were taken are distantly related, but the gene encoded by the sequences is highly conserved across lineages. Horizontal gene transfer, especially between otherwise divergent bacteria, can also confound outgroup usage.

In this research, three sequences from GenBank (*D. dichotoma*, *D. pulchella* and *D. fasciola*) were used as outgroups in the analysis of psbA sequences and three sequence (*D. dichotoma*, *D. pulchella* and *D. dichotoma* v. *linearis*) in that of rbcL.

The techniques used to obtain phylogenetic trees can be classified into the wide categories of distance based and character based methods. Only those used in phylogenetic analysis of this study will be here described. Distance based methods of phylogenetic analysis explicitly rely on a measure of genetic distance between the sequences being classified, and therefore they require a multiple sequence alignment as an input. Distance is often defined as the fraction of mismatches at aligned positions, with gaps either ignored or counted as mismatches. Distance methods attempt to construct an all-to-all matrix from the sequence query set describing the distance between each sequence pair. From this is constructed a phylogenetic tree that places closely related sequences under the same interior node and whose branch lengths closely reproduce the observed distances between sequences. Distance matrix methods may produce either rooted or unrooted trees, depending on the algorithm used to calculate them. They are frequently used as the basis for progressive and

iterative types of multiple sequence alignment. The main disadvantage of distance-matrix methods is their inability to efficiently use information about local high-variation regions that appear across multiple subtrees. Among distance based techniques several other methods can be distinguished. Neighbor-joining (NJ) methods apply general data clustering techniques to sequence analysis using genetic distance as a clustering metric. The simple neighbor-joining method produces unrooted trees, but it does not assume a constant rate of evolution (i.e., a molecular clock) across lineages.

The most widely used techniques among characters based methods are probably parsimony and maximum likelihood methods. Parsimony (P) is a method of identifying the potential phylogenetic tree that requires the smallest total number of evolutionary events to explain the observed sequence data. Some ways of scoring trees also include a "cost" associated with particular types of evolutionary events and attempt to locate the tree with the smallest total cost. This is a useful approach in cases where not every possible type of event is equally likely - for example, when particular nucleotides or amino acids are known to be more mutable than others. The most naive way of identifying the most parsimonious tree is simple enumeration, considering each possible tree in succession and searching for the tree with the smallest score. However, this is only possible for a relatively small number of sequences or species and, consequently, a number of heuristic search methods for optimization have been developed to locate a highly parsimonious tree, if not the most optimal in the set. Most such methods involve a steepest descent-style minimization mechanism operating on a tree rearrangement criterion.

The maximum likelihood (ML) method uses standard statistical techniques for inferring probability distributions to assign probabilities to particular possible phylogenetic trees. The method requires a substitution model to assess the probability of particular mutations; roughly, a tree that requires more mutations at interior nodes to explain the observed phylogeny will be assessed as having a lower probability. This is broadly similar to the maximum-parsimony method, but maximum likelihood allows additional statistical flexibility by permitting varying rates of evolution across both lineages and sites. In fact, the method requires that evolution at different sites and along different lineages must be statistically independent. Maximum likelihood is thus well suited to the analysis of distantly related sequences, but because it formally requires search of all possible combinations of tree topology and branch length, it is computationally expensive to perform on more than a few sequences.

The bootstrap (Felsenstein, 1985) is a simulation methodology created to evaluate the significance of measurements obtained with all the different methods of studying molecular evolution. It corresponds to performing a certain number of resamplings in the multialignment. If for each simulated multialignment, the corresponding phylogenetic tree is constructed, the percentage of simulated trees, which supports the topology behind each node of the tree, can be calculated. At the end a consensus tree can be build, reporting the most representative topology, among those generated by simulation, in which a significance coefficient is attributed to each node, corresponding to the percentage of supporting simulations. In general, only nodes with a bootstrap percentage higher than 50% are considered significant (see Attimonelli *et al*., 1997).

For the sequences of psbA and rbcL genes of the target species of this study, Neighbor Joining distance tree (with Kimura 2-parameter model) and Maximum parsimony tree were computed using Phylip v.3.6 package (Felsenstein, 1989; http://evolution.genetics.washington.edu/phylip.html) and Maximum likelihood tree (HKY model) was obtained using PAUP* v. 4.04 (Swofford, 2003; http://paup.csit.fsu.edu/). The program Modeltest version 3.06 (Posada & Crandall, 1998; http://darwin.uvigo.es/software/modeltest.html) was employed to selected TVM+I model in Maximum Likelihood. In computing trees, the option of 10000 bootstrap replicates has been selected. The resulting multitude of plausible trees is best expressed by a network which displays alternative potential evolutionary paths in the form of cycles.

In this work it is also used a method (called median joining, MJ; Bandelt et al. 1999) for constructing networks from recombination-free population data that combines features of Kruskal's (1956) algorithm for finding minimum spanning trees by favouring short connections, and Farris's (1970) maximum-parsimony (MP) heuristic algorithm, which sequentially adds new vertices called ''median vectors'', except that MJ method does not resolve ties. In this study, two MJ networks were produced with the program Network v. 4.5 (http://www.fluxustechnology.com/NETW4500.exe): one with all obtained psbA sequences and another with all obtained rbcL gene sequences.

4.3. Results

Once checked genomic DNA extraction (Fig. 54), PCR products for psbA marker were loaded on agarose minigel which yielded a fragment of 932 bp (Fig 55A).

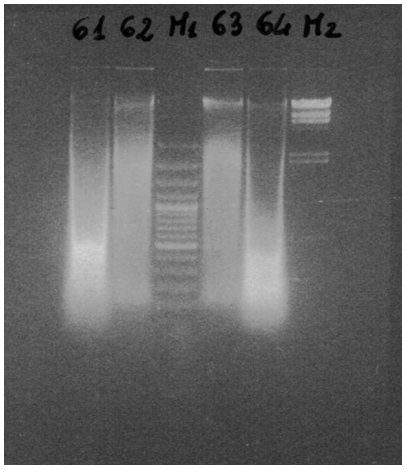
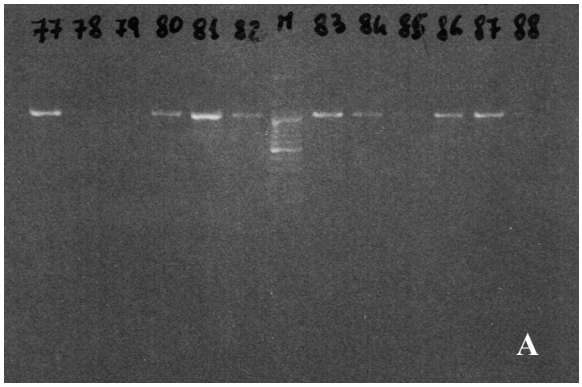
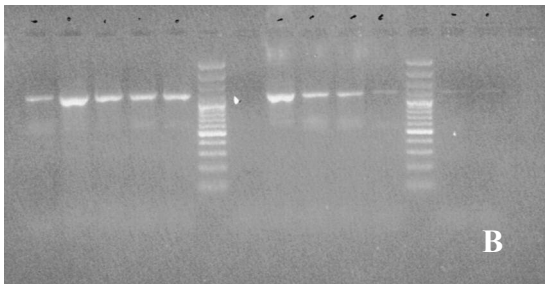


Figure 54: Genomic DNA.

Each pair of primers for rbcL (Fig. 55B and 55C) yielded a fragment of 1200; combining both pairs of primers a 1494 bp fragment was obtained.





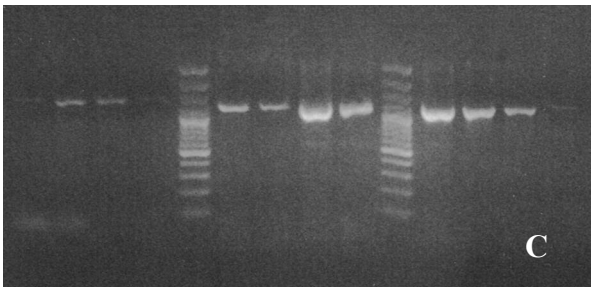


Figure 55: A) PCR fragments for psbA; B) PCR fragments for rbcL with primers pair 68F and 80R; C) PCR fragments for rbcL with primers pair 96F and S3r.

Statistics of both markers are showed in Table 19. RbcL was twice longer than psbA; also a double number of sequences and identical sites for rbcL were analyzed compared to psbA. However the ratio of A and T frequencies were three time higher in rbcL than psbA, suggesting a stronger DNA structure in the first marker.

Table 19: Statistics for psbA and rbcL fragments

	psbA	***rbcL***
Length	932	1,494
Sequences	15	28
Identical Sites	822 (88.2%)	1,279 (85.6%)
Bases Frequencies:		
A	3,527	13,595
C	2,478	6,111
G	2,799	8,296
T	5,149	13,473
?	27	3

The DNA polymorphism is showed in Fig. 56 and Table 20 for both psbA and rbcL. psbA showed a higher polymorphism compared to rbcL (Fig. 56) almost in the first part of the fragment, while rbcL was more conserved. This is clear also in Fig. 57 were alignments for psbA are characterized by a high number of different nucleotide variation whereas in rbcL (Fig. 58) variation is much lower.

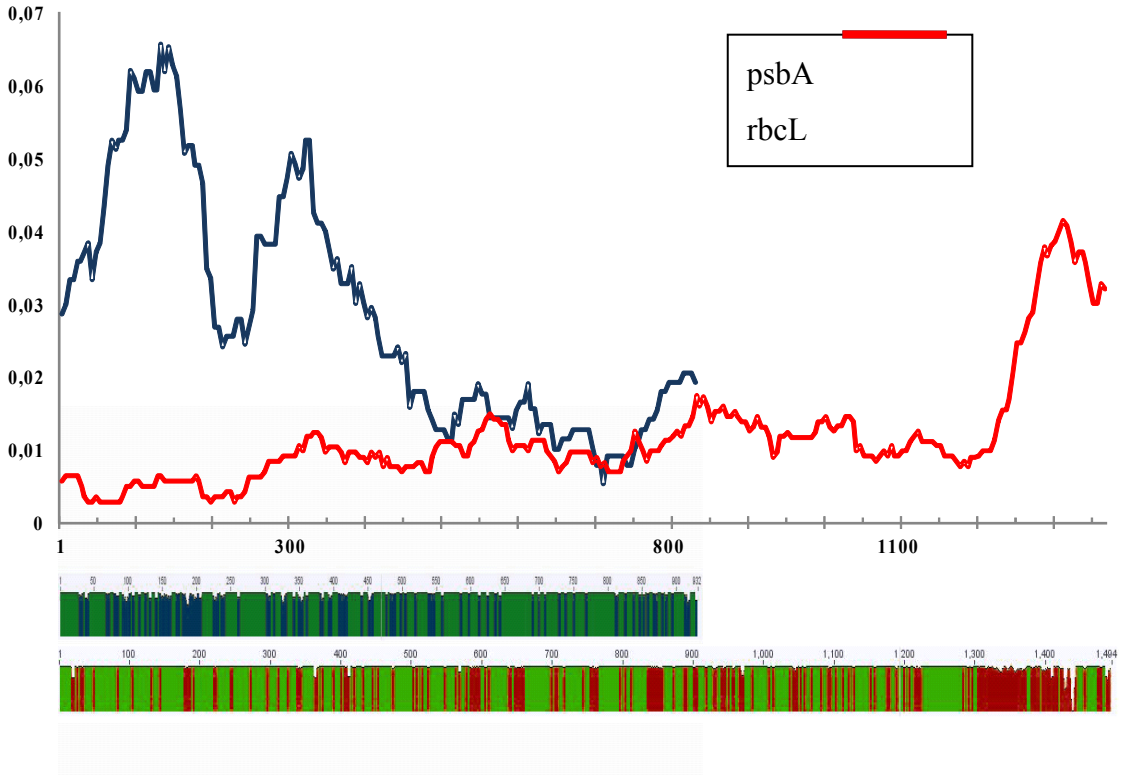


Figure 56: DNA polymorphism for both psbA and rbcL.

Table 20: DNA Polymorphism over fragment length for psbA and rbcL

	psbA	*rbcL*
Number of sequences	15	28
Number of sites	932	1494
Number of polymorphic (segregating) sites (S)	107	184
Total number of mutations (Eta)	112	193
Number of Haplotypes (h)	12	5
Nucleotide diversity (Pi)	0,02744	0,01203

Mean Evolutionary Diversity (D) for the entire population in psbA was 0.024 with a SE = 0.003.

The analysis involved 12 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 932 positions in the final dataset for psbA.

In rbcL, D was 0.013 with SE = 0.001; the analysis involved 28 nucleotide sequences. There were a total of 1494 positions in the final dataset.

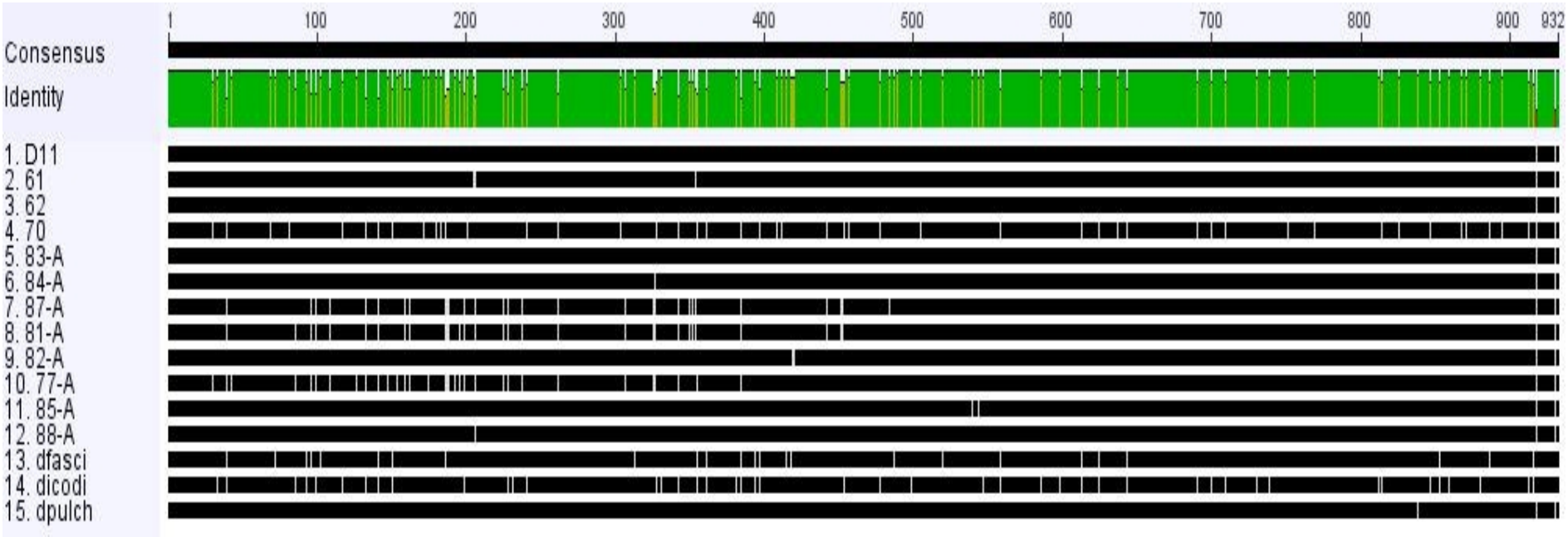


Figure 57: Alignment for psbA fragments plotted over a consensus sequence. The Identity green bar show the single base variability along the fragment length.

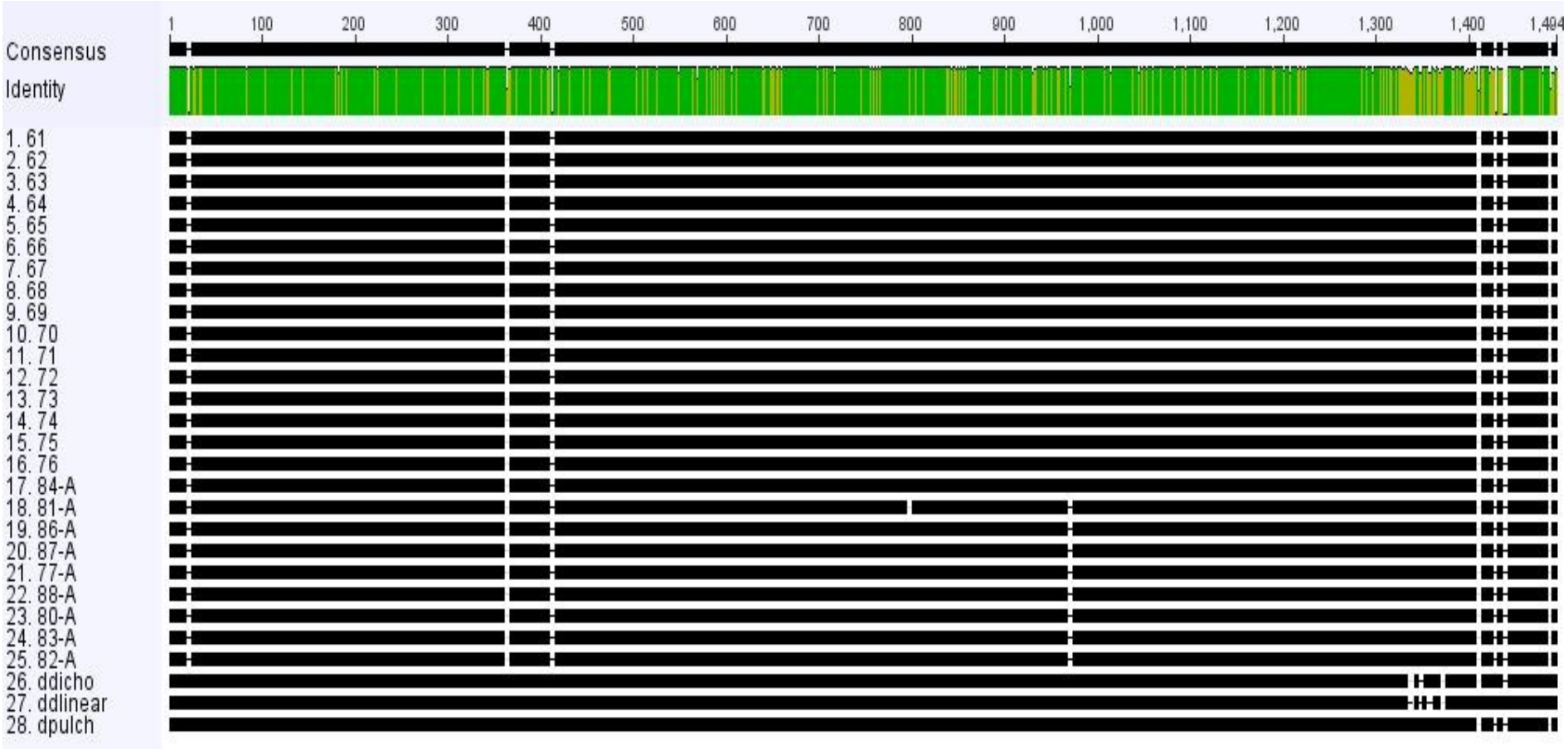


Figure 58: Alignment for rbcL fragments over a consensus sequence. The Identity green bar show the single base variability along the fragment length.

.

Median Joining Network revealed four haplotypes for psbA (Fig. 59A); three of them constituted a principal haplogroup (H1). Two haplotypes were present in both acidified and not-acidified environment, while one just in not-acidified environment. However no difference in distribution of morphotypes was reported in both environments.

Moreover a strong genetic identity of the haplogroup with *D. pulchella* (sensu Tronholm *et al*., 2010a) is clear.

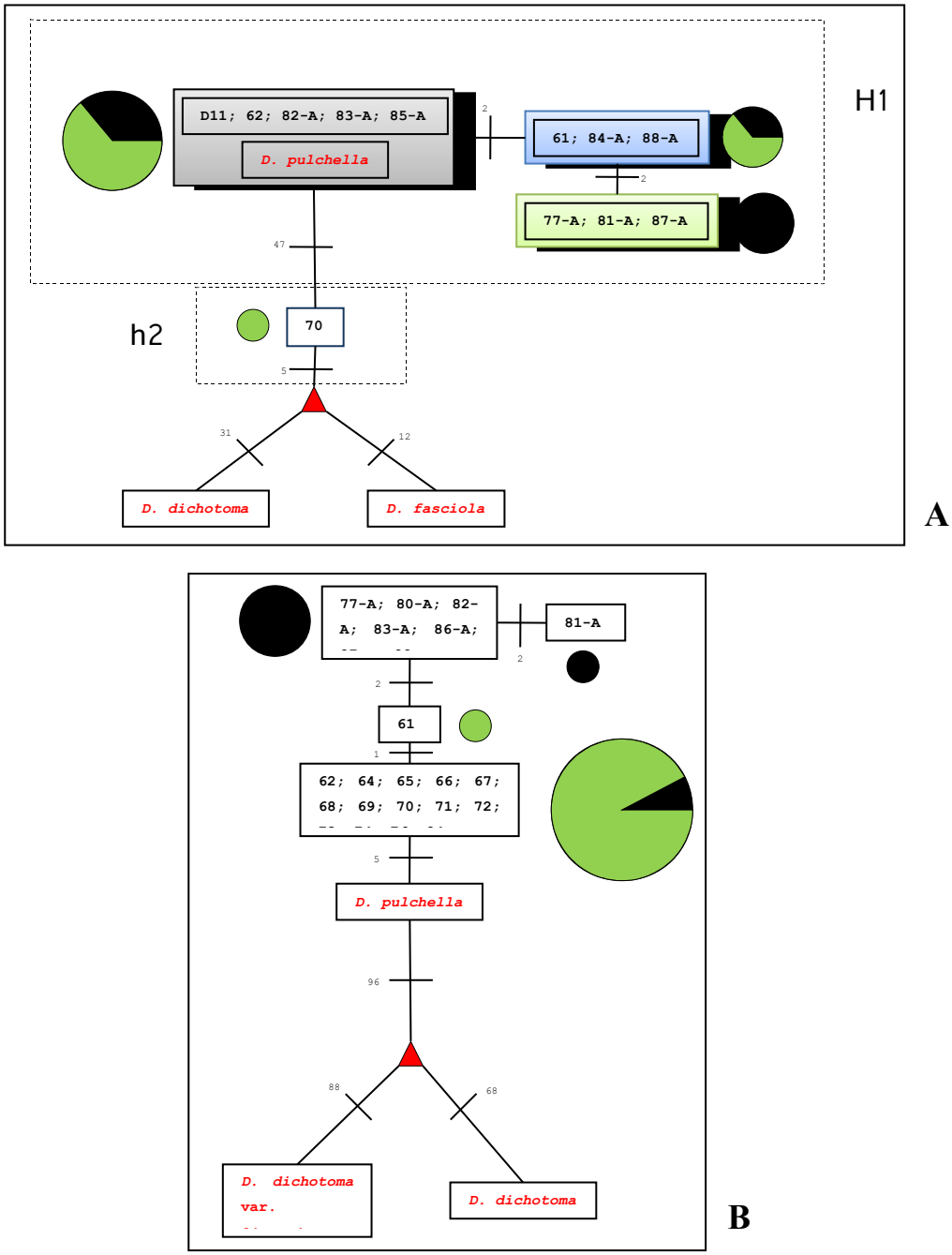


Figure 59: Median Joining Network drawn on the basis of psbA sequences (A) and rbcL (B). Circles radius proportional to haplotype frequency. In green, haplotypes of the acidified area; in black, haplotypes from the non-acidified area.

Four haplotypes were detected with the analysis of rbcL fragments (Fig. 59B). They were different distributed between the two pH environments. Two haplotypes were reported in the acidified environment while three haplotype were recorded at normal pH (8.1). Two haplotypes were exclusive of the normal pH environment whereas just one was reported in the acidified zone.

No difference was recorded with both markers between the genotype and the three identified morphotypes.

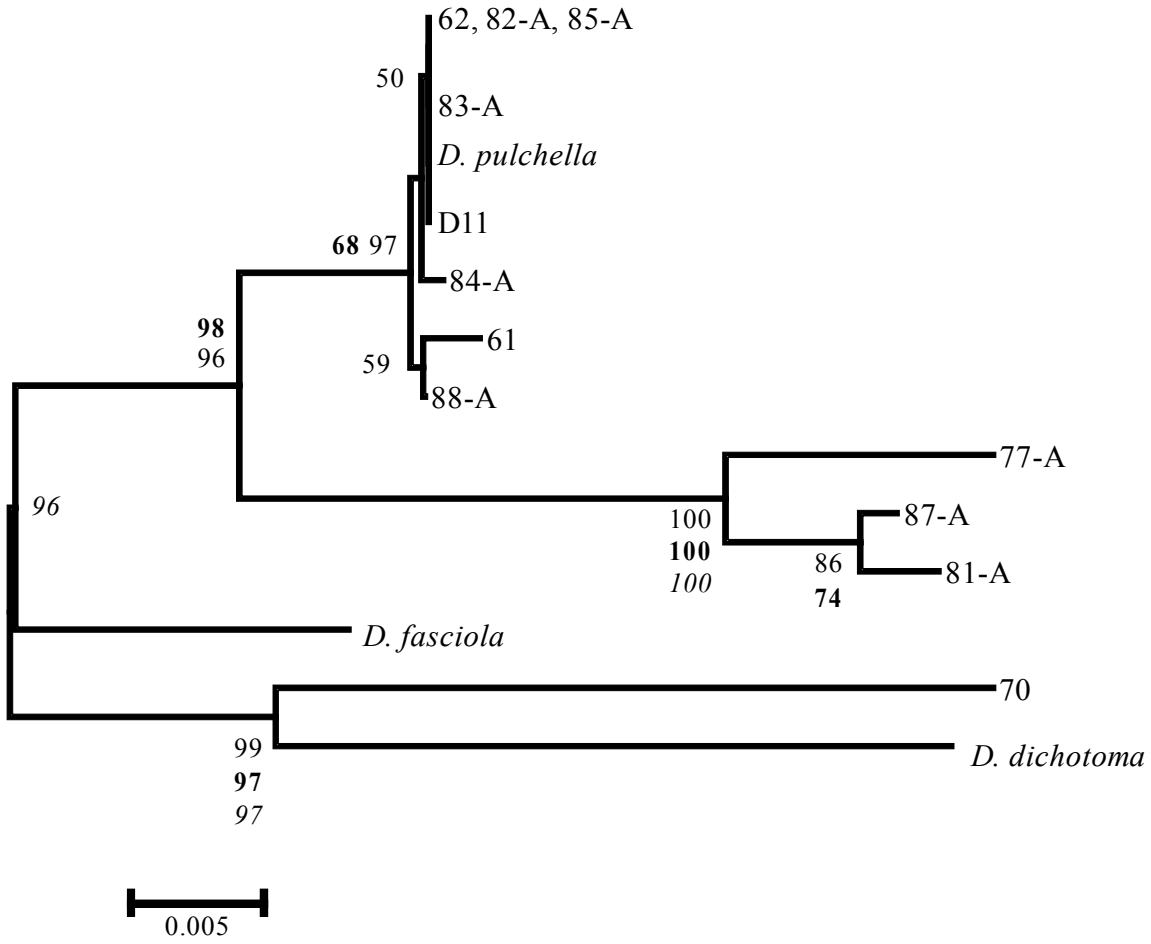


Figure 60: Neighbor-joining (Kimura-2 parameter as a nucleotide substitution model), Parsimony (P) and Maximum Likelihood (ML) tree after the analysis of psbA sequences of *D. dichotoma* morphotypes. Significative bootstrap values (10000 replicates) are given at the node of each branch (numbers in italic are from P, numbers in bold are from ML.

In Figure 60 is showed a tree grouping three different phylogenetic analyses for psbA: Neighbour-joining, Parsimony and Maximum Likelihood. Very similar results were obtained with all three analyses with the Median-Joining Networks supporting the results of the latter.

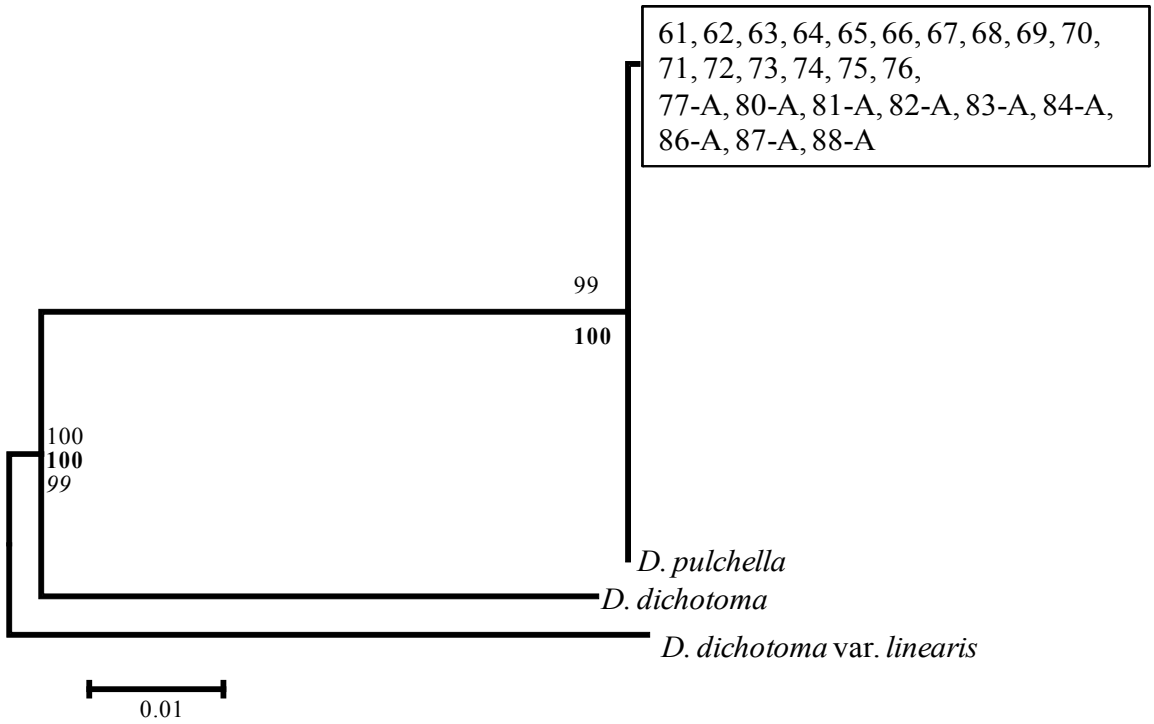


Figure 61: Neighbor-joining (Kimura-2 parameter as a nucleotide substitution model), Parsimony (P) and Maximum Likelihood (ML) tree after the analysis of rbcL sequences of *D. dichotoma* morphotypes. Significative bootstrap values (10000 replicates) are given at the node of each branch (numbers in italic are from P, numbers in bold are from ML.

Phylogenetic analyses for rbcL (Fig. 61) (Neighbour-joining, Parsimony and Maximum Likelihood) gave a similar pattern to the Median-Joining Network also, supporting the results of the latter.

5. General discussion and conclusions

5.1. Macroalgal community changes

5.1.1. Natural substrate community

Research into the effects of ocean acidification on benthic marine habitats has been largely restricted to tropical reefs (Hoegh-Guldberg *et al*.. 2007; Anthony *et al.*, 2008; Kuffner et al. 2008; Manzello *et al.,* 2008; De'ath *et al*., 2009) while in temperate benthic systems research focus has centred on individual species or small species groups (e.g. Dupont *et al*., 2008; Parker et al., 2009; Thomsen et al. 2010). The present study is the first to detail how the changes in marine carbonate chemistry that result from increasing CO_2 levels can result in complex effects in macroalgal habitats, affecting the structure of rocky shore communities. Naturally acidified areas are useful as they encompass the life histories of interacting organisms and include the feedbacks and indirect effects that occur within natural marine systems but which are difficult to replicate in mesocosm and laboratory conditions (Thomsen *et al*. 2010). This work adds to similar field studies on the effects of ocean acidification on seagrass algal epiphytes (Martin *et al*., 2008), foraminiferan community structure (Dias *et al*., 2010) invertebrate settlement (Cigliano *et al*., 2010), and bryozoan calcification (Rodolfo-Metalpa *et al*., 2010) all of which indicate major ecological shifts as CO_2 levels rise.

Our macroalgal data offer some cause for relief as they show that many seaweeds can clearly continue to photosynthesise even at extremely high CO_2 levels. Various Rhodophyta (e.g. *Chondracanthus acicularis*), Ochrophyta (e.g. *Sargassum vulgare*) and Chlorophyta (*Chaetomorpha linum*) are able to grow at CO_2 levels that far exceed those predicted due to human emissions. A cautionary note is needed when interpreting these results, as one of the problems with using CO_2 vents to predict the effects of ocean acidification is that they only affect localised areas. It is possible that some of the species present at the vents are not able to complete their life history in high CO_2 conditions but recruit into the acidified areas from populations outside. The filamentous Rhodophyta *Polysiphonia scopulorum*, for example, decreased in reproductive capacity as CO_2 levels increased, even though it was present in the most acidified area. Surprisingly, we found that some of the Ochrophyta and Rhodophyta (i.e. *Dictyota dichotoma* and *Osmundea truncata*) appeared to grow and reproduce best at the highest CO_2 levels found within the vents. The resilience of a diverse range of Ochrophyta to high CO_2 conditions offers hope that kelp forests of the world may survive the ongoing acidification of the coming decades. However, preliminary experimental work suggests that

increased CO_2 levels may be detrimental to kelp forest habitats as they encourage the growth of algal competitors (Connell & Russel 2010).

Examination of macroalgal communities living near CO_2 vents at mean pH 7.8 provides us with insights as to what to expect as the global oceans acidify due to human CO_2 emissions. Fortunately, 95% of the macroalgal species present in our study were able to tolerate mean pH 7.8, even though diel fluctuations in pH at these volcanic vents was greater and more rapid than would be expected due to ocean acidification (Hall-Spencer *et al*., 2008; Riebesell, 2008).

However, our *in situ* observations give support to concerns that, when water acidifcation is combined with a high wave exposure, the effects on the community structure are not so strike. In fact, a slightly but not significant decrease in species richness was recorded along the pH gradient on the North side, characterized by higher levels of hydrodynamic energy than the southern side.

Given the widespread concerns over the fate of calcified algae in the coming decades (Kuffner *et al*., 2008; Nelson, 2009) it is noteworthy that several calcitic and aragonitic species of algae were able to settle and grow at the high CO_2 levels found near the vents. *Peyssonnelia squamaria* and *Hydrolithon cruciatum* increased in abundance at mean pH 7.8, replacing other calcified species that were lost from the system. The fact that algae with high Mg-calcite skeletons, the most soluble form, can survive high CO_2 conditions near the vents offers hope that this vulnerable group will not disappear, as some predict, but that calcareous species which are more tolerant of high CO_2 levels will replace those species that are lost, at least until carbonate saturation will be above 1. However, there are caveats; the mean pH 7.8 data show a 25% loss in biodiversity of calcareous algae and a major reduction in cover of all the erect articulated genera (the calcitic *Jania rubens*, *Corallina officinalis, Corallina elongata, Amphiroa rigida, Amphiroa criptarthrodia* and the aragonitic *Halimeda tuna*). Low abundances and bleached *Corallina elongata* have also been recorded at acidified volcanic vents in the Azores (Couto *et al*., 2010). Erect calcareous algae are often important for calcareous sediment production and typically host a high biodiversity of associated fauna and flora (Nelson, 2009). Erect calcareous species may be more sensitive to corrosive short-term drops in pH caused by spikes in CO_2 levels at the vents than the crustose calcareous forms which may benefit from chemical buffering in the benthic boundary layer. As a group, the calcareous algae exhibited a decrease in abundance and a decrease in reproductive capacity from normal pH conditions to mean pH 7.8. Our data on the selective effects of high CO_2 levels on seaweed reproduction are preliminary and worthy of further investigation given that

some parts of an organism's life-history can be more vulnerable to the effects of ocean acidification than others (e.g. Ellis et al. 2009).

Whilst our observations show that the vast majority of algal species can tolerate the levels CO_2 predicted to occur this century, and that even calcified species continue to grow, we recorded a simplification of the macroalgal community and increased dominance by a few species at mean pH 7.8. Both erect and crustose calcified species were largely replaced by non-calcified algae (e.g. *Hildenbrandia rubra*, *Dictyota dichotoma* and *Osmundea truncata*) that are presumably better adapted to the changes in carbonate chemistry that result from elevated CO_2 levels. Ochrophyta remained the dominant, high-biomass, canopy-forming species throughout the CO_2 gradient but *Cystoseira amentacea* var. *stricta* was absent below 7.8 pH whereas *Sargassum vulgare* was confined to the most acidified sector. These two genera often dominate Mediterranean phytobenthic assemblages but have steadily declined in abundance since the 1940s (Thibaut et al. 2005). In the Gulf of Naples, where our study took place, the loss of canopy-forming brown algae has been correlated with increased pollution levels, increased water turbidity, and increased temperature (Buia et al. 2007). The quite unique occurrence of *Sargassum vulgare* in the most acidified zone (Buia, unpublished data), might relate to a lack of grazing by sea urchins which are highly susceptible to ocean acidification (Miles *et al*., 2007) and completely absent from the Ischia CO_2 vents (Hall-Spencer *et al*., 2008).

An analysis of shifts in algal categories along the pH gradient revealed that of the crustose, erect and turf-forming algae, it was the turfs that declined most in diversity and abundance along the pH gradient, at least in the Southern side, despite the fact that these are usually considered to be opportunistic species that are resilient to ocean acidification (Kleypas *et al.,* 2006). Connel and Russel (2010) demonstrated in mesocosm experiments that increased CO_2 levels (550 ppm) had no effect on the cover of turf algae at ambient temperature (17°C) but that turf cover increased considerably when increased CO_2 levels were combined with elevated temperatures. Our data suggest that in autumn, on a shallow Mediterranean rocky shore at mean temperature of 21-22°C and about 1000 ppm CO_2 (S2) causes a significant fall in the richness and cover of turf species that are present at background CO_2 levels. The nature of CO_2 induced shifts in macroalgal community structure are likely to vary with biogeographic region, but the fact that CO_2 enrichment can cause significant shifts in the abundance of whole morphological groups indicates that rising CO_2 levels will alter local ecosystems, including food web dynamics and the cycling of carbon and nutrients.

The morpho-functional approach (Littler & Littler, 1980) was applied in order to understand which were the relationships between seaweed forms and physiological processes and their responses to pH pressure.

Our results support the hypothesis that water acidification affects the richness of the communities, both in terms of species and morpho-functional groups. However, we cannot confirm that the algal response to water acidification moves to face towards a reduction in the thallus complexity. Infact, apart from all calcareous species (from semi-calcareous to articulated calcareous groups) totally laking in the most acidified sectors, the algal groups formed by those more structured and complex thalli are most abundant and/or dominant in the most acidified sectors. The leathery *Sargassum vulgare*, the corticated laminar *Dictyota dichotoma* and the terete *Osmundea truncata* are dominant species in the South side while the non-calcareous Hildebrandia crouaniorum is dominant in the North side. Despite their complexity, their forms are different: the first two species are erect while the third is incrusting. Being the North side more exposed than the South, the temporal variations in the chemical composition of the water is more relevant; as a consequence, the primary factor in determining the community structure on this side seems to be the water movement than the pH values and other morphological traits (e.g. size) could be more relevant in terms of response to wave action (Carpenter & Williams, 1993). The high cover in N3 of the incrusting *Hildebrandia crouaniorum* and of two very small filamentous species (*Antithamnion cruciatum* and *Spermothamnion repens*) belonging to the simplest group (AG1) seems to confirm that species with similar sizes might respond similarly to wave forces and pH independently of the structural complexity of their thallus.

The occurrence of more complex thallus in the most acidified South sector could be related to their better regulation of cellular homeostasis in a calmer hydrodynamic regime. As no studies were carried out to find out which are the effects of seawater acidification on cellular and physiological mechanisms, further studies are needed to understand how acidification is effective on physiological mechanisms (e.g. nutrients uptake, membrane transport or intracellular pH regulation) to confirm this hypothesis.

5.1.2. Artificial substrate communities

Many laboratory studies have shown that the early life history stages of several animal organisms are negatively impacted by acidifed seawater, including work on echinoderms, crustaceans and molluscs (Kurihara & Shirayama, 2004; Kurihara *et al*., 2004, 2007; Dupont *et al*., 2008; Kurihara & Ishimatsu, 2008; Ellis *et al*., 2009; Findlay *et al*., 2009). The potential shifts in benthic recruitment that may result from water acidification on early life history changes has been recently studied by Cigliano *et al* (2010) in the same site around the Castello Aragonese where CO_2 vents occur. These authors found a reduction in the overall biodiversity of the invertebrates along with the reduction in pH, consistent with data on epiphytes of *P. oceanica* leaves (Martin *et al*., 2008) and on my PhD thesis results on algal communities. On the opposite, are very few the studies on the impact of water acidification on the algal recruitment (Aegian, 1995; Mackenzie & Agegian, 1989). These earlier studies each have one or more experimental limitations such as use of closed systems, small water volumes, artificial or low irradiance, lack of treatment replication, and/or very short incubation times. Studies on outdoor flow-through mesocosms have been then conducted by Kuffner *et al*. (2008) that observed a severe inhibition in the recruitment and growth of coralline algae, and by Jockiel *et al*. (2008) on the settlement of crustose rhodolith spores and communities development. An 86% relative reduction in acidified mesocosms was recorded.

As there are no studies under natural pH variations, my data represent the first contribute on the impact of water acidification on the early stage of macroalgal succession *in situ.* It is a very preliminary report in which the cover of the single species has been taken into account.

Bar tiles represented a situation of resources availability that can be exploited by organisms. According to facilitation model, pioneer species are the first species to establish, modifying the environment that will be later colonized by late-successiona species eliminating the pioneer ones.

According to Connell and Slatyer (1977), mechanisms of succession could be incorporated into three alternative models: facilitation, inhibition, and tolerance. The classical explanation of the ecological mechanism of community change during succession is the so-called *facilitation model*. This theory suggests that the recently disturbed situation is first exploited by certain pioneer species that are most capable of reaching and establishing on the site. These initial species modify the site, making it more suitable for invasion by other species, for example, by carrying out the earliest stages of soil development. Once

established, the later-successional species eliminate the pioneers through competition. This ecological dynamic proceeds through a progression of stages in which earlier species are eliminated by later species, until the climax stage is reached, and there is no longer any net change in the community.

Another proposed mechanism of succession is the *tolerance model*. This concept suggests that all species in the succession are capable of establishing on a newly disturbed site, although with varying successes in terms of the rapid attainment of a large population size and biomass. In contrast with predictions of the facilitation model, the early occupants of the site do not change environmental conditions in ways that favor the subsequent invasion of later-successional species. Rather, with increasing time, the various species sort themselves out through their differing tolerances of the successionally increasing intensity of biological stresses associated with competition. In the tolerance model, competition-intolerant species are relatively successful early in succession when site conditions are characterized by a free availability of resources. However, these species are eliminated later on because they are not as competitive as later species, which eventually develop a climax community.

A third suggested mechanism of succession is the *inhibition model*. As with the tolerance model, both earlyand later-successional species can establish populations soon after disturbance. However, some early species make the site less suitable for the development of other species. For example, some plants are known to secrete toxic biochemicals into soil (these are called allelochemicals), which inhibit the establishment and growth of other species. Eventually, however, the inhibitory species die, and this creates opportunities that later-successional species can exploit. These gradual changes eventually culminate in development of the climax community.

The very short time of this study did not allow detecting the climax phase of the different communities. As usual in nature, multiple environmental controls can affect species performance and interactions in ways not predictable because of nonadditive (synergistic or antagonistic) effects (Lotze & Worm, 2002).

Our data indicate that the settlemt of early colonizing species was mainly drived by both pH gradient and wave exposition. A succession of encrusting, filamentous, and corticated forms has been recorded. After two months, encrusting species were the dominant group: calcareous forms at normal pH conditions whilst soft forms occurred at acidified sites. Among the second forms it is to worth to note the presence of the brown Myrionema sp., a characteristic epiphytic species of Posidonia oceanica (Mazzella *et al*., 1989), which occurs all around the castello Aragonese (Buia *et al*, 2000). This species represents an early

colonizing species of the posidonia blades, together with diatoms and coralline crustose species, mainly during the early-spring time, when the epiphytic cover is scarse. The presence of this species has to be related to its temporal abundance in the surrounding habitat. Another soft crustose species appears eliminating the brown alga but only at normal pH conditions, whereas the thin, filamentous *Chaetomorpha* became dominant in the most acidified zone, without bias for the site exposition. At the end of the experiment, the presence of the corticate *Dyctiota* is developped only at normal and medium ph conditions, even if this species is widespread on natural substrata.

The high differentiation in species composition along the pH gradient, affected highly the early community structure during the short experiment period. Even though relatively more structured community was observed in the Northern side compared to the South after 2 months, then a turning over was observed on the community complexity of the two sides in the following months. This could be explained by the higher hydrodinamism in the North which may initially favour the presence of more germling stage on the substrate but later only fewer species may growth in higher water movements. In the Southern side a community complexity level along the pH gradient was maintained during the 4 months experiment, where a poorer diversity and structure was observed at low pH which increased with higher pH mean values.

Although climacic community was not reached after 4 months a clear complexity increasing along time was generally observed.

Encrusting calcareous species were the discriminant group along the acidification gradient. Coralline algae are generally the dominant group on bottom layer of the community which competes for the primary space with non-calcareous encrusting and thiny filamentous brown algae; on the other hand they favour the colonization of the top layers by other erect species (Littler & Littler, 1985). The higher abundance of thiny brown and green algae in the acidified environment may cause by a combination of two principal factors. The first one was the pH which interferes with the development of calcareous encrusting species which they compete with; on the other hand the much reduced precence of grazers in low pH environment, constituted principally by small gastropods and sea urchins, allowed the non-calcified species' overgrowth (Worm & Lotze, 2006).

Changes in the early stages of colonization will likely interfere with final community structure leading to variation in the community functions for the entire food web. Further studies are need to understand later mechanisms which lead to the final community stage.

5.2. Different responses in ecophysiological traits in target algal species along a natural pH gradient

The analysis of photochemistry by means of fluorescence parameters has been a powerful mean to assess *in situ* the light transformation efficiency and the absorbed light use in both photochemical and non-photochemical processes of the target species *S. vulgare*, *J. rubens* and *D. dichotoma* v. *intricata*. Results obtained in laboratory studies showed significant higher values of Φ_{PSII}, ETR and qP for Phaeophyceae, compared to Corallinaceae. The higher photochemical activity of Pheaophyceae could be related for these species to a better efficiency of light absorption and utilization. The higher values of qN index for Corallinaceae, compared to other algae family, indicate that the calcareous species are able to dissipate safely the absorbed light excess through non-radiative processes.

Different intrinsic features between Phaeophyceae and Corallinaceae were evidenced also by comparison of the PSII maximum photochemical efficiency (F_v/F_m). In particular, Phaeophyceae showed significant higher values compared to Corallinaceae, indicating a higher potentiality of light conversion at the reaction centres.

Unfortunately, laboratory data, even if were useful to point out important intrinsic features of the different investigated species, did not allow a correlation with pH. In order to obtain a more complete outline, field studies have been carried out in order to assess the effects of the acidification on photosynthetic apparatus.

The values of photochemical indexes Φ_{PSII}, qP and qN measured *in situ* for *D. dichotoma* both at normal pH (8.1) and low pH did not show any difference, suggesting no influence of pH. A similar pattern was found in *S. vulgare* (living at low pH) proving a comparable capability to convert light energy during photosynthetic process.

The species *J. rubens* showed the highest values of qP and qN compared to Phaeophyceae for all PPFD range examined, differently from the laboratory results. A possible reason could be related to the habitat where this species lives. The site of *J. rubens* is located in a longer irradiated area compared to those where *S. vulgare* and *D. dichotoma* grow. Thus photosynthetic apparatus is likely adapted to higher irradiances with the consequence of a higher photochemical activity and higher capacity to dissipate thermally the excess of light energy.

This hypothesis is confirmed by the comparison of RLC; in fact higher $rETR_{max}$ value was detected for *D. dichotoma* of Normal pH environment (other than *J. rubens*) compared to *D. dichotoma* of Low pH and *S. vulgare*.

Higher rETRmax values might indicate a higher rate of enzymatic reactions (Gèvaert *et al*., 2003) and/or a higher number of reaction centres (RCs) (Lobban *et al*., 1985).

From a physiological point of view, alpha is related to a) light harvesting efficiency and to b) RCs number and enzymatic reaction rate (Lobban *et al*., 1985).

Alpha was higher for *D. dichotoma* grows in normal pH while showed lower values in *J. rubens* and *D. dichotoma* var. *intricata* grows at low pH.

The highest value of E_k (saturating irradiance) was observed for *J. rubens* confirming that *J. rubens* is adapted to live at higher irradiances compared to *D. dichotoma* and *S. vulgare*.

Both *D. dichotoma* from different pH environments and *S. vulgare* showed a similar maximum photochemical efficiency demonstrating that population living at low pH did not show a stressful status of phosynthetic apparatus in response to acidified environment. On the contrary, *J. rubens* exhibited a lower efficiency in light conversion according to findings by other authors for Rhodophyta (Hanelt *et al*., 1993; Saroussi & Beer, 2007).

Transplanting experiments allowed us to assess the potentiality of acclimation or adaptation level of *D. dichotoma* and *S. vulgare* which live in the acidified environment and *J. rubens* forced to a lower pH environment. Moreover, the activity assay of the enzyme PARP allowed a further investigation on cellular damage due to pH variation. The immunopositive signal of 140kDa could represent the dimeric form of the protein. The molecular weight (about 80kDa) of *alga* PARP seems be very similar to homologues of animal PARP-2 in *Arabdopsis* and maize (Babiychuk *et al*., 1998).

The photochemical behavior of the species *Sargassum vulgare* was not influenced by growth in different pH environments as demonstrated by comparable values of quantum yield of PSII electron transport and photochemical quenching. Moreover, *S. vulgare* at different pH values maintained a higher non-photochemical quenching indicating independently from pH, a good capacity to dissipate thermally the excess of light energy at photosystems.

This result is supported by $rETR_{max}$ and alpha values which did not change in different pH conditions. However a decrease of E_k was reported in transplanted thalli in both pH conditions compared to wild thalli. This change is maybe related to an early reaching of the light saturation point.

The F_v/F_m was one of the most important indexes to assess suddently the level of acclimation capability of the target species in different pH environments.

Results suggest that *Sargassum vulgare* at low pH, did not suffer the transplanting as showed by comparable results of F_v/F_m values between wild thalli and transplanted thalli in native environment. However this species exhibited stress signals at normal pH condition (8.1). The significant F_v/F_m decrease is often associated to a photoinibition dynamic or cronical, evidenced by a decrease of the effective quantum yield of PSII and an increase in non-photochemical quenching (Hader & Figueroa, 1997; Hader *et al.*, 2001). In this study, the acidification could have induced an awarness of photochemical apparatus increasing its susceptibility to photoinhibitory damage risks. In this framework, as no decrease in the quantum yield of PSII was found, but an increase in qN was observed, the reduction of maximal photochemical efficiency could be ascribed to a dynamic photoinhibition maybe induced by a built up of a pH-gradient across the thylacoid membrane which could be caused by an alteration of the ATP-ase functioning.

In addition to photoinibition, several others mechanisms may be proposed including scattering of excessive solar energy from excited chlorophylls via carotenoids, causing inactivation of PSII reaction centers (Hader & Figueroa, 1997). As regards pigment content, transplanted thalli of *S. vulgare* at normal pH condition did not show any variation in total chlorophyll and carotenoids content compared to wild thalli of low pH. This indicates that photosynthetic apparatus in terms of light harvesting complexes maintains its stability even under pH values different from that of origin.

These results suggest that *S. vulgare* appears well adapted to low pH environment but its photosynthetic apparatus is not able to acclimatize at different pH. This species probably needs more time than the two weeks established for the experiment, to reach the acclimation.

J. rubens is a species totally absent in the area at lowest pH, because of its sensitivity to acidification. After just one week thalli dissolved their calcium carbonate in S3, where pH is less than 7. Transplanting in low pH environment (S2 = 6.78) caused a stressful status in *J. rubens*' photochemical apparatus, confirmed by the strong decrease of Fv/Fm values. This physiological response was associated with morphological change since transplanted thalli became weaker to touch after three weeks; this suggests that even in the intermediate zone, this species could not well overcome the carbonate dissolution. However, since *J. rubens* is also present with natural populations in S2 with more calcified thalli, it could be very interesting to compare photosynthetic performance and the physiological differences into the calcification mechanism between normal and mid pH populations. It is to be pointed out that this species was not threatened by transplanting since no stressful signal (see F_v/F_m results) was detected in transplanted thalli of native environment compared to wild thalli.

Photoinibition may be the response of *J. rubens* to acidification; consistently with this observation, in this species has been observed a decline in PSII photochemical efficiency (Φ_{PSII}) and a rise of qN. In thalli at lower pH, $rETR_{max}$ and *alpha* also decreased but not E_k suggesting the occurrence of a decrease in the electron transport rate and/or a loss of the RC. Most probably the loss of calcium carbonate from thallus has rendered this species more exposed to irradiance and UV with a consequent photodamage of PSII reaction centers and subsequent proteolysis of the D1 protein (Hader & Figueroa, 1997). The decrease of total chlorophyll and carotenoid contents found in transplanted thalli of low pH may be consistent with the occurrence of photodamages to reaction centres. On the other hand, similar results reported for PARP activity suggest that the presence of carbonate in *J. rubens*' thalli serves as buffering power to avoid cellular damage induced by acidification.

Unfortunately the loss of the transplanted thalli of *D. dichotoma* from lower pH environment made us unable to define the response of this species to acidification. However, since natural populations of both pH origins have the same physiological behaviour as demonstred by laboratory fluorescence measurements, it may be supposed that *D. dichotoma* transplanting from normal pH to low pH may reflects the same acclimation capability of *D. dichotoma* from low pH.

Transplanted thalli in lower pH condition decreased their electron transport rate and the yield or, maybe, their RC number. Moreover also light harvesting efficiency decreased even though it was already observed higher efficiency of wild thalli from normal pH compared to those from lower pH.

Comparable results for the photochemical indexes F_v/F_m, qP and qN between transplanted thalli in both pH environments, indicated *Dictyota dichotoma*'s ability to adapt its photosynthetic apparatus to different pH. This result is supported also by the similar PARP activity in different treatments of *D. dichotoma*. However the significant decrease of F_v/F_m in transplanted thalli compared to wild thalli suggests that *D. dichotoma* appears very sensitive to transplanting which likely causes a mechanical stress, due to lower thickness of the thallus compared to *S. vulgare* and *J. rubens*.

5.3. Genetic Diversity along a ph gradient

Molecular data evidenced how psbA and rbcL had a different meaning into the differentiation of the haplotypes. In fact, psbA has a strong value in discriminating different morphotypes as reported in literature (Tronholm *et al*., 2010a) and it is used for molecular taxonomy.

Analyzing psbA, haplogroup H1 may be a variety of *D. pulchella*, while the haplotype h2 may be a variety of *D. dichotoma* e/o *D. fasciola*.

On the contrary, rbcL showed a distinct separation among the identified haplotypes in different pH environments; the lower number of haplotypes present in the acidified area may represent an environmental selection, giving to this marker a possible importance to highlight adaptation to acidification.

Results of molecular data showed missed association among different morphotypes and genetic variability in *Dictyota* complex.

Generally a stressed environment, e.g. submitted to acidification, favours a selective pressure on a population characterized by a high phenotypical plasticity.

The supposed presence of a species with Atlantic-tropical and sub-tropical origins (*D. pulchella*), may derive by a past Mediterranean colonization after post-glaciation events and then acidified environment may have supported a bottleneck effect through the selection among the most suitable genes to the stressed environment.

Molecular data evidenced how the genus *Dictyota*, characterized by a morphological and genetic plasticity, was a great model to study the response to the selective pressure of acidification in marine organisms, setting the basis to study the adaptation to acidification by using a classical morphological approach integrated with a comparative analysis of the environmental factors associated to the intrinsic genome capability to adapt in different environments.

Recently Tronholm *et al*. (2010a) revised the taxonomy of the genus *Dictyota* along the European coasts. They concluded that only six clades represented by *D. dichotoma*, *D. fasciola*, *D. spiralis*, *D. implexa*, *D. mediterranea* and the new described species *D. cyanoloma*. To distinguish them, they used psbA marker combined with a morphometric approach. *D. dichotoma* showed the highest morphological variability ranging from broad to nearly filamentous thalli. *D. implexa* groups in this classification all the species with narrow axes (*D. pusilla*, *D. divaricata* and *D. dichotoma* v. *intricata*). The strong morphological variation of these two latter species will requires a genetical confirmation on the less typical forms. It should be underlined that also a specimen recognized as *D. pulchella* (coming from

Bermuda) is contained in *D. implexa*. So at this point the status of *D. pulchella* remains uncertain now because of the amphi-Atlantic distribution of the *D. implexa* which had not to be possible to confirm since no specimens of *D. pulchella* from the type locality has been included in the analysis (Tronholm *et al*., 2010a). The correspondence of sequences between the species studied in this work and the *D. pulchella* has to be confirmed in the future. Now it is possible to state that the different morphological forms identified as different taxa, are just a single species.

Yñiguez *et al*. (2010) studied the environmental effects on the morphology of *Dictyota* as an organism composed of modules (dichotomies) capable to adjust size, shape and resource allocation to their local environment. So, species can create a continuum between two forms: the "phalanx" or consolidating compact strategy, usually adapted in high light environment and the "guerilla" (i.e. elongated) or explorer spreading strategy, usually living in low light environment (Lovett- Doust, 1981). However not only the light, although very important, plays a driving factors to lead in changing morphology, but also temperature for growth and reproduction, water movement to allow an optimal uptaking of nutrients and to avoid structural damage at the same time, grazing pressure and epiphytes growth. Especially in *Dictyota* they did not found any morphological change to light variation due to the large range of optimum light condition of the genus; rather species respond more to disturbance (in this case herbivores and high water motion) which causes damage and as consequence, growth of adventitious branches. In fact, they found smaller and hemispherical thalli in low environment and high disturbance; vice versa in high nutrient and low disturbance the thalli were larger but hemispherical (Yñiguez *et al*., 2010).

According to these results, it can be argued that high morphology variability found at Castello along the pH gradient may be a result of just a morphological adaptation of one species.

The morphotype identified as *D. pulchella*, very abundant and with the larger size compared to the other two species seems to be the form that better adapt to the lower pH condition. In the future it would be interesting to investigate the expression pattern which allows such growth, compared to other forms, in this peculiar environment.

5.4. Conclusions

To conclude, our field observations show that a diverse range of macroalgal species are resilient to even greater changes in seawater carbonate chemistry than those predicted to occur due to anthropogenic CO_2 emissions over the coming century. However, our observations show that we need to plan for shifts in community structure and the loss of biodiversity because some algal species are intolerant of increased CO_2 levels whilst others thrive. Many macroalgal species have reduced abundance at mean pH 7.8 and further work is required to determine the mechanisms that cause this. Changes in inorganic carbon chemistry may disrupt a range of processes (e.g. calcification, reproduction, membrane transport and cellular physiology) putting some species at a competitive disadvantage. Research effort has been a worry with the detrimental effects of ocean acidification on marine organisms but we also need to better understand which marine organisms will proliferate as CO_2 levels increase. Volcanic vents show that some invasive macroalgae can tolerate persistent high CO_2 levels and they show what ecological shifts can be expected in vegetated marine ecosystems (Hall-Spencer et al. 2008).

Results in the present work support the important idea to adopt an integrated approach to assess the deterioration in structure and function of vegetated communities in response to acidification.

Acidification may strongly affect both the distribution and the abundances of macroalgal forms. Absence of calcareous algae likely has a key role to the decrease in diversity and complexity of new-born communities into acidified environment.

However, calcareous macroalgae will not be the only vulnerable species. In fact, what is emerged by photochemical analysis is that photosynthesis is highly affected by pH variation and most of the species, which live now in the sea, may not have such a physiological plasticity in photochemical processes to overcome so fast changes in marine pH.

Genetic plasticity of genus *Dictyota* may explain the physiological adaptability to acidified environment as demonstrated by fluorescence analysis. This result contributes to confirm that genetic species diversity is the strength in evolutionary processes in response to global change.

Molecular results open new perspectives to study involved genes in the evolution of the genus *Dictyota* as well as functional genes involved into the adaptive response to ocean acidification.

6. REFERENCES

AFONSO CARILLO J., SANSON M., 2000. Algas, hongos y fanerogamas marinas de las Islas Canarias - Clave analitica. Servicio de Publicaciones Universidad de La Laguna, 254 pp.

AGEGIAN CR ,1985. The biogeochemical ecology of Porolithon gardineri (Foslie). Ph.D. thesis, University of Hawaii, p 178

AIROLDI, L., BECK, M.W., 2007. Loss, status and trends for coastal marine habitats of Europe.Oceanography and Marine Biology: an Annual Review 45: 347-407.

ALTHAUS F.R., RICHTER C., 1987. In "ADPribosylation of Proteins: Enzymology and Biological Significance". Springer-Verlag (Berlin).

AME' J.C., ROLLI V., SCHREIBER V., NIEDERGANG C., ET AL., 1999. PARP-2, a novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase. J.Biol.Chem. 274: 17860-17868.

AMOR Y., BABIYCHUK E., INZÈ D., LEVINE A., 1998. The involvement of poly (ADPribose) polymerase in the oxidative stress responses in plants, FEBS Lett., 440: 1-7.

ANDERSEN R.A., 2004. Biology and systematics of heterokont and haptophyte algae American Journal of Botany, 91 (10): 1508-1522.

ANDERSEN R.A., 2004. Biology and systematics of heterokont and haptophyte algae. American Journal of Botany, 91: 1508-1522.

ANTHONY K.R.N., KLINE D.I., DIAZ-PULIDO G., DOVE S., HOEGH-GULDBERG O., 2008. Ocean acidification causes bleaching and productivity loss in coral reef builders. Proc. National Academy of Science, USA 105: 17442-17446.

ATTIMONELLI M., PESOLE G., QUAGLIARELLO E., SACCONE C., 1997. Principi di bioinformatica, Gnocchi, Napoli.

AVISE J. C., ARNOLD J., BALL R.M, BERMINGHAM E., LAMB T., NEIGEL J.E., REEB C.A., SAUNDERS N.C., 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Ann. Rev. Ecol. Syst., 18: 489-522.

AVISE J.C., 1994. Molecular Markers. Natural History and Evolution. Kluwer Academic Publishers, Boston: 511 pp.

BABIYCHUK E, COTTRILL P.B., STOROZHENKO, S, FUANGTHONG M., CHEN, Y, O'FARRELL, M.., VAN MONTAGU M, INZE D, AND KUSHNIR S., 1998. Higher plants possess two structrurally different poly(ADP-ribose) polymerases. Plant J,.15: 635-645

BABIYCHUK E., COTTRILL P.B., STOROZHENKO S., FUANGTHONG M., CHEN Y., O' FARRELL M.K., VAN MONTAGU M., INZÈ D., KUSHNIR S.,1998. Higher plants possess two structurally different poly(ADP-ribose) polymerases. The Plant Journal, 15: 635-645.

BAKER N.R., BOWYER J.R., 1994. Photoinibition of photosynthesis: from molecular mechanisms to the field. Bios Scientific Publisher, Oxford, pp. 471.

BALATA D., PIAZZI L., CINELLI F., 2004. A comparison among assemblages in areas invaded by Caulerpa taxifolia and C. racemosa on a subtidal Mediterranean rocky bottom. Marine Ecology, 25: 1-13.

BALLESTEROS E., 1986. Métodos de análisis estructural en comunidades naturales, en particular del fitobentos. Oecologia Aquatica, 8: 117-131.

BALLESTEROS E., TORRAS X., PINEDO S, GARCÍA M., MANGIALAJO L., TORRES DE M. 2007. A new methodology based on littoral community cartography for the implementation of the European Water Framework Directive. Mar. Pollut. Bull., 55: 172-180.

BALLESTEROS E., TORRAS X., PINEDO S., GARCIA M., MANGIALAJO L., DE TORRES M., 2007. A new methodology based on littoral community cartography for the implementation of the European Water Framework Directive. Marine Pollution Bulletin, 55: 172-180.

BAROTT K., SMITH J., DINSDALE E., HATAY M., SANDIN S., ROHWER F., 2009. Hyperspectral and Physiological Analyses of Coral-Algal Interactions. PLOS ONE, 4 (11): e8043.

BARSANTI L., GUALTIERI P., 2006. Algae- Anatomy, Biochemistry and Biotechnology. Taylor & Francis, 301 pp.

BASSON P. W., HARDY J.T., LAKKIS V., 1976. Ecology of marine macroalgae in relation to pollution along the coast of Lebanon. Acta Adr., 18 (19): 307-325.

BEACH K., WALTERS L., BORGEAS H., SMITH C., COYERD J., VROOM P., 2003. The impact of Dictyota spp. on Halimeda populations of Conch Reef, Florida Keys. Journal of Experimental Marine Biology and Ecology, 297: 141– 159.

BEER S., AXELSSON L., 2004. Limitation in the use of PAM fluorometry for measuring photosynthetic rates of macroalgae at high irradiances. Journal of Phycology, 39 (1): 1-7.

BEER S., BJÖRK M., 2000. Measuring rates of two tropical seagrasses by pulse amplitude modulated (PAM) fluorometry. Aquatic Botany, 66: 69-76.

BEER S., BJÖRK M., GADEMANN R., RALPH P., 2001. Measurements of photosynthetic rates in seagrasses. In: Short F.T., Coles R.G. (Eds.). Global Seagrass Research Methods. Elsevier Science, pp. 183-198.

BEER, S. & KOCH, E., 1996. Photosynthesis of marine macroalgae in globally changing CO2 environments. Marine Ecology Progress Series 141: 199-204.

BEGON M., HARPER J. L., TOWNSEND C. R., 1998. Ecologia. Individui, popolazioni, comunità. Zanichelli Editore, 4a edizione, 854 pp.

BELSHER T., 1974. Séquence des effets d'un égoût urbain en function de l'éloignement de la source de pollution, sur les peuplement photophiles de mode battue (fraction algale): premiers resultats. Bull. Soc. phycol. Fr., 19.

BELSHER T., BOUDOURESQUE C. F., 1976. L'impact de la pollution sur la fraction algale des peuplements benthiques de Méditerranée. Estratto da: Atti della Tavola Rotonda Internazionale "La biologia marina per la difesa e la produttività del mare": 216-260.

BENEDETTI-CECCHI L., PANNACCIULLI F., BULLERI F., MOSCHELLA P.S., AIROLDI L., RELINI G., CINELLI F., 2001. Predicting the consequences of anthropogenic disturbance: large-scale effects of loss of canopy algae on rocky shores. Marine Ecology Progress Series, 214: 137-150.

BERNECKER A., 2008. Marine Benthic Algae, p. 109-117; Species list: CD p. 17-70. In I.S Wehrtmann & J. Cortés (eds). Marine Biodiversity of Costa Rica, Central America. Monographiae Biologicae, Vol. 86. Springer and Business Media B.V., Berlin, Germany.

BERNIE D., LOWE J., TYRRELL T., LEGGE O., 2010. Influence of mitigation policy on ocean acidification. Geophysical Research Letters, 37: L15704. (doi: 10.1029/2010GL043181)

BICKFORD D., LOHMAN D., SODHI N. S., NG P. K. L., MEIER R., WINKER K., INGRAM K., DAS I., 2007. Cryptic species: a new window on diversity and conservation. Trends Ecol. Evol., 22: 148-155.

BILGER W., SCHREIBER U., BOCH M., 1995. Determination of the quantum efficiency of photosystem II and of non-photochemical quencing of chlorophyll fluorescence in the field. Oecologia, 102: 425-432.

BIRKY C.W. JR., 1995. Uniparental inheritance of mitochondrial and chloroplast genes: Mechanisms and evolution. Proceedings of the National Academic of Science, 92: 11331-11338.

BJÖRK M., UKU J., WEIL A., BEER S., 2000. Photosynthesis tolerance to dessiccation of tropical intertidal seagrasses. Marine Ecology Progress Series, 191: 121-126.

BJORKMAN O., DEMMING B., 1987. Photon yield of O2 evolution and chlorophyll fluorescence at 77k among vascular plants of diverse origins. Planta, 170: 489-504.

BLANDIN P., 1986. Bioindicateurs et diagnostic des systèmes écologiques. Bull. Ecol., 17 (4):215-307.

BOROWITZKA M. A.,1972. Intertidal algal species diversity and the effect of pollution, Aust. J. mar. Freshwat. Res., 23: 73-84.

BOROWITZKA, M.A. AND LARKUM, A., 1976. Calcification in the green algal Halimeda, III, The sources of inorganic carbon for photosynthesis and calcification and a model of the mechanisms of calcification. Journal of Experimental Botany 27: 879-893.

BOROWITZKA, M.A., 1981. Photosynthesis and calcification in the articulated coralline alga Amphiroa anceps and A. foliaceae. Marine Biology 62: 17-23.

BOURY-ESNAULT N., SOLÉ-CAVA A.M., THORPE J.P., BOURY-ESNAULT N., 1992. Genetic and cytological divergence between colour morphs of the Mediterranean sponge Oscarella lobularis Schmidt (Porifera, Demospongiae, Oscarellidae). Journal of Natural History, 26: 271–284.

BRADBURY M., BAKER N.R., 1981. Analysis of the slow phase of the in vivo chlorophyll fluorescence induction curve. Changes in the redox state of photosystem II electron acceptors and fluorescence emission from photosystem I and II. Biochimica and Biophysica Acta, 635: 542-551.

BRUNO J.F., BERTNESS M.D., 2001. Habitat modification and facilitation in benthic marine communities. In: Bertness M.D., M.E. Hay, and S.D. Gaines (eds.). Marine Community Ecology. Sinauer, Sunderland, MA, pages 201-218.

BUIA M.C., PORZIO L., PATTI F.P., 2007. The Application of the "CARLIT method" to assess the ecological status of coastal waters in the Gulf of Naples. In: Pergent-Martini, C., El Asmi, S., Le Ravallec, C. (Eds.), Proceedings of the third Mediterranean Symposium on Marine Vegetation. CAR/ASP publ., Tunis: 253-254.

BULLERI F.; BENEDETTI-CECCHI L., 2008. Facilitation of the introduced green alga Caulerpa racemosa by resident algal turfs: experimental evaluation of underlying mechanisms Marine Ecology Progress Series, 364: 77-86

BURTON R.S., 1983. Protein polymorhisms and genetic differentiation of marine invertebrate populations. Marine Biology Letters, 4: 193-206.

BURTON R.S., FIELDMAN M.V., 1982. Population genetics of coastal and estuarine invertebrates: does larval behaviour influence population structure? In: Kennedy ES (ed) Estuarine comparisons. Academic Press, New York, pp 537-551.

BUSCHMANN C., 1999. Photochemical and non-photochemical quencing coefficients of the chlorophyll fluorescence: comparison of variation and limits. Photosynthetica, 37 (2): 217-224.

CABIOC'H J., FLOC'H J.Y., LE TOQUIN A., BOUDOURESQUE C.F., MEINESZ A., VERLAQUE M., 2006. Guide des algues des mers d'Europe. Delachaux et Niestlé, Paris, 272 pp.

CALDEIRA K., WICKETT M.E., 2003. Anthropogenic carbon and ocean pH. Nature, 425 (6956): 365-365.

CAPUTO P., 1998. The usage of molecular data in phylogenetic systematics. Memorie del Museo Civico di Storia Naturale di Verona (2° serie) Sezione Scienze della Vita N. 13 - 1998 Atti 1° Colloquio Nazionale di Sistematica Cladistica: 33-39.

CARPENTER RC; WILLIAMS SL, 1993. Effects of algal turf canopy height and microscale substratum topography on profiles of flow speed in a coral forereef environment, Limnology And Oceanography, 38: 687-694

CARR H., BJÖRK M., 2003. A methodologica comparison of photosynthetic oxigen evolution and estimated electron transport rate in tropical Ulva (Chlorophyceae) species under different light and inorganic carbon conditions. Journal of Phycology, 39: 1125-1131.

CAVALIER-SMITH T., 2007. Evolution and relationships of algae: major branches of the tree of life. In: Brodie J. and J Lewis (eds.), Unravelling the algae. The past, present and the future of algal systematics. The Systematics Association, Special Volume Series 75, CRC Press London

CAVENDER-BARES J., BAZZAZ F.A., 2004. From leaves to Ecosystems: Using Chlorophyll fluorescence to assess photosynthesis and plant function in ecological studies. In: George Papaqeorgiou and Govindjee (eds.) Chlorophyll a Fluorescence a Signature of Photosynthesis, Springer, page 737-755.

CHIARUGI A.,2002. Poly-ADPR Polymerase:killer or conspirator? The suicide hypothesis revisited. Trends Pharm. Sc. 23: 122-129.

CHOI C.G, TAKEUCHI Y., TERAWAKI T.,SERISAWA Y.,OHNO M.,SOHN C.H., 2002. Ecology of seaweeds beds on two types of artificial reef. Journal of Applied Phycology, 14: 343-349.

CIGLIANO M.M., GAMBI M.C., RODOLFO-METALPA R., PATTI F.P., HALL-SPENCER J.M. 2010. Effects of ocean acidification on invertebrate settlement at volcanic CO2 vents. Marine Biology, DOI: 10.1007/s00227-010-1513-6

CLARIDGE M.F., DAWAH H.A., WILSON M.R., 1997. Species: the units of biodiversity. London: Chapman and Hall, pp. 439.

CLARKE K.R., WARWICK R.M., 2001. Change in marine communities: an approach to statistical analysis and interpretation, 2nd ed. PRIMER-E Press, Plymouth.

COGNETTI G., COGNETTI G., 1992. Inquinamenti e protezione del mare. Edizioni Calderini, 318 pp.

CONNELL S.D., RUSSELL B.D., 2010. The direct effects of increasing CO2 and temperature on non-calcifying organisms: increasing the potential for phase shifts in kelp forests. Proceedings of the Royal Society B, 277: 1409-1415 (doi:10.1098/rspb.2009.2069).

CORMACI M., DURO A., FURNARI G., 1982. Considerazioni sugli elementi fitogeografici della flora algale della Sicilia. Naturalista Siciliano, ser. IV, 6 (1): 7-14.

CORMACI M., FURNARI G., 1999. Changes of the benthic algal flora of the Tremiti Islands (southern Adriatic) Italy. Hydrobiologia, 398-399: 75-79.

CORMACI M., FURNARI G., GIACCONE G., 2004. Macrophytobentos. Biol. Mar. Medit., 11 (Suppl. 1): 217-246.

COUTO R.P., NETO A.I., RODRIGUES A.S., 2010. Metal concentration and structural changes in Corallina elongata (Corallinales, Rhodophyta) from hydrothermal vents. Marine Pollution Bulletin, 60: 509–514.

COYNE, J.A., 1992. Genetics and speciation. Nature, 6, 355 (6360): 511-515.

D'AMOURS D., DESNOYERS S., D'SILVA I., POIRER G.G., 1999. Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. Biochem. J. 342: 249-268.

DARCY-HALL T.L., HALL S.R, 2008. Linking limitation to species composition: importance of inter- and intra-specific variation in grazing resistance. Oecologia, 155: 797-808.

DAWSON S.P., DENNISON W.C., 1996. Effects of ultraviolet and photosynthetically active radiation on five seagrasses species. Marine Biology, 125: 629-638.

DE BLOCK M., VERDUYN C., DE BROUWER D., CORNELISSEN M., POLY, 2005. (ADP-ribose) polymerase in the plants affects energy homeostasis, cell death and stress tolerance, Plant J., 41(1): 95-106.

DE CLERCK O., LELIAERT F., VERBRUGGEN H., LANE C.E., CAMPOS DE PAULA J., PAYO D.A., COPPEJANS E., 2006. A revised classification of the Dictyotae (Dictyotales, Phaeophyceae) based on rbcL and 26S ribosomal DNA sequence analyses. Journal of Phycology, 42: 1271-1288.

DE CLERCK, O., 2003. The genus Dictyota in the Indian Ocean. Opera Botanica Belgica 13: 1-205, 54 figs, 12 tables.

DE REVIER B., 2002. Biologie et phylogenie des algues. Belin, Paris, 351 pp.

DE'ATH G., LOUGH J.M., FABRICIUS K.E., 2009. Declining coral calcification on the Great Barrier Reef. Science, 323: 116-119.

DELLA CROCE N., CATTANEO VIETTI R., DANOVARO R., 1997. Ecologia e protezione dell'ambiente marino costiero. UTET, 426 pp.

DELWICHE C.F., 2007. Algae in the warps and weave of life:bound of plastids. In: Brodie J. and J Lewis eds, Unravelling the algae. The past, present and the future of algal systematics. The Systematics Association, Special Volume Series 75, CRC Press London.

DEMMIG-ADAMS B., 1990. Carotenoids and photoprotection in plants. A role for the xantophiyll zeaxanthin. Biochimica and Biophysica Acta, 1020: 1-24.

DIAS B.B., HART M.B., SMART C.W., HALL-SPENCER J.M., 2010. Modern seawater acidification: the response of foraminifera to high-CO2 conditions in the Mediterranean Sea. Journal of the Geological Society, 167: 843-846.

DIAZ-PULIDO G., McCOOK L., 2008. Macroalgae (Seaweeds). In: Chin, A., (Ed.), The State of the Great Barrier Reef. On-line, Great Barrier Reef Marine Park Authority, Townsville. Viewed on http://www.gbrmpa.gov.au/corp_site/info_services/publications/sotr/downloads/SORR_Macroalgae.pdf

DIEZ I., SECILLA A., SANTOLARIA A., GOROSTIAGA J. M., 1999. Phytobenthic intertidal community structure along an environmental pollution gradient. Mar. Poll. Bull., 38(6): 463-472.

DOUCET-CHABEAUD G., GODON C., BRUTESCO C., DE MURCIA G., KAZMAIER M., 2001. Ionising radiation induces the expression of PARP-1 and PARP-2 gene in Arabidopsis. Mol. Genet. Genomics, 265(6): 954-963.

DOYLE J.J., DOYLE J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull., 19: 11-15.

DRAISMA S.G.A., PRUD'HOMME VAN REINE W.F., STAM W.T., OLSEN J.L., 2001. A reassessment of phylogenetic reltionships within the Phaeophyceae based on RUBISCO large subunit and ribosomal DNA sequences. Journal of Phycology, 37: 586-603.

DUPONT S, HAVENHAND J, THORNDYKE W, PECK L, THORNDYKE M., 2008. Near-future level of CO_2-driven ocean acidiWcation radically aVects larval survival and development in the brittlestar *Ophiothrix fragilis*. Mar Ecol Prog Ser, 373: 285–294

DUPONT S., HAVENHAND J., THORNDYKE W., PECK L., THORNDYKE M. 2008. Near-future level of CO2-driven ocean acidification radically affects larval survival and development in the brittlestar Ophiothrix fragilis. Marine Ecology Progress Series, 373: 285-294.

ELLIS RP, BERSEY J, RUNDLE SD, HALL-SPENCER JM, SPICER JI, 2009. Subtle but signiWcant eVects of CO2 acidiWed sea water on embryos of the intertidal snail, *Littorina obtusata*. Aquat Biol 5:41–48

EPRON D., DREYER E., BREDA N., 1992. Photosynthesis of oak trees (Quercus petrae (Matt) Liebl.) during drought stress under field conditions: diurnal course of net CO2 assimilation and photochemical efficiency of photosystem II. Plant. Cell and Environment, 15: 809-820.

FABRY V.J., SEIBEL B.A., FEELY R.A., ORR J.C., 2008. Impacts of ocean acidification on marine fauna and ecosystem processes. ICES Journal of Marine Science, 65: 414–432. (doi:10.1093/icesjms/fsn048)

FABRY, V.J., SEIBEL, B.A., FEELY, R.A., ORR, J.C., 2008. Impacts of ocean acidification on marine fauna and ecosystem processes. – ICES Journal

FALACE A., CURIEL D., SFRISO A., 2009. Study of the macrophyte assemblages and application of phytobenthic indices to assess the Ecological Status of the Marano-Grado Lagoon (Italy). Marine Ecology-an Evolutionary Perspective, 30 (4): 480-494.

FALKOWSKI P.G., RAVEN J.A., 1997. Aquatic Photosynthesis. Blackwell Science, pp. 375.

FEELY R.A., SABINE C.L., LEE K., BERELSON W., KLEYPAS J., FABRY V.J., MILLERO F.J., 2004. Impact of anthropogenic CO2 on the CaCO3 system in the oceans. Science, 305: 362–366. (doi:10.1126/science.1097329)

FEELY R.A., SABINE C.L., LEE K., BERELSON W., KLEYPAS J., FABRY, V.J., MILLERO F.J., 2004. Impact of anthropogenic CO2 on the CaCO2 system in the ocean. Science, 305: 362–366.

FELSENSTEIN J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39: 783-791.

FINDLAY HS, KENDALL MA, SPICER JI, WIDDICOMBE S., 2009. Future high CO2 in the intertidal may compromise adult barnacle *Semibalanus balanoides* survival and embryonic development rate. Mar Ecol Prog Ser, 389: 193–202

FU F.X., MULHOLLAND M.R., GARCIA N.S., BECK A., BERNHARDT P.W., WARNER M.E., SANUDO-WILHELMY S.A., HUTCHINS D.A., 2008. Interactions

between changing pCO2, N2 fixation, and Fe limitation in the marine unicellular cyanobacterium Crocosphaera. Limnol. Oceanography, 53: 2472–2484.

FURNARI G., CORMACI M., SERIO D., 1999. Catalogue of the benthic marine macroalgae of the Italian coast of the Adriatic Sea. Bocconea, 12: 1-214.

FURNARI G., GIACCONE G., CORMACI M., ALONGI G., CATRA M., NISI A., SERIO D., 2010. Macrophytobenthos. Biologia Marina Mediterranea, 17 (suppl. 1): 801-828.

FUTUYMA D.J., MAYER G.C., 1980. Non-allopatric speciation in animals. Systematic Zoology, 29: 254-271.

GAMON J.A., PEARCY R.W., 1989. Leaf movement, stress avoidance and photosynthesis in Vitis californica. Oecologia, 79: 475-481.

GAO K., ARUGA Y., ASADA K., ISHIHARA T., AKANO T., KIYOHARA M., 1993. Calcification in the articulated coralline alga Corallina pilufera, with special reference to the effect of elevated CO2 concentration. Marine Biology, 117: 129-132.

GAO K., ARUGA Y., ASADA K., ISHIHARA T., AKANO T., KIYOHARA M., 1991. Enhanced growth of the red alga Porphyra yezoensis Ueda in high CO2 concentrations. Journal of Applied Phycology 3: 355-362.

GAO K., ARUGA Y., ASADA K., KIYOHARA M., 1993b. Influence of enhanced CO2 on growth and photosynthesis of the red algae Gracilaria sp. and G. chilensis. Journal of Applied Phycology 5: 563-571.

GAO K.S., JI Y., ARUGA Y., 1999. Relationship of CO2 concentrations to photosynthesis of intertidal macroalgae during emersion. Hydrobiologia, 399: 355-359.

GAO, K., ARUGA, Y., ASADA, K., ISHIHARA, T., AKANO, T. AND KIYOHARA, M. 1993. Calcification in the articulated coralline alga Corallina pilulifera, with special reference to the effect of elevated CO2 concentration. Marine Biology 117: 129-132.

GENTY B., BRIANTAIS J.-M., BAKER N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence.Biochim. Biophys. Acta, 990: 87-92.

GENTY B., BRIANTAIS J.M., BAKER N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica and Biophysica Acta, 990: 87-92.

GENTY B., HARBISON J., BRIANTAIS J.M., BAKER N.R., 1990. The relationship between non-photochemical quencing of chlorophyll fluorescence and the rate of photosystem II photochemistry in leaves. Photosynthesis Research, 28: 249-257.

GÈVAERT F., CRÉACH A., DAVOULT D., MIGNÉ A., LEVAVASSEUR G., ARZEL P., HOLL A.C., LEMOINE Y., 2003. Laminaria saccarina photosynthesis measured in situ: photoinhibition and xanthophyll cycle during a tidal cycle. Marine Ecology Progress Series, 247: 43-50.

GIACCONE G., ALONGI G., PIZZUTO F., 1994. La vegetazione marina bentonica fotofila del Mediterraneo: II. Infralitorale e circalitorale. Proposte di aggiornamento. Bollettino Accademia Gioenia Sci. Nat. Catania, 27:111-157

GIORDANO M., BEARDALL J., RAVEN J.A., 2005. CO2 concentrating mechanisms in algae: mechanism, environmental modulation, and evolution. Annual Review of Plant Biology 56: 99–131

GIORDANO M., BEARDALL J., RAVEN J.A., 2005. CO2 concentrating mechanisms in algae: Mechanisms, Environmental Modulation, and Evolution. Annual Review of Plant Biology, 56: 99-131.

GITAY H., NOBLE I.R., CONNELL J. H, 1999. Deriving functional types for rain-forest trees. Journal of Vegetation Science, 10: 641–650.

GONZALEZ-DAVILA M., SANTANA-CASIANO J.M., RUEDA M.J., LLINAS O., GONZALEZ E.F., 2003. Seasonal and interannual variability of sea-surface carbon dioxide species at the European Station for Time Series in the Ocean at the Canary Islands (ESTOC) between 1996 and 2000. Global Biogeochemical Cycles 17, article number 1076

GRASSLE J.F., MACIOLEK N.J., 1992. Deep-sea species richness: Regional and local diversity estimates from quantitative bottom samples. American Naturalist, 139: 313-341.

GREEN P.J., KAY S.A., LAM E. CHUA, N-HIn vitro DNA footprinting, in Plant molecular biology manual (Gelvin, S. B.& Schilperoort, R. A., eds) Supplement 1, B11, pp. 1-2, Kluwer Academic Publishers. Dordrecht

GROOM Q.J., BAKER N.R., 1992. Analysis of light-induced depressions of photosynthesis in leaves of a wheat crop during the winter. Plant Physiology, 100 (3): 1217-1223.

GUIDETTI P., 2006. Marine Reserves re-establish lost predatory interactions and cause community Changes in Rocky Reefs. Ecological Applications, 16 (3): 963-976

GUINOTTE J.M., FABRY V.J., 2008. Ocean acidification and its potential effects on marine ecosystems. Annals N.Y. Academy of Science, 1134: 320-342. (doi:10.1196/annals.1439.013)

GUIRY M.D., GUIRY G.M., 2010. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org.

HADER D.P., FIGUEROA F., 1997. Photoecophysiology of marine macroalgae. Photochemistry and Photobiology, 66 (1): 1-14.

HADER D.P., LEBERT M., FIGUEROA F., JIMÉNEZ C., VIÑEGLA B., PEREZ-RODRIGUEZ E., 1998. Photoinhibition in Mediterranean macroalgae by solar radiation measured on site by PAM fluorescence. Aquatic Botany, 61: 225-236.

HADER D.P., LEBERT M., HELBLING E.W., 2001. Photosynthetic performance of marine macroalgae measured in Patagonia on site. Trends in Photochemistry & Photobiology, 8: 145-152.

HADER D.P., LEBERT M., SINHA R.P., BARBIERI E.S., HELBLING E.W., 2002. Role of protective and repair mechanisms in the inhibition of photosynthesis in marine macroalgae. Photochemical & Photobiological Science, 1: 809-814.

HALL-SPENCER J.M., RODOLFO-METALPA R., MARTIN S., RANSOME E., FINE M., TURNER S.M., ROWLEY S.J., TEDESCO D., BUIA M.C., 2008. Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. Nature, 454: 96- 99.

HALL-SPENCER J.M., RODOLFO-METALPA R., MARTIN S., RANSOME E., FINE M., TURNER S.M., ROWLEY S.J., TEDESCO D., BUIA M-C., 2008. Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. Nature, 454: 96- 99.

HAMEL G., 1939. Phéophycées de France. Paris, pp. 345-347.

HANELT D., HUPPERTZ K., NULTSCH W., 1993. Daily course of photosynthesis and photoinhibition in marine macroalgae investigated in the laboratory and field. Marine Ecology Progress Series, 97: 31-37.

HE J., CHEE C.W., GOH C.J., 1996. 'Photoinhibition' of Heliconia under natural tropical conditions: the importance of leaf orientation for light interception and leaf temperature. Plant, Cell and Environment, 19: 1238-1248.

HENDRICKS I.E., DUARTE C.M., 2010. Ocean acidification: Separating evidence from judgment - A reply to Dupont et al. Estuar. Coastal and Shellfish Science, 89: 186-190.

HEY J., 2001. Genes, Categories and Species. Oxford, England: Oxford University Press.

HEY J., WAPLES R.S., ARNOLD M.L., BUTLIN R.K., HARRISON R.G., 2003. Understanding and confronting species uncertainty in biology and conservation. Trends in Ecology and Evolution, 18: 597-603.

HILLIS D. M., MORITZ C., MABLE B. K., 1996. Molecular Systematics, Sinauer Associates. Sunderland, Massachusetts.

HINGA K.R., 2002. Effects of pH on coastal phytoplankton. Marine Ecology Progress Series, 238: 281–300

HOEGH-GULDBERG O., MUMBY P.J., HOOTEN A.J., STENECK R.S., GREENFIELD P., GOMEZ E., HARVELL C.D., SALE P.F., EDWARDS A.J., CALDEIRA K., KNOWLTON N., EAKIN C.M., IGLESIAS-PRIETO R., MUTHIGA N., BRADBURY R.H., DUBI A., HATZIOLOS M.E., 2007. Coral reefs under rapid climate change and ocean acidification. Science, 318: 1737-1742. (doi:10.1126/science.1152509)

HORTON P., RUBAN A., 1994. The role of light harvesting complex II in energy quenching. In: Baker N.S., Bowyer J.R. (eds) Photoinhibition of Photosynthesis: from Molecular-Mechanisms to the Field. Bios Scientific Publishers, Oxford: 111-128.

HOWARD D.J., BERLOCHER S.H., 1998. Endless Forms: Species and Speciation. Oxford, England: Oxford University Press, pp. 470.

HUANG Q., SHEN H.M., 2009. To die or to live: the dual role of poly(ADP-ribose) polymerase-1 in autophagy and necrosis under oxidative stress and DNA damage. Autophagy, 5(2): 273-276.

HWANG I.K., KIM H.S., LEE W.J., 2005. Polymorphism in the brown alga Dictyota dichotoma (Dictyotales, Phaeophyceae) from Korea. Marine Biology, 147: 999-1015.

IGLESIAS-RODRIGUEZ M.D., HALLORAN P.R., RICKABY R.E.M., HALL I.R., COLMENERO-HIDALGO E., GITTINS J.R., GREEN D.R.H., TYRREL T., GIBBS S.J., VON DASSOW P., REHM E., ARMBRUST E.V., BOESSENKOOL K.P., 2008. Phytoplankton calcification in a high-CO2 world. Science, 320: 336-340.

IPCC (2001). The third assessment report of the Intergovernmental Panel on Climate Change (IPCC). Cambridge University Press: Cambridge, UK, and New York, USA.

IPCC (INTERGOVERNMENTAL PANEL ON CLIMATE CHANGE), 2007. Climate Change 2007 Synthesis Report. Cambridge University Press, New York.

IPCC (Intergovernmental panel on climate Change), 2007. Climate Change 2007 Synthesis Report. Cambridge University Press, New York.

IRVING A D , CONNELL S D, 2006. Physical disturbance by kelp abrades erect algae from the understorey. Mar ecol prog ser, 324:127-137.

ISRAEL A., HOPHY M. 2002. Growth, photosynthetic properties and Rubisco activities and amounts of marine macroalgae grown under current and elevated seawater CO2 concentration. Global Change Biology 8: 831-840.

IVESA L., LYONS D.M., DEVESCOVI M., 2009. Assessment of the ecological status of north-eastern Adriatic coastal waters (Istria, Croatia) using macroalgal assemblages for the European Union Water Framework Directive. Aquatic Conservation-Marine And Freshwater Ecosystems, 19 (1): 14-23.

JACOBSON M.K., JACOBSON, E.L., 1999. Discovering new ADP-ribose polymer cycles: protecting the genome and more. Trends Biochem Sci. 24: 415-417.

JOHNSON G.M., SCHOLES J.D., GRIME J.P., HORTON P., 1989. Fluorescence responses on step changes in irradiance by plants from different light habitats. In: Baltscheffsky M. (Ed.) Proceedings of the VIII International Congress on Photosynthesis. Kluwer Academic Publisher, pp. 361-364.

JOHNSTON A. M., MABERLY S. C., RAVEN J. A., 1992. The acquisition of inorganic carbon by four red macroalgae. Oecologia, 92: 317-326.

JOKIEL P.L., RODGERS K.S., KUFFNER I.B., ANDERSSON A.J., COX E.F., MACKENZIE F.T., 2008. Ocean acidification and calcifying reef organisms: a mesocosm investigation. Coral Reefs, 27: 473-483. (doi:10.1007/s00338-008-0380-9)

KARUKSTIS K.K., 1991. Chlorophyll fluorescence as a physiological probe to the photosynthetic apparatus. In: H. Sheer, (ed.), Chlorophylls. CRC Press Inc., Boca Raton pp. 769-795.

KAUTSKY H., APPEL W., AMANN H., 1960. Chlorophyllfluorescenz und kohlensaureassimilation. Biochemische Zeitschrift, 322: 277–292.

KICKHOEFER V.A., SIVA A.C., KEDERSHA N.L., INMAN E.M., ET AL. (1999) The 193-kDa vault protein, V-PARP, is a novel poly(ADP-ribose) polymerase. J.Cell Biol., 146: 917-928.

KIMURA M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16: 111-120.

KLEYPAS J.A., FEELY R.A., FABRY V.J., LANGDON C., SABINE C.L., ROBBINS L.L., 2006. Impacts of ocean acidification on coral reefs and other marine calcifiers: a guide for future research. Contribution No. 2897 from NOAA/Pacific Marine Environmental Laboratory http://www.fedworld.gov/onow/

KNOWLTON N., 1986. Cryptic and sibling species among the decapod Crustacea. J. Crustac. Biol., 6: 356–363.

KNOWLTON N., 1993. Sibling species in the sea. Annu. Rev. Ecol. Syst., 24: 189-216.

KNOWLTON N., WEIGT L.A., SOLORZANO L.A., MILLS D.K., BERMINGHAM E., 1993. Divergence in proteins, mitochondrial DNA, and reproductive compatibility across the Isthmus of Panama. Science, 260, 5114: 1629-1632.

KOK B.A., 1984. A critical consideration of the quantum yield of Chlorella photosynthesis. Enzymologia, 13: 1-56.

KRANZ S.A., SÜLTEMEYER D., RICHTER K.U., ROST B., 2009. Carbon acquisition in Trichodesmium: the effect of pCO2 and diurnal changes. Limnology and Oceanography, 54: 548-559.

KRAUSE G.H., WEIS E., 1991. Chlorophyll fluorescence and photosynthesis: the basics. Annual Review of Plant Physiology and Plant Molecular Biology. 42: 313-349.

KROEKER K.J, PORZIO L., BUIA M.C., SCIPIONE M.B., GAMBI M.C., in preparation. Divergent responses within a marine community to in-situ ocean acidification.

KROEKER K.J., KORDAS R.L., CRIM R.N., SINGH G.G., 2010. Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecology Letters, 13: 1419-1434. (doi: 10.1111/j.1461-0248.2010.01518.x)

KÜBLER J.E., JOHNSTON A.M., RAVEN J.A., 1999. The effects of reduced and elevated CO2 and O2 on the seaweed Lomentaria articulata. Plant Cell. Environment, 22: 1303–1310. (doi:10.1046/j.1365-3040.1999.00492.x).

KÜBLER, J.E., JOHNSTON, A.M. AND RAVEN, J.A. 1999. The effects of reduced and elevated CO2 and O2 on the seaweed Lomentaria articulata. Plant, Cell and Environment 22: 1303-1310.

KUFFNER I.B, ANDERSSON A.J, JOKIEL P.L, RODGERS K.S, MACKENZIE F.T., 2008. Decreased abundance of crustose coralline algae due to ocean acidification. Nature Geoscience, 1:114–117

KUFFNER I.B., ANDERSSON A.J., JOKIEL P.L., RODGERS K.S., MACKENZIE F.T., 2008. Decreased abundance of crustose coralline algae due to ocean acidification. Nature Geoscience, 1:114-117.

KURIHARA H, ISHIMATSU A., 2008. eVects of high CO2 seawater on the copepod (*Acartia tsuensis*) through all life stages and subsequent generations. Mar Pollut Bull, 56: 1086–1090

KURIHARA H, KATO S, ISHIMATSU A, 2007. Effects of increased seawater *p*CO2 on early development of the oyster *Crassostrea gigas*. Aquat Biol, 1: 91–98

KURIHARA H, SHIMODE S, SHIRAYAMA Y, 2004. Sub-lethal effects of elevated concentration of CO2 on planktonic copepods and sea urchins. J Oceanogr, 60:743–750

KURIHARA H, SHIRAYAMA Y, 2004. Effects of increased atmospheric CO2 on sea urchin early development. Mar Ecol Prog Ser, 274: 161–169

LANGDON, C., BROECKER, W.S., HAMMOND, D.E., GLENN, E., FITZSIMMONS, K., NELSON, S.G., PENG, T.-S., HAJDAS, I. AND BONANI, G. 2003. Effect of elevated CO2

on the community metabolism of an experimental coral reef. Global Biogeochemical Cycles 17: 10.1029/2002GB001941.

LANGER G., NEHRKE G., PROBERT I., LY J., ZIVERI P., 2009. Strain specific responses of Emeliana huxleyi to changing seawater chemistry. Biogeosciences, 6: 2637-2646.

LAVERY P, VANDERKLIFT M., 2002. A comparison of spatial and temporal patterns in epiphytic macroalgal assemblages of the seagrasses Amphibolis griffithii and Posidonia coriacea. Marine Ecology Progress Series, 236: 99-112

LE CORGUILLÉ G., PEARSON G., VALENTE M., VIEGAS C., GSCHLOESSL B., CORRE E., BAILLY X., PETERS A.F., JUBIN C., VACHERIE B., COCK J.M., LEBLANC C., 2009. Plastid genomes of two brown algae, Ectocarpus siliculosus and Fucus vesiculosus: further insights on the evolution of red-algal derived plastids. BMC Evolutionary Biology, 9: 253-267.

LEISHMAN M.R.,WESTOBY M., 1992. Classifying plants into groups on the basis of associations of individual traits—evidence from Australian semi-arid woodlands. Journal of Ecology, 80: 417–424.

LEVINE H. G., 1984. The use of seaweeds for monitoring coastal waters. In: Shubert E. (Ed.), Algae as ecological indicators, Academic Press, London, 189-210.

LEVITAN D.R., GROSBERG R.K., 1993. The analysis of paternity and maternity in the arine hydrozoan Hydractinia symbiolongicarpus using randomly amplified polymorphic DNA (RAPD) markers. Mol. Ecol., 2: 315-326.

LEWIS L.A., MCCOURT R.M., 2004. Green algae and the origin of land plants. American Journal of Botany, 91: 1535-1556.

LICHTENTALER K.H., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods in Enzymology, 148: 350-382.

LITTLER D.S., LITTLER M.M., BROOKS B.L., 2000. Checklist of marine algae and seagrasses from the ponds of the Pelican Cays, Belize. Atoll Research Bulletin, 474: 184.

LITTLER M. M., LITTLER D. S., 1980. The evolution of thallus form and survival strateqes in benthic marine macroalgae; field and laboratory test of a functional form model. Am. Nat., 116: 25-44

LITTLER M. M., MURRAY S. N., 1975. Impact of sewage on the distribution, abundance and community structure of rocky intertidal macro-organisms. Mar. Biol., 30: 277-291.

LITTLER M., LITTLER D.S., 1984. Relationships between macroalgal functional form groups and substrata stability in a subtropical rocky-intertidal system. Journal of Experimental Marine Biology and Ecology, 74: 13-34.

LOBBAN C.S., HARRISON P.J., DUNCAN M.J., 1985. The Physiological Ecology of Seaweeds. Cambridge University Press, pp. 4-34.

LONGSTAFF B.J., KILDEA T., RUNCIE J.W., CHESHIRE A., DENNISON W.C., HURD C., KANA T., RAVEN J.A., LARKUM A.W.D., 2002. An in situ study of photosynthetic oxigen exchange and electron transport rate in the marine macroalgae Ulva lactuca (Chlorophyta). Photosynthesis Research, 74: 281- 293.

LOTZE , WORM, 2002.Complex interactions of climatic and ecological controls on macroalgal recruitment. Limnol Oceanogr, 47(6):1734-1741.

LÜNING K., 1990. Seaweeds: their environment, biogeography and ecophysiology. John Wiley & Sons, New York, 527 pp.

MACKENZIE F.T, AGEGIAN C.,1989. Biomineralization and tentative links to plate tectonics. In: Crick R (ed) *Origin, evolution, and modern aspects of biomineralization in plants and animals*. Plenum Press, New York, pp 11–28

MANGIALAJO L., CHIANTORE M., CATTANEO-VIETTI R., 2008. Loss of fucoid algae along a gradient of urbanisation, and structure of benthic assemblages. Marine Ecology-Progress Series, 358: 63-74.

MANGIALAJO L., CHIANTORE M., CATTANEO-VIETTI R., 2008. Loss of fucoid algae along a gradient of urbanisation, and structure of benthic assemblages. Marine Ecology Progress Series, 358: 63–74.

MANZELLO D.P., KLEYPAS J.A., BUDD D.A., EAKIN C.M., GLYNN P., LANGDON C., 2009. Poorly cemented coral reefs of the eastern tropical Pacific: possible insights into reef development in a high CO2 world. Proceedings of National Academy of Science USA, 30: 10450-10455.

MARINO G, DI MARTINO V., GIACCONE G., 1999. La vegetazione marina della Penisola Maddalena (Siracusa, Sicilia S-E). Bollettino Accademia Gioenia Sci. Nat. Catania, 31: 235-287.

MARTIN S., GATTUSO J.P., 2009. Response of Mediterranean coralline algae to ocean acidification and elevated temperature. Global Change Biology, 15: 2089–2100. (doi:10.1111/j.13652486.2009.01874.x)

MARTIN S., RODOLFO-METALPA R., RANSOME E., ROWLEY S., BUIA M.C., GATTUSO J.P., HALL-SPENCER J.M., 2008. Effects of naturally acidified seawater on seagrass calcareous epibionts. Biol. Lett., 4, 689-692.

MARTIN S., RODOLFO-METALPA R., RANSOME E., ROWLEY S., BUIA M.C., GATTUSO J.P., HALL-SPENCER J.M., 2008. Effects of naturally acidified seawater on seagrass calcareous epibionts. Biology Letters, 4: 689-692.

MAXWELL K., JOHNSON G.N., 2000. Chlorophyll fluorescence- a practical guide. Journal of Experimental Botany, 51 (345): 659-668.

MAYDEN R.L., 2002. On biological species, species concepts and individuation in the natural world Fish. Fish., 3: 171-196.

MAYR, E. 1942. Systematics and the origin of species. Columbia University Press, New York.

MCINTOSH, L., POULSEN, C., BOGORAD, L., 1980. Chloroplast gene sequence for the large subunit of ribulose bis-phosphatecarboxylase of maize. Nature, 288 (5791): 556-560.

MENÉNDEZ, M., MARTINEZ, M. & COMÍN, F.A., 2001. A comparative study of the effect of pH and inorganic carbon resources on the photosynthesis of three floating macroalgae species of a Mediterranean coastal lagoon. Journal of Experimental Biology and Ecology 256: 123-136.

MEYER O., 1993. Functional Groups of Microorganisms. In Schulze, E. D. & H. A. Mooney (eds), Biodiversity and Ecosystem Function. Ecological Studies. Springer- Verlag, Berlin: 67–96.

MICHELI F., HALPERN B.S., 2005. Low functional redundancy in coastal marine assemblages. Ecology Letters, 8: 391-400.

MIDDELBOE A. L., HANSEN P. J., 2007. High pH in shallow macroalgal habitats. Marine Ecology Progress Series, 338: 307-317.

MILES H., WIDDICOMBE S., SPICER J., HALL-SPENCER J.M., 2007. Effects of anthropogenic seawater acidification on acid-base balance in the sea urchin Psammechinus miliaris. Marine Pollution Bulletin, 54: 89-96.

MORITZ C., HILLIS D.M., 1996. Molecular Systematics: Context and Controversies. In Hillis, D. M., Moritz, C., Mable, B. K. (eds), Molecular Systematics, Sinauer Associates. Sunderland, Massachusetts, pp. 1-13.

MULLIS K.B., FALOONA F.A., 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Meth. Enzymol., 155: 335-350.

MURPHY R.W., SITES J.W., BUTH D.G. Jr., HAUFLER C.H., 1996. Proteins: Isozyme Electrophoresis. In: Hillis D.M., Moritz C., Mable B.K. (eds.), Molecular Systematics. Sinauer Associates. Sunderland, Massachusetts, 655 pp.

MURRAY S. N., LITTLER M. M., 1978. Patterns of algal succession in a perturbated marine intertidal community. J. Phycol., 14: 506-512.

NEI M., KUMAR S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

NELSON W.A., 2009. Calcified macroalgae – critical to coastal ecosystems and vulnerable to change: a review. Marine and Freshwater Research, 60: 787-801. (doi:10.1071/MF08335)

OBERWINKLER F., 1993. Evoultion of Functional Groups in Basidiomycetes. (Fungi). In Schulze, E. D. & H. A. Mooney (eds), Biodiversity and Ecosystem Function. Ecological Studies. Springer-Verlag, Berlin

of Marine Science, 65: 414–432.

OGREN E., SJOSTROM M., 1990. Estimation of the effect of photoinhibition on the carbon gain in leaves of a willow canopy. Planta, 181: 560-567.

ORFANIDIS S., PANAYOTIDIS P., STAMATIS N., 2001. Ecological evaluation of transitional and coastal waters : a marine benthic macrophytes- based model. Mediterranean Marine Science, 2/2: 45-65.

ORFANIDIS S., PANAYOTIDIS P., STAMATIS N., 2001. Ecological evaluation of transitional and coastal waters: a marine benthic macrophytes- based model. Mediterranean Marine Science, 2: 45-65.

OTTE D. and ENDLER J.A., 1989. Speciation and its Consequences. Sinauer, Sunderland, MA.

PALACIOS S.L., ZIMMERMAN R.C., 2007. Response of eelgrass Zostera marina to CO2 enrichment: possible impacts of climate change and potential remediation of coastal habitats. Marine Ecology Progress Series, 344: 1-13.

PALUMBI, S.R., 1992. Marine speciation on a small planet. Trends in Ecology and Evolution, 7: 114-118.

PALUMBI, S.R., 1994. Genetic Divergence, Reproductive Isolation, and Marine Speciation. Annual Review of Ecology and Systematics, 25: 547-572.

PALUMBI, S.R., 1996. Nucleic acids II: the polimerase chain reaction. In: Hillis D.M., Moritz C., Mable B.K. (eds.), Molecular systematics, Sinauer Associates, Sunderland, Massachusetts, pp. 205-247.

PANAYOTIDIS P., FERETOPOULOU J., MONTESANTO B., 1999. Benthic vegetation as an ecological quality descriptor in an eastern Mediterranean coastal area (Kalloni Bay, Aegean Sea, Greece). Estur. Coast. Shelf Sci., 48: 205-214.

PANAYOTIDIS P., MONTESANTO B., ORFANIDIS S., 2004. Use of low-budget monitoring of macroalgae to implement the European Water Framework Directive. Journal of Applied Phycology, 16 (1): 49-59

PARKER L.M., ROSS P.M., O'CONNOR W.A., 2009. The effect of ocean acidification and temperature on the fertilisation and embryonic development of the Sydney rock oyster Saccostrea glomerata (Gould 1850). Global Change Biology, 15: 2123–2136. (doi:10.1111/j.1365-2486.2009.01895.x)

PATRICK J., KEELING 2004. Diversity and evolutionary history of plastids and their hosts. American Journal of Botany, 91(10): 1481–1493.

PATTERSON C., 1988. Homology in classic and molecular biology. Mol. Biol. Evol., 5: 603-625.

PAULAY, G., 1990. Effects of late Cenozoic sea-level fluctuations on the bivalve faunas of tropical oceanic islands. Paleobiology, 16: 415-434.

PERES J. M., PICARD J., 1964. Nouveau Manuel de Bionomie Benthique de la mer Méditerranée. Rec. Trav. Station Marine d'Endoume, 3 (47): 5-137.

PERGENT G., 1991. Les indicateurs écologiques de la qualité du milieu marin en Méditerranée. Océanis, 17 (4): 341-350.

PERGENT G., 1991. Les indicateurs écologiques de la qualité du milieu marin en Méditerranée. Océanis, 17: 341-350.

PHILLIPS J.C., KENDRICK G.A., LAVERY P.S., 1997. A test of a functional group approach to detecting shifts in macroalgal communities along a disturbance gradient. Marine Ecology Progress Series, 153: 125-138.

PLATT T., GALLEGOS C. L., HARRISON W. G.,1980. Photoinhibition of photosynthesis in natural assemblages of phytoplankton. J. Mar. Res., 38: 687-701.

PODANI J., 2001. Syn-tax 2000. Computer program for data analysis in Ecology and Systematics. Scientia Publishing, Budapest.

PORZIO, L., BUIA, M.C. & SPENCER-HALL, J. (accepted) Natural pH gradient effects on a shallow macroalgal community. Journal of Experimental Marine Biology and Ecology.

POWLES S.B., 1984. Photoinhibition of photosynthesis induced by visible light. Annu. Rev. Plant Physiol., 35: 15-44.

PRESSEY R.L., HUMPHERIES C.J., MARGULES C.R., VANE-WRIGHT R.I., WILLIAMS P. H, 1993. Beyond opportunism: key principles for systematic reserve selection. Trends in Ecology and Evolution, 8: 124–128.

QUICK W.P., HORTON P., 1984 Studies on the induction of chlorophyll fluorescence in barley protoplasts. I. Factors affecting the observation of oscillations in the yield of chlorophyll fluorescence and the rate of oxygen evolution. In: Proceedings of the Royal Society of London B, 220, 361–370.

RALPH P.J., GADEMANN R., 2005. Rapid light curves: A powerful tool to assess photosynthetic activity. Aquatic Botany, 82: 222-237.

RALPH PJ, GADEMANN R., LARKUM A.W.D., KÜHL M., 2002. Spatial heterogeneity in active fluorescence and PSII activity of coral tissuee. Marine Biology, 141:539-646.

RAVEN J. A., 2003. Inorganic carbon concentrating mechanisms in relation to the biology of algae. Photosynthesis Research, 77: 155-171.

RAVEN J.A., GEIDER R.J., 2003. Adaptation, Acclimation and Regulation in Algal Photosynthesis. In: Larkum A.W., Douglas S.E., Raven J.A. (Eds.). Photosynthesis in Algae, Kluwer Academic Publisher, pp. 385-412.

REISKIND J. B., BEER S., BOWES G., 1989. Photosynthesis, photorespiration and ecophysiological interactions in marine macroalgae. Aquatic Botany, 34: 131-152.

RIEBESELL U., 2008. Climate change – acid test for marine biodiversity. Nature, 454: 46-47.

RIEBESELL U., SCHULZ K.G., BELLERBY R.G.J., BOTROS B., FRITSCHE P., MEYERHÖFER M., NEILL C., NONDAL G., OSCHLIES A., WOHLERS J., ZÖLLNER E., 2007. Enhanced biological carbon consumption in a high CO2 ocean. Nature, 450: 545-549.

RIEDL R., 1991. Fauna e flora del Mediterraneo. Franco Muzzio Editore, 777 pp.

RODRIGUEZ-PRIETO C., POLO L., 1996. Effects of sewage pollution in structure and dynamics of the community of Cystoseira mediterranea (Fucales, Phaeophyceae). Sci. Mar., 60 (2-3): 253-263.

RODRIGUEZ-PRIETO C., POLO L., 1996. Effects of sewage pollution in structure and dynamics of the community of Cystoseira mediterranea (Fucales, Phaeophyceae). Scientia Marina, 60: 253-263.

RUMPHO M.E., MUJER C.V., ANDREWS D.L., MANHART, J.R., PIERCE S.K., 1994. Extraction of DNA from mucillagenous tissues of a sea slug (Elysia chlorotica). Biotechniques, 17: 1097-1102.

RUNCIE J.W., RIDDLE M.J., 2004. Measuring variability in chlorophyll-fluorescence-derived photosynthetic parameters in situ with a programmable multi-channel fluorometer. Functional Plant Biology, 31: 559-562.

RUSSEL P. J., 1998. Genetica, III edizione, EdiSES, Napoli.

SAEZ A. G., LOZANO E., 2005. Body doubles. Nature, 433: 111.

SALMASO N, PADISAK J, 2007. Morpho-Functional Groups and phytoplankton development in two deep lakes (Lake Garda, Italy and Lake Stechlin, Germany). Hydrobiologia, 578:97-112.

SAMBROOK J., FRITSCH E.F., MANIATIS T., 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York, 1759pp.

SAMPER-VILLARREAL J., BERNECKER A., WEHRTMANN I.S., 2008. Inventory of macroalgal epiphytes on the seagrass Thalassia testudinum (Hydrocharitaceae) in Parque Nacional Cahuita, Caribbean coast of Costa Rica. Revista de Biologia Tropical, 56 (Suppl. 4): 163-174.

SANDERS H. L., 1968. Marine benthic diversity: a comparative study. Am. Nat., 102: 243-282.

SANTELICES B, UGARTE R., 1987. Algal life-history strategies and reistence to digestion. Marine Ecology Progress Series, 35:267-275.

SAROUSSI S., BEER S., 2007A. Alpha and quantum yield of aquatic plants derived from PAM fluorometry: Uses and misuses. Aquatic Botany, 86: 89-92.

SAROUSSI S., BEER S., 2007B. Acclimation of macroalgae as reflected in photosynthetic parameters derived from PAM fluorometry, and possible implications for abundance patterns. Marine Ecology, 28: 377-383.

SATTARIAN, A., 2006. Contribution to the biosystematics of Celtis L. (Celtidaceae) with special emphasis on the African species. PhD thesis Wageningen University, Wageningen, 142 pp.

SAUNDERS G.W., HOMMERSAND H.M, 2004. Assessing red algal supraordinal diversity and taxonomy in the context of contemporary systematic data. American Journal of Botany, 91: 1494-1507.

SCHELTEMA, R.S., WILLIAMS, I.P., 1983. Long distance dispersal of planktonic larvae and the biogeography and evolution of some Polynesian and western Pacific mollusks. Bulletin of Marine Science, 33: 545-565.

SCHNETTER R., HÖRNIG I., WEBER-PEUKERT G., 1987. Taxonomy of some North Atlantic Dictyota species (Phaeophyta). Hydrobiologia, 151/152: 193-197.

SCHOPF, T.J.M., 1979. The role of biogeographic provinces in regulating marine faunal diversity through geologic time. In: Gray J., Boucot A.J. (eds) Historical Biogeography, Plate

Tectonics, and the Changing Environment. Oregon State University Press, Corvallis pp. 449-457.

SCHREIBER U., 2004. Pulse-Amplitude-Modulation (PAM) Fluorometry and Saturation Pulse Method: An Overview. In: George Papaqeorgiou and Govindjee (eds.) Chlorophyll a Fluorescence a Signature of Photosynthesis, Springer, page 279-319.

SCHREIBER U., SCHLIWA U., BILGER W., 1986. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quencing with a new type of modulation fluorimeter. Photosynthesis Reasearch, 10: 51-62.

SEMESI I. S., KANGWE J., BJÖRK M. 2009. Alteration in seawater pH and CO2 affect calcification and photosynthesis in the tropical coralline alga, Hydrolithon sp. (Rhodophyta). Estuarine, Coastal and Shelf Science 84: 337-341.

SERIO, D., ALONGI, G., CATRA, M., CORMACI, M., FURNARI, G., 2006. Changes in the benthic algal flora of Linosa Island (Straits of Sicily, Mediterranean Sea). Botanica Marina, 49: 135-144.

SERREZE M. C., 2010. Understanding Recent Climate Change. Conservation Biology, 24, (1): 10–17.

SHIPLEY B.,KEDDY P.A., MOORE D.R.J.,LEMKY K., 1989. Regeneration and establishment strategies of emergent macrophytes. Journal of Ecology, 77: 1093–1110.

SMITH S., GIRIAT I., SCHMITT A., DE LANGE, T., 1998. Tankyrase, a poly(ADP-ribose) polymerase at human telomers. Science, 282: 1484-1487.

SNELL, T.W. & HAWKINSON, C.A., 1983. Behavioral reproductive isolation among populations of the rotifer Brachionus plicatilis. Evolution, 37: 1294–1305.

SODEN B. J., WETHERALD R. T., STENCHIKOV G. L., ROBOCK A., 2002. Global cooling after the eruption ofMount Pinatubo: a test of climate feedback by water vapor. Science, 296: 727–730.

SOKOLOV E.P., 2000. An improved method for DNA isolation from mucopolysaccharideriche molluscan tissues. J. Moll. Stud., 66: 573-575.

SOLÉ CAVA, A.M., BOURY-ESNAULT, N., VACELET, J., THORPE, J.P., 1992. Biochemical genetic divergence and systematics in sponges of the genera Corticium and Oscarella (Demospongiae: Homoscleromorpha) in the Mediterranean Sea. Marine Biology, 113: 299-304.

SOLTAN D., VERLAQUE M., BOUDOURESQUE C. F., FRANCOUR P., 2001. Changes in macroalgal communities in the vicinity of a Mediterranean sewage outfall after the settings up of a treatment plant. Mar. Poll. Bull., 42(1):59-70.

STAUFFER J.R. Jr., MCKAYE K.R., 2001. The naming of cichlids. Journal of Aquariculture and Aquatic Sciences , 9: 1-16.

STENECK R.S., 1983. Escalating herbivory and resulting adaptive trends in calcareous algae. Paleobiology, 9:45–63.

STENECK R.S., DETHIER M.N., 1994. A functional group approach to the structure of algal-dominated communities. Oikos, 69:476–498.

STENECK R.S., WATLING L. (1980) FEEDING capabilities and limitation of herbivorous molluscs: a functional group approach. Marine Biology, 68, 299-319.

SUNDBY C., CHOW W.S., ANDERSON J.M., 1993. Turnover of the photosystem II D1 protein in higher plants under photoinhibitory and non□photoinhibitory irradiance. Journal of Biological Chemistry, 268: 25476–25482.

TAMURA K., DUDLEY J., NEI M., KUMAR S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-1599.

THE ROYAL SOCIETY, 2005. Ocean acidification due to increasing atmospheric carbon dioxide. Policy document 12/05. The Royal Society, London.

THE ROYAL SOCIETY, 2005. Ocean acidification due to increasing atmospheric carbon dioxide. Policy document 12/05. The Royal Society, London.

THIBAUT T., PINEDO S., TORRAS X., BALLESTEROS E., 2005. Long-term decline of the Fucales (Cystoseira spp. and Sargassum spp.) in the Albères coast (France, North-western Mediterranean). Mar. Poll. Bull., 50: 1472-1489.

THIBAUT T., PINEDO S., TORRAS X., BALLESTEROS E., 2005. Long-term decline of the Fucales (Cystoseira spp. and Sargassum spp.) in the Albères coast (France, North-western Mediterranean). Marine Pollution Bulletin, 50: 1472-1489.

THOMSEN J., GUTOWSKA M.A., SAPHÖRSTER J., HEINEMANN A., TRÜBENBACJ K., FIETZKE J., HIEBENTHAL C., EISENHAUER A., KÖRTZINGER A., WAHL M., MELZNER F., 2010. Calcifying invertebrates succeed in a naturally CO2 enriched coastal habitat but are threatened by high levels of future acidification. Biogeosciences Discuss., 7: 5119-5156. (doi:10.5194/bgd-7-5119-2010)

TRONHOLM A., SANSON M., AFONSO-CARILLO J., VERBRUGGEN H., DE CLERCK O., 2010b. Niche partitioning and the coexistence of two cryptic Dictyota (Dictyotales, Phaeophyceae). Journal of Phycology, 46: ***

TRONHOLM A., STEEN F., THYBERGEIN L., LELIAERT F., VERBRUGGEN H., RIBERA SIGUAN M.A., DE CLERCK O., 2010a. Species delimitation, taxonomy and

biogeography of Dictyota in Europe (Dictyotales, Phaeophyceae). Journal of Phycology, 46: ***

USSEGLIO-POLATERA P., BOURNAUD M., RICHOUX P., TACHET H., 2000. Biological and ecological traits of benthic freshwater macroinvertebrates: relationships and definition of groups with similar traits. Freshwater Biology, 43: 143–164.

VALLADARES F., PEARCY R.W., 1997. Interactions between water stress, sun–shade acclimation, heat tolerance and photoinhibition in the sclerophyll Heteromeles arbutifolia. Plant, Cell and Environment, 20: 25–36.

VAN DEN HOECK C., MANN D.G., JAHNS H.M., 1995. Algae. An introduction to Phycology. Cambridge University Press, 623 pp.

VAN KOOTEN, O., SNEL, J.F.H., 1990. The use of chlorophyll fluorescence nomenclature in plant stress physiology. Photosynthesis Research, 25: 147-150.

WEBB W.L., NEWTON M., STARR D., 1974. Carbon dioxide exchange of Alnus rubra: a mathematical model. Oecologia, 17: 281-291.

WINNEPENNINCKX B., BACKELJAU T., WACHTER R. De, 1993. Extraction of high molecular weight DNA from molluscs. Trends Gen., 9: 407.

WOTTEN J.T., PFISTER C.A., FORESTER J.D., 2008. Dynamic patterns and ecological impacts of declining ocean pH in a high-resolution multi-year dataset. Proc. Natl. Acad. Sci. USA, 105: 18848-18853

WRAY J.L., 1977. Calcareous Algae. Elsevier Scientific Publishing, Amsterdam, 185 pp.

YING W., ALANO C.C., GARNIER P., SWANSON R.A., 2005. NAD+ as a metabolic link between DNA damage and cell death, J. Neurosci. Res., 79: 216-223.

YÑIGUEZ A.T., MCMANUS J.W., COLLADO-VIDES L., 2010. Capturing the dynamics in benthic structures: environmental effects on morphology in the macroalgal genera Halimeda and Dictyota. Marine Ecology Progress Series, 411: 17-32.

7. APPENDIXES

APPENDIX I

LIST OF SPECIES BY THEIR MEAN PERCENTAGE IN THREE SECTORS ALONG A ROCKY SHORE PH GRADIENT IN THE SOUTHERN SIDE, RECORDED IN SCRAPINGS IN 2007 (nc= negligible cover; numbers correspond to the analysis shown in Fig. 19).

Species	S1 pH=8.1	S2 pH=7.8	S3 pH=6.7	see Fig. 19	Species	S1 pH=8.1	S2 pH=7.8	S3 pH=6.7	see Fig. 19
Jania rubens	69.22	10.09	-	18	*Spermothamnion strictum*	0.04	0.01	-	nc
Valonia utricularis	28.5	2.57	0.12	45	*Corallinaceae*	0.04	0.14	-	nc
Flabellia petiolata	20.33	66.22	-	42	*Polysiphonia denudata*	0.04	-	-	nc
Amphiroa rigida	10.28	3.56	-	2	*Ceramium codii*	0.03	0.01	-	4
Phymatolithon cfr *lenormandii*	7.79	2.94	-	26	*Crouania attenuata*	0.03	-	-	nc
Dictyota dichotoma	6.09	1.34	44.17	31	*Gelidium minusculum*	0.03	0	-	nc
Laurencia obtusa	5.69	-	-	nc	*Cladophora dalmatica*	0.03	0.02	-	39
Peyssonnelia polymorpha	4.22	1.5	-	23	*Cladophora sericea*	0.03	-	-	nc
Peyssonnelia squamaria	3.87	8.86	-	24	*Dasya baillouviana*	0.03	-	-	nc
Sphacelaria tribuloides	3.67	0.9	-	35	*Anotrichium tenue*	0.02	0.05	-	nc
Lobophora variegata	3.6	8.39	-	nc	*Ceramium diaphanum*	0.02	-	0.02	nc
Stypocaulon scoparium	3.56	1	0.14	36	*Monosporus pedicellatus*	0.02	-	-	nc
Hildenbrandia rubra	3.18	4.44	26.28	15	*Meredithia microphylla*	0.02	-	-	nc
Phyllophora crispa	3.07	-	-	25	*Halopteris filicina*	0.02	-	-	nc
Neogoniolithon brassica-florida	2.9	0.22	-	nc	*Chaetomorpha linum*	0.02	0.01	0.3	38
Lithophyllum sp.	2.69	-	-	19	*Cladophora flexuosa*	0.02	-	-	nc
Hydrolithon cruciatum	2.53	18.53	-	17	*Titanoderma pustulatum*	0.02	0.16	-	nc
Bryopsis plumosa	2.33	0.37	-	37	*Cladostephus spongiosus*	0.02	-	2.57	30
Dictyota spiralis	2.31	1.67	-	32	*Sphacelaria cirrosa*	0.02	0.18	0.09	nc
Corallina elongata	1.87	0.44	-	7	*Antithamnion cruciatum*	0.01	0.02	0.42	3
Mesophyllum sp.	1.83	4.61	-	20	*Centroceras clavulatum*	0.01	-	-	nc
Padina pavonica	1.67	0.92	-	33	*Cladophora socialis*	0.01	-	-	nc
Cystoseira amentacea var. *stricta*	1.61	-	-	nc	*Acrothamnion preissii*	-	-	0.01	nc
Griffithsia phyllamphora	1.11	1.27	-	nc	*Aglaothamnion bipinnatum*	-	0.03	-	1
Pseudochlorodesmis furcellata	1.01	1.14	-	44	*Aglaothamnion diaphanum*	-	0.03	-	nc
Polysiphonia scopulorum	0.91	0.42	0.03	28	*Antithamnion sp.*	-	0.02	-	nc
Gelidiella pannosa	0.7	0.31	-	11	*Ptilothamnion sphaericum*	-	0.01	-	nc
Herposiphonia secunda f. *tenella*	0.6	0.06	-	13	*Spermothamnion repens*	-	-	0.01	nc
Lophosiphonia cristata	0.59	0.57	-	nc	*Amphiroa criptarthrodia*	-	0.33	-	nc

Heterosiphonia crispella	0.57	0.14	-	14	*Pterocladiella capillacea*	-	-	0.5	29
Corallina officinalis	0.56	-	-	nc	*Chondracanthus acicularis*	-	-	4.96	6
Nitophyllum punctatum	0.48	0.16	-	21	*Peyssonnelia armorica*	-	-	0.17	nc
Cladophora rupestris	0.44	-	-	41	*Peyssonnelia bornetii*	-	0.33	-	nc
Herposiphonia secunda	0.42	-	-	nc	*Peyssonnelia* cfr *rubra*	-	0.02	-	nc
Halimeda tuna	0.38	0.19	-	nc	*Phyllophora sicula*	-	0.01	-	nc
Gelidium bipectinatum	0.29	0.18	-	12	*Contarinia squamariae*	-	0.03	-	nc
Ceramium flaccidum	0.28	0.1	-	5	*Herposiphonia sp.*	-	0.01	-	nc
Ceramium circinatum	0.26	-	-	nc	*Cladophora laetevirens*	-	0.01	-	nc
Cladophora prolifera	0.24	0.64	-	nc	*Cladophora pellucida*	-	0.12	0.37	40
Osmundea truncata	0.22	0.07	4.7	22	*Choreonema thuretii*	-	0.01	-	nc
Champia parvula	0.2	0.21	-	nc	*Lithophyllum incrustans*	-	1.22	-	nc
Peyssonnelia dubyi	0.2	-	-	nc	*Phymatolithon lenormandii*	-	0.36	-	nc
Pneophyllum fragile	0.18	0.06	-	27	*Dasya hutchinsiae*	-	0.01	-	nc
Falkenbergia sp.	0.16	0.04	-	10	*Polysiphonia fibrata*	-	0.02	-	nc
Parvocaulis parvulum	0.12	0.06	-	43	*Feldmannia irregularis*	-	0.01	-	nc
Dasya rigidula	0.12	-	-	9	*Dictyopteris polypodioides*	-	0.01	-	nc
Hydrolithon boreale	0.11	0.04	-	16	*Dictyota dichotoma var. intricata*	-	-	0.26	nc
Chondrophycus papillosus	0.07	-	-	nc	*Sargassum vulgare*	-	-	22.17	34
Hydrolithon farinosum	0.06	0.03	-	nc	*Sphacelaria rigidula*	-	0.01	-	nc
Titanoderma sp.	0.06	0.11	-	nc	*Sphacelaria sp.*	-	0.01	-	nc

APPENDIX II

LIST OF SPECIES AND THEIR MEAN PERCENTAGE IN SECTORS OF BOTH SOUTHERN, NORTHERN SIDE AND THE SECOND CONTROL C3 RECORDED IN SCRAPINGS IN 2008.

	S1	S2	S3	N1	N2	N3	C3
Jania rubens	19.375	6.25	0	0	0.05	0	8.5
Lobophora variegata	15.625	0.6	0	0.25	0.05	0.025	0.15
Amphiroa rigida	9.2	10.25	0	0.05	0.375	0	0
Phymatolithon lenormandii	8.375	5.375	0	2.25	3.375	0	3
Valonia utricularis	7.75	2.375	4	0.225	0.6	3.575	3.1
Flabellia petiolata	7.75	11	0	42.25	33.5	11.5	2
Hildenbrandia crouaniorum	7.625	28.25	63.2	1.025	5.5	31.625	2
Phymatolithon cfr. lenormandii	4	0	0	0	0	0	0
Bryopsis plumosa	3.625	0.775	0	4	0.95	0.975	3
Dictyota fasciola	2.875	2	0	0	2.5	7	0
Dictyota dichotoma	2.375	0	8.8	1.075	0.575	1.375	3.35
Padina pavonica	2.375	0.625	0	0	0	0	0.35
Sphacelaria tribuloides	2.15	1.5	0	0	0	0.175	0.25
Hydrolithon farinosum	2.125	0	0	0.075	0.425	0	0.1
Stypocaulon scoparium	1.75	1.9	0.75	0	0.875	13.4	0.75
Griffithsia phyllamphora	1.55	0	0	0	0.125	0	0
Hydrolithon cruciatum	1.5	0.1	0	1.925	0.625	0	0.1
Peyssonnelia squamaria	1.425	2.95	0	5.075	8.125	2.75	11.5
Dasya corymbifera	1.4	1.1	0	0	0.025	0.225	0
Lithophyllum incrustans	1.375	0.125	0	1.45	2	0	7.25
Dictyota dichotoma var. intricata	1.25	8.125	89	0	1.75	0.5	0
Caulerpa prolifera	1.25	9	5	0	0	0	4.25
Lophosiphonia cristata	1.25	1.7	0	0	0	0	0
Pseudochlorodesmis furcellata	1.25	0.625	0	1.55	0.125	0	0.65
Polysiphonia scopulorum	1.15	0.075	0	0.125	0	0	0.1
Peyssonnelia armorica	1	0.075	0	0	0.05	0	0
Titanoderma corallinae	1	0	0	0.275	0.35	0	2.75
Griffithsia opuntioides	0.875	0.325	0.075	0.125	0	0	0
Herposiphonia tenella	0.675	0.1	0	0	0	0.25	0.05
Cladophora coelothrix	0.65	0.625	0	0.25	0.1	0	3.75
Sphacelaria cirrosa	0.625	0.875	0	0	0	0	2.5
Gelidiella pannosa	0.6	0.9	0.175	0.35	0.075	0.05	0
Phyllophora crispa	0.575	0.575	35.9	0.4	1.4	4	0.6
Peyssonnelia rosa-marina	0.575	1.25	0	1.25	0.85	0	0
Laurencia tenera	0.525	0.075	0	0	0	0	0
Hydrolithon sp.	0.5	0.05	0	0.125	0	0	0
Bryopsis cupressoides	0.45	0	0	0	0	0	0
Champia parvula	0.425	0.275	0	0	0.025	0	0.25
Titanoderma pustulatum	0.425	0.05	0	1.35	0	0	3.9
Ceramium flaccidum	0.375	0.2	0	0	0	0.025	0
Zonaria tournefortii	0.375	0	0	0	0	0	0.05
Ceramium codii	0.325	0.075	0	0.05	0	0	0.1
Nitophyllum punctatum	0.275	0.3	0	0.05	0.025	0.025	0.55
Falkenbergia sp.	0.275	0.6	0	0.275	0.975	1.6	0.15
Cyanophyta	0.25	0.375	0	0.025	0.05	0.3	0
Heterosiphonia crispella	0.225	0.05	0	0.275	0.025	0.175	0.1
Parvocaulis parvulum	0.2	0.025	0	0	0	0	0
Pterocladiella capillacea	0.15	0.175	1	0.9	0	1.25	0.25

	S1	S2	S3	N1	N2	N3	C3
Chaetomorpha linum	0.125	0.125	1.075	0.05	0.025	0.3	0.05
Cladophora laetevirens	0.125	0.05	0	0	0	0.05	0
Titanoderma cfr. pustulatum	0.125	0.075	0	0	0.325	0	0
Dasya rigidula	0.125	0	0	0	0	0	0
Peyssonnelia bornetii	0.125	0	0	2.125	0	0.125	24.75
Osmundea truncata	0.1	0.1	2	0	0	0.075	0
Lomentaria clavaeformis	0.1	0	0	0.1	0	0	0
Cladophora pellucida	0.1	0	0	0	0	0	0
Gelidium spinosum	0.075	0.675	0	0	0	0	0
Sargassum vulgare	0.05	1.25	44.375	0	0	0	0
Peyssonnelia polymorpha	0.05	0.45	0	0.375	0	0	21.9
Pneophyllum fragile	0.05	0.05	0	0	0	0	0.1
Antithamnion cruciatum	0.025	0.875	1.725	0	0	15.25	0.05
Ceramium diaphanum	0.025	0.05	0.025	0	0	0.075	0.05
Ceramium circinatum	0.025	0.025	0	0	0	0	0
Aglaothamnion diaphanum	0.025	0	0	0	0	0	0.1
Spermothamnion strictum	0.025	0	0	0	0	0	0
Monosporus pedicellatus	0.025	0	0	0	0	0	0
Chondrophycus papillosus	0.025	0	0	0	0	0	0
Dasya hutchinsiae	0.025	0	0	0	0	0.025	3.5
Spermothamnion repens	0.025	0	0	0	0	16.5	0
Pleonosporium borreri	0.025	0	0	0	0.025	0	0
Nemacystus flexuosus var. gyraudi	0.025	0	0	0	0	0	0
Cladostephus spongiosum	0	0.1	3.575	0	0	0.775	0
Cladophora prolifera	0	2	62.025	0.15	0	0.125	3.5
Peyssonnelia dubyi	0	0	0.1	0.825	1.1	0	0
Gelidium pusillum	0	0	0.15	0	0	0	0
Chondracanthus acicularis	0	0	1.6	0	0	0.175	0
Phyllophora sicula	0	0	0.125	0	0	0	0
Sphacelaria sp.	0	0.275	0.025	0.025	0	0	0.15
Gymnogongrus griffithsiae	0	0.05	0.05	0	0	0.125	0
Polysiphonia elongata	0	0	0.025	0	0	0	0
Herposiphonia secunda	0	1.475	0	0	0	0	0.1
Corallina officinalis	0	0.5	0	0	0	0	0
Dasya baillouviana	0	0.025	0	0	0	0	0.5
Dasya ocellata	0	0.05	0	0	0	0	0
Corallina elongata	0	3.25	0	0.775	51.25	1.75	0.2
Phyllophora pseudoceranoides	0	2.125	0	0	0	0	0
Dictyopteris polypodyoides	0	0.05	0	0.05	0.025	0	0
Halimeda tuna	0	0.025	0	0	0	0	2.75
Halydictyon mirabile	0	0.025	0	0	0	0	0
Callithamnion corymbosum	0	0.025	0	0	0	0	0
Spongites fruticulosus	0	0.875	0	0	0	0	0
Codium bursa	0	0	0	0	0	0	0
Cutleria multifida	0	0	0	0.125	1.75	29.375	0
Acrochetium daviesii	0	0	0	0	0	0.025	0
Titanoderma sp.	0	0	0	0	0	0.025	0
Halopteris filicina	0	0	0	0.3	0.225	3.5	0.05
Aglaothamnion cordatum	0	0	0	0	0	0.025	0
Aglaothamnion sp.	0	0	0	0	0	0.025	0
Titanoderma cistoseirae	0	0	0	0	0.025	0	0
Ralfsia verrucosa	0	0	0	0	2	0	0
Aglaothamnion tenuissimum v. tenuissimum	0	0	0	0.025	0.15	0	0
Amphiroa beauvoisii	0	0	0	0.025	0.375	0	2

Rhodophyllis divaricata	0	0	0	0	0.025	0	0
	S1	**S2**	**S3**	**N1**	**N2**	**N3**	**C3**
Pterocladiella melanoidea	0	0	0	0	0.15	0	0
Acrosorium venulosum	0	0	0	0.025	0.25	0	0
Griffithsia sp.	0	0	0	0.125	0	0	1.15
Hydrolithon farinosum v. calychodyctium	0	0	0	0.025	0	0	0
Contarinia squamariae	0	0	0	0.025	0	0	0
Aglaothamnion bipinnatum	0	0	0	0.275	0	0	0.2
Challithamniella tingitana	0	0	0	0.125	0	0	0
Asparagopsis sp.	0	0	0	0	0	0	0.05
Gelidium bipectinatum	0	0	0	0	0	0	1
Colpomenia sinuosa	0	0	0	0	0	0	0.05
Sphacelaria rigidula	0	0	0	0	0	0	0.05

APPENDIX III

LIST OF SPECIES AND THEIR MEAN PERCENTAGE IN SECTORS OF BOTH SOUTHERN, NORTHERN SIDE AND THE SECOND CONTROL C3 RECORDED IN MAY, JUNE AND JULY 2008 DURING COLONIZATION EXPERIMENT.

	after 2months							after 3 months							after 4 months						
	C3	**S1**	**S2**	**S3**	**N1**	**N2**	**N3**	**C3**	**S1**	**S2**	**S3**	**N1**	**N2**	**N3**	**C3**	**S1**	**S2**	**S3**	**N1**	**N2**	**N3**
Antithamnion cruciatum																					+
Blastophysa rhizophus																	+				
Callythamnion corymbosum																			+		
Ceramium diaphanum																					
Ceramium flaccidum																			+	+	
Chaetomorpha linum				+	+			+		+	+	+	+	+	+		+	+	+	+	+
Champia parvula																				+	
Chondracanthus acicularis																					
Cianobatteri	+						+	+			+			+	+	+	+	+	+	+	+
Cladophora coelothrix															+						
Cladophora socialis		+	+		+			+						+	+	+	+	+	+	+	+
Cladosiphon sp.						+															
Cladostephus spongiosus											+			+				+			+
Corallina sp.					+			+		+		+	+			+	+		+	+	
Cruoria cruoriaeformis																	+				
Cutleria multifida gametophyte	+	+			+	+															
Cutleria multifida sporophyte					+	+	+					+	+	+							+
Dasya rigidula																			+	+	
Diatomee	+	+			+		+	+	+	+	+			+	+	+	+	+	+	+	+
Dictyota dichotoma									+			+	+	+		+	+		+	+	+
Dictyota fasciola										+											
Dictyota sp.																			+	+	+
Dinoflagellate									+	+	+			+	+	+	+	+	+	+	+
Enteromorpha sp.							+							+							+
Falkenbergia sp.																			+	+	
Feldmannia sp.	+	+		+	+	+	+	+			+	+	+	+		+		+	+	+	+

	after 2months							after 3 months							after 4 months						
	C3	**S1**	**S2**	**S3**	**N1**	**N2**	**N3**	**C3**	**S1**		**C3**	**S1**	**S2**	**S3**	**N1**	**N2**	**N3**	**C3**	**S1**		**C3**

Griffithsia sp.																+							
Gyraudia sphacelarioides	+				+	+	+	+				+	+	+	+				+				+
Hincksia sp.													+						+				
Hydrolithon farinosum	+	+	+		+			+	+	+			+	+		+	+	+		+	+		
Lobophora variegata																				+	+	+	
Lophosiphonia cristata																	+			+			
Microspongium tenuissimum								+	+	+	+	+	+	+									
Myrionema sp.	+	+	+	+	+	+	+	+	+		+		+				+	+		+	+		
Nemacystus flexuosus													+										
Nemacystus sp.				+										+									
Osmundea truncata													+	+			+		+	+			
Padina pavonica																+							
Palmophyllum?			+		+	+		+	+	+		+	+				+	+		+	+		
Peyssonnelia dubyi																				+			
Pneophyllum fragile								+															
Polysiphonia scopulorum																	+						
Pseudolithoderma adriaticum								+								+	+	+	+				
Ralfsia verrucosa																			+	+			+
Spatoglossum solieri																							
Sphacelaria cirrosa			+						+	+		+	+			+	+	+			+		
Sphacelaria rigidula	+																						+
Sphacelaria sp.	+					+		+	+	+	+	+			+	+	+	+	+	+	+		+
Sphacelaria tribuloides								+							+	+	+						+
Stilophora sp.																			+	+			
Titanoderma mediterraneum	+	+	+		+			+	+	+		+	+			+	+	+		+	+		

APPENDIX IV

LIST OF SPECIES RECORDED IN 2007 AND 2008 AT CASTELLO WITH CATEGORIES AND ALGAL GROUPS.

				2007			2008						
	Species	CG	AG	S1 pH=8.1	S2 pH=7.8	S3 pH=6.7	S1 pH=8.1	S2 pH=7.8	S3 pH=6.7	N1 pH=8.1	N2 pH=7.8	N3 pH=7.0	C3 pH=8.1
1	*Acrothamnion preissii*	T	1			*							
2	*Acrochetium daviesii*	T	1									*	
3	*Acrosorium venulosum*	T	1							*	*		
4	*Aglaothamnion bipinnatum*	T	1		*					*			*
5	*Aglaothamnion cordatum*	T	1									*	
6	*Aglaothamnion diaphanum*	T	1		*		*						*
7	*Aglaothamnion sp.*	T	1									*	
8	*Aglaothamnion tenuissimum var. tenuissimum*	T	1							*	*		
9	*Amphiroa beauvoisii*	E	12							*	*		*
10	*Amphiroa criptarthrodia*	E	12		*								
11	*Amphiroa rigida*	E	12	*	*		*	*		*	*		
12	*Anotrichium tenue*	T	1	*	*								
13	*Antithamnion cruciatum*	T	1	*	*	*	*	*	*			*	*
14	*Antithamnion sp.*	T	1		*								
15	*Asparagopsis sp.*	E	2										*
16	*Bryopsis cupressoides*	T	1				*						
17	*Bryopsis plumosa*	T	1	*	*		*	*		*	*	*	*
18	*Callithamnion corymbosum*	T	1					*					
19	*Caulerpa prolifera*	E	4				*	*	*				*
20	*Centroceras clavulatum*	T	1	*									
21	*Ceramium circinatum*	T	1	*			*	*					
22	*Ceramium codii*	T	1	*	*		*	*		*			*
23	*Ceramium diaphanum*	T	1	*		*	*	*	*			*	*
24	*Ceramium flaccidum*	T	1	*	*		*	*				*	
25	*Chaetomorpha linum*	T	1	*	*	*	*	*	*	*	*	*	*
26	*Challithamniella tingitana*	T	1							*			
27	*Champia parvula*	T	4	*	*		*	*			*		*
28	*Chondracanthus acicularis*	E	7			*			*			*	

29	*Chondrophycus papillosus*	E	7	*				*							
30	*Choreonema thuretii*	C	10		*										

continued

				2007			2008						
	Species	**CG**	**AG**	**S1 pH=8.1**	**S2 pH=7.8**	**S3 pH=6.7**	**S1 pH=8.1**	**S2 pH=7.8**	**S3 pH=6.7**	**N1 pH=8.1**	**N2 pH=7.8**	**N3 pH=7.0**	**C3 pH=8.1**
31	*Cladophora coelothrix*	T	1				*	*		*	*		*
32	*Cladophora dalmatica*	T	1	*	*								
33	*Cladophora flexuosa*	T	1	*									
34	*Cladophora laetevirens*	T	1		*		*	*				*	
35	*Cladophora pellucida*	E	1		*	*	*						
36	*Cladophora prolifera*	E	1	*	*			*	*	*		*	*
37	*Cladophora rupestris*	E	1	*									
38	*Cladophora sericea*	T	1	*									
39	*Cladophora socialis*	T	1	*									
40	*Cladostephus spongiosus*	E	7	*		*		*	*			*	
42	*Colpomenia sinuosa*	C	6										*
43	*Contarinia squamariae*	C	6		*					*			
44	*Corallina elongata*	E	12	*	*			*		*	*	*	*
45	*Corallina officinalis*	E	12	*				*					
46	*Corallinaceae ind.*	C	11	*	*								
47	*Crouania attenuata*	C	1	*									
48	*Cutleria multifida*	C	5							*	*	*	
49	*Cyanophyta*	T	1				*	*		*	*	*	
50	*Cystoseira amentacea var. stricta*	E	8	*									
51	*Dasya baillouviana*	T	2	*				*					*
52	*Dasya corymbifera*	T	2				*	*			*	*	
53	*Dasya hutchinsiae*	T	2		*		*					*	*
54	*Dasya ocellata*	T	2					*					
55	*Dasya rigidula*	T	2	*			*						
56	*Dictyopteris polypodioides*	E	6		*			*		*	*		
57	*Dictyota dichotoma*	E	6	*	*	*	*		*	*	*	*	*
58	*Dictyota dichotoma var. intricata*	E	6			*	*	*	*		*	*	
59	*Dictyota fasciola*	E	6				*	*			*	*	
60	*Dictyota spiralis*	E	6	*	*								
61	*Falkenbergia sp.*	T	1	*	*		*	*		*	*	*	*

62	*Feldmannia irregularis*	T	1			*								
	continued			**2007**			**2008**							
	Species	**CG**	**AG**	**S1 pH=8.1**	**S2 pH=7.8**	**S3 pH=6.7**	**S1 pH=8.1**	**S2 pH=7.8**	**S3 pH=6.7**	**N1 pH=8.1**	**N2 pH=7.8**	**N3 pH=7.0**	**C3 pH=8.1**
63	*Flabellia petiolata*	E	9	*	*		*	*		*	*	*	*
64	*Gelidiella pannosa*	T	7	*	*		*	*	*	*	*	*	
65	*Gelidium bipectinatum*	T	7	*	*								*
66	*Gelidium minusculum*	T	7	*									
67	*Gelidium pusillum*	T	7						*				
68	*Gelidium spinosum*	T	7				*	*					
69	*Griffithsia opuntioides*	T	1				*	*	*	*			
70	*Griffithsia phyllamphora*	T	1	*	*		*				*		
71	*Griffithsia sp.*	T	1							*			*
72	*Gymnogongrus griffithsiae*	T	7					*	*			*	
73	*Halimeda tuna*	E	9	*	*			*					*
74	*Halopteris filicina*	E	7	*						*	*	*	*
75	*Halydictyon mirabile*	T	1					*					
76	*Herposiphonia secunda*	T	2	*				*					*
77	*Herposiphonia secunda f. tenella*	T	2	*	*		*	*				*	*
78	*Herposiphonia sp.*	T	2		*								
79	*Heterosiphonia crispella*	T	2	*	*		*	*		*	*	*	*
80	*Hildenbrandia crouaniorum*	C	5				*	*	*	*	*	*	*
81	*Hildenbrandia rubra*	C	5	*	*	*							
82	*Hydrolithon boreale*	C	10	*	*								
83	*Hydrolithon cruciatum*	C	10	*	*		*	*		*	*		*
84	*Hydrolithon farinosum*	C	10	*	*		*			*	*		*
85	*Hydrolithon farinosum var. calychodyctium*	C	10							*			
86	*Hydrolithon sp.*	C	10				*	*		*			
87	*Jania rubens*	E	12	*	*		*	*			*		*
88	*Laurencia obtusa*	T	7	*									
89	*Laurencia tenera*	T	7				*	*					
90	*Lithophyllum incrustans*	C	11		*		*	*		*	*		*
91	*Lithophyllum sp.*	C	11	*									
92	*Lobophora variegata*	C	6	*	*		*	*		*	*	*	*
93	*Lomentaria clavaeformis*	T	4				*			*			

94	*Lophosiphonia cristata*	T	2	*	*		*	*						

continued

				2007			2008						
	Species	**CG**	**AG**	**S1 pH=8.1**	**S2 pH=7.8**	**S3 pH=6.7**	**S1 pH=8.1**	**S2 pH=7.8**	**S3 pH=6.7**	**N1 pH=8.1**	**N2 pH=7.8**	**N3 pH=7.0**	**C3 pH=8.1**
95	*Meredithia microphylla*	C	6	*									
96	*Mesophyllum sp.*	C	11	*	*								
97	*Monosporus pedicellatus*	T	1	*			*						
98	*Nemacystus flexuosus var. gyraudi*	T	1				*						
99	*Neogoniolithon brassica-florida*	C	11	*	*								
100	*Nitophyllum punctatum*	T	3	*	*		*	*		*	*	*	*
101	*Osmundea truncata*	T	7	*	*	*	*	*	*			*	
102	*Padina pavonica*	E	9	*	*		*	*					*
103	*Parvocaulis parvulum*	T	4	*	*		*	*					
104	*Peyssonnelia armorica*	C	5			*	*	*			*		
105	*Peyssonnelia bornetii*	C	9		*		*			*		*	*
106	*Peyssonnelia dubyi*	C	9	*					*	*	*		
107	*Peyssonnelia polymorpha*	C	11	*	*		*	*		*			*
108	*Peyssonnelia rosa-marina*	C	11				*	*		*	*		
109	*Peyssonnelia cfr rubra*	C	9		*								
110	*Peyssonnelia squamaria*	C	9	*	*		*	*		*	*	*	*
111	*Phyllophora crispa*	E	6	*			*	*	*	*	*	*	*
112	*Phyllophora pseudoceranoides*	E	6					*					
113	*Phyllophora sicula*	E	6		*				*				
114	*Phymatolithon cfr lenormandii*	C	11	*	*		*						
115	*Phymatolithon lenormandii*	C	11		*		*	*		*	*		*
116	*Pleonosporium borreri*	C	1				*				*		
117	*Pneophyllum fragile*	C	10	*	*		*	*					*
118	*Polysiphonia denudata*	T	2	*									
119	*Polysiphonia elongata*	T	2						*				
120	*Polysiphonia fibrata*	T	2		*								
121	*Polysiphonia scopulorum*	T	2	*	*	*	*	*		*			*
122	*Pseudochlorodesmis furcellata*	T	1	*	*		*	*		*	*		*
123	*Pterocladiella capillacea*	T	7			*	*	*	*	*		*	*
124	*Pterocladiella melanoidea*	T	7								*		
125	*Ptilothamnion sphaericum*	T	1		*								

continued

				2007			2008						
				S1	S2	S3	S1	S2	S3	N1	N2	N3	C3
	Species	**CG**	**AG**	**pH=8.1**	**pH=7.8**	**pH=6.7**	**pH=8.1**	**pH=7.8**	**pH=6.7**	**pH=8.1**	**pH=7.8**	**pH=7.0**	**pH=8.1**
126	*Ralfsia verrucosa*	C	5								*		
127	*Rhodophyllis divaricata*	T	6								*		
128	*Sargassum vulgare*	E	8			*	*	*	*				
129	*Spermothamnion repens*	T	1			*	*					*	
130	*Spermothamnion strictum*	T	1	*	*		*						
131	*Sphacelaria cirrosa*	T	2	*	*	*	*	*					*
132	*Sphacelaria rigidula*	T	2		*								*
133	*Sphacelaria sp.*	T	2		*			*	*	*			*
134	*Sphacelaria tribuloides*	T	2	*	*		*	*				*	*
135	*Spongites fruticulosus*	C	11					*					
136	*Stypocaulon scoparium*	E	7	*	*	*	*	*	*		*	*	*
137	*Titanoderma pustulatum*	C	11	*	*								
138	*Titanoderma cfr. pustulatum*	C	11				*	*			*		
139	*Titanoderma cistoseirae*	C	11								*		

continued

				2007			2008						
				S1	S2	S3	S1	S2	S3	N1	N2	N3	C3
	Species	**CG**	**AG**	**pH=8.1**	**pH=7.8**	**pH=6.7**	**pH=8.1**	**pH=7.8**	**pH=6.7**	**pH=8.1**	**pH=7.8**	**pH=7.0**	**pH=8.1**
142	*Titanoderma sp.*	C	11	*	*							*	
143	*Valonia utricularis*	T	4	*	*	*	*	*	*	*	*	*	*
144	*Zonaria tournefortii*	E	6				*						*

APPENDIX V

SIMPER ANALYSIS BETWEEN SECTORS SECTORS AT THE SAME TIME ON COLONIZATION DATA

Group C3-may

Average similarity: 60.72

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Myrionema sp.	63.89	38.43	4.46	63.29	63.29
Hydrolithon farinosum	0.94	12.61	4.36	20.78	84.07
Feldmannia sp.	14.78	6.90	0.58	11.36	95.42

Group S1-may

Average similarity: 70.03

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Myrionema sp.	50.56	50.75	13.49	72.48	72.48
Titanoderma mediterraneum	0.39	13.51	10.33	19.29	91.76

Group S2-may

Average similarity: 76.99

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Myrionema sp.	40.56	45.47	15.82	59.06	59.06
Sphacelaria cirrosa	0.61	13.71	3.50	17.81	76.87
Titanoderma mediterraneum	0.56	13.51	5.74	17.54	94.42

Group S3-may

Average similarity: 77.06

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Feldmannia sp.	61.11	48.49	3.85	62.93	62.93
Myrionema sp.	10.33	28.56	7.87	37.07	100.00

Group N1-may

Average similarity: 57.41

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Myrionema sp.	20.78	18.37	4.65	32.00	32.00
Cutleria multifida sporophyte	1.39	9.54	4.86	16.62	48.61
Palmophyllum?	25.56	7.83	0.58	13.65	62.26
Titanoderma mediterraneum	0.17	6.50	7.69	11.32	73.58
Hydrolithon farinosum	0.44	6.50	7.69	11.32	84.89

Group N2-may

Average similarity: 88.12

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Feldmannia sp.	70.56	23.83	8.64	27.05	27.05
Myrionema sp.	38.89	20.42	19.52	23.18	50.22
Palmophyllum?	13.33	12.44	3.07	14.11	64.33
Gyraudia sphacelarioides	2.11	9.29	10.73	10.54	74.87

Group N3-may

Average similarity: 61.22

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Feldmannia sp.	75.56	33.53	6.80	54.77	54.77
Cianobatteri	26.67	16.99	4.90	27.76	82.52
Myrionema sp.	4.11	6.31	0.58	10.30	92.82

Group C3-june

Average similarity: 64.01

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Microspongium tenuissimum	28.11	15.13	4.01	23.64	23.64
Myrionema sp.	10.11	12.08	10.45	18.88	42.52
Titanoderma mediterraneum	8.00	10.71	5.23	16.74	59.25
Hydrolithon farinosum	3.50	9.04	5.07	14.13	73.38
Diatomee	1.28	7.41	8.81	11.57	84.96

Group S1-june

Average similarity: 64.68

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Palmophyllum?	10.22	18.02	6.22	27.86	27.86
Hydrolithon farinosum	4.00	13.84	3.20	21.41	49.27
Titanoderma mediterraneum	2.22	12.97	6.87	20.05	69.32
Sphacelaria sp.	0.50	9.43	5.32	14.58	83.90
Microspongium tenuissimum	10.00	4.78	0.58	7.39	91.30

Group S2-june

Average similarity: 64.84

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Sphacelaria cirrosa	5.11	17.62	7.36	27.17	27.17
Microspongium tenuissimum	11.83	16.85	6.83	25.99	53.16
Hydrolithon farinosum	0.44	8.28	6.97	12.77	65.93
Corallina sp.	0.17	7.80	7.36	12.03	77.96

Group S3-june

Average similarity: 86.70

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Feldmannia sp.	61.67	21.51	14.03	24.81	24.81
Chaetomorpha linum	17.28	15.57	18.14	17.96	42.77
Microspongium tenuissimum	8.06	12.47	11.10	14.38	57.15
Myrionema sp.	7.78	11.28	15.36	13.01	70.16
Sphacelaria sp.	0.83	6.34	27.38	7.31	77.47

Group N1-june

Average similarity: 76.82

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Palmophyllum?	55.00	19.20	7.12	24.99	24.99
Cutleria multifida sporophyte	4.11	10.79	7.42	14.05	39.04
Microspongium tenuissimum	6.83	9.96	11.17	12.96	52.00
Hydrolithon farinosum	2.67	9.43	7.86	12.27	64.27
Titanoderma mediterraneum	1.72	8.79	8.11	11.44	75.71

Group N2-june

Average similarity: 71.72

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Palmophyllum?	29.11	16.66	9.18	23.22	23.22
Microspongium tenuissimum	9.56	11.83	2429.71	16.49	39.72
Gyraudia sphacelarioides	5.33	10.10	7.44	14.08	53.79
Cutleria multifida sporophyte	6.06	8.96	2.21	12.50	66.29
Chaetomorpha linum	1.50	7.75	15.70	10.81	77.10

Group N3-june

Average similarity: 81.39

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Feldmannia sp.	82.78	19.65	22.09	24.14	24.14
Microspongium tenuissimum	6.22	9.59	10.80	11.78	35.92
Gyraudia sphacelarioides	5.89	9.54	11.59	11.73	47.64
Chaetomorpha linum	5.39	7.44	3.73	9.14	56.78
Cianobatteri	1.78	7.41	23.64	9.11	65.89

Group C3-july

Average similarity: 70.69

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Titanoderma mediterraneum	66.67	19.13	7.81	27.07	27.07
Pseudolithoderma adriaticum	32.22	15.09	3.25	21.35	48.41
Hydrolithon farinosum	26.67	14.95	6.95	21.14	69.56
Dinoflagellate	21.94	9.46	9.91	13.38	82.94
Diatomee	3.11	8.45	7.86	11.95	94.89

Group S1-july

Average similarity: 62.31

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Titanoderma mediterraneum	32.78	11.31	6.64	18.15	18.15
Hydrolithon farinosum	11.56	10.53	20.65	16.91	35.05
Cladophora socialis	15.44	9.62	12.72	15.45	50.50
Sphacelaria cirrosa	3.89	7.09	21.10	11.38	61.88
Pseudolithoderma adriaticum	4.56	6.21	5.00	9.96	71.85

Group S2-july

Average similarity: 55.43

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Titanoderma mediterraneum	20.78	11.79	5.14	21.26	21.26
Hydrolithon farinosum	6.00	7.89	5.33	14.24	35.50
Cianobatteri	1.50	6.45	2.80	11.64	47.14
Chaetomorpha linum	3.94	5.70	2.56	10.29	57.43
Palmophyllum?	26.11	4.64	0.58	8.37	65.80

Group S3-july

Average similarity: 57.03

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Chaetomorpha linum	23.33	10.08	6.56	17.68	17.68
Diatomee	4.83	7.63	4.68	13.37	31.05
Dinoflagellate	4.50	7.50	3.74	13.15	44.20
Feldmannia sp.	48.33	7.33	0.58	12.85	57.05
Cianobatteri	2.33	6.58	8.74	11.54	68.59

Group N1-july

Average similarity: 67.77

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Palmophyllum?	83.89	15.92	19.14	23.49	23.49
Titanoderma mediterraneum	6.89	7.04	4.71	10.39	33.89
Chaetomorpha linum	5.67	6.54	4.51	9.65	43.53
Diatomee	3.83	6.18	10.49	9.11	52.65
Sphacelaria sp.	2.78	5.46	4.71	8.06	60.71

Group N2-july

Average similarity: 70.67

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%

Palmophyllum?	45.56	13.53	9.96	19.15	19.15
Dictyota dichotoma	28.89	11.00	9.41	15.56	34.71
Sphacelaria cirrosa	5.67	7.96	23.37	11.26	45.97
Lobophora variegata	6.44	6.81	3.96	9.64	55.61
Chaetomorpha linum	4.00	6.07	3.91	8.60	64.21

Group N3-july

Average similarity: 63.27

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Feldmannia sp.	60.56	12.35	3.37	19.52	19.52
Chaetomorpha linum	34.44	11.78	7.88	18.61	38.14
Dinoflagellate	4.33	6.57	34.08	10.38	48.52
Diatomee	2.61	6.47	18.74	10.23	58.75
Cianobatteri	1.22	5.52	34.08	8.73	67.48

MAY

Groups C3-may & S1-may

Average dissimilarity = 33.41

	Group C3-may	Group S1-may				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Feldmannia sp.	14.78	0.11	10.04	1.46	30.05	30.05
Cutleria multifida gametophyte	0.44	0.11	3.39	0.93	10.15	40.20
Hydrolithon farinosum	0.94	0.44	3.38	0.76	10.12	50.33
Titanoderma mediterraneum	0.33	0.39	3.37	0.87	10.10	60.43
Diatomee	0.44	0.11	3.25	0.93	9.71	70.14

Groups S1-may & S2-may

Average dissimilarity = 29.87

	Group S1-may	Group S2-may				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Sphacelaria cirrosa	0.00	0.61	8.28	4.02	27.71	27.71
Hydrolithon farinosum	0.44	0.22	4.19	1.17	14.03	41.74
Cladophora socialis	0.11	0.22	3.54	0.89	11.86	53.60
Palmophyllum?	0.00	0.56	3.38	0.67	11.32	64.92
Cutleria multifida gametophyte	0.11	0.00	2.53	0.67	8.47	73.39

Groups C3-may & S3-may

Average dissimilarity = 51.52

	Group C3-may	Group S3-may				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%

Feldmannia sp.	14.78	61.11	12.94	0.96	25.11	25.11
Myrionema sp.	63.89	10.33	9.12	2.19	17.70	42.81
Hydrolithon farinosum	0.94	0.00	8.16	2.69	15.85	58.66
Chaetomorpha linum	0.00	5.56	4.71	0.65	9.14	67.79
Titanoderma mediterraneum	0.33	0.00	3.91	1.25	7.59	75.39

Groups S1-may & S3-may

Average dissimilarity = 61.60

	Group S1-may	Group S3-may				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Feldmannia sp.	0.11	61.11	24.53	3.95	39.82	39.82
Myrionema sp.	50.56	10.33	9.08	2.35	14.74	54.55
Titanoderma mediterraneum	0.39	0.00	7.38	6.12	11.98	66.53
Hydrolithon farinosum	0.44	0.00	5.74	1.30	9.31	75.85
Chaetomorpha linum	0.00	5.56	5.43	0.67	8.81	84.66

Groups S2-may & S3-may

Average dissimilarity = 67.72

	Group S2-may	Group S3-may				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Feldmannia sp.	0.00	61.11	26.18	4.86	38.66	38.66
Sphacelaria cirrosa	0.61	0.00	8.02	3.68	11.84	50.50
Titanoderma mediterraneum	0.56	0.00	7.75	5.18	11.45	61.95
Myrionema sp.	40.56	10.33	7.44	2.24	10.99	72.94
Chaetomorpha linum	0.00	5.56	5.28	0.67	7.80	80.74

Groups S1-may & N1-may

Average dissimilarity = 50.35

	Group S1-may	Group N1-may				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Palmophyllum?	0.00	25.56	11.47	1.24	22.78	22.78
Feldmannia sp.	0.11	25.67	7.68	0.93	15.25	38.04
Cutleria multifida sporophyte	0.00	1.39	7.19	4.51	14.28	52.32
Myrionema sp.	50.56	20.78	3.96	1.55	7.86	60.18
Gyraudia sphacelarioides	0.00	0.22	3.14	1.32	6.24	66.42

Groups S2-may & N1-may (similar pH)

Average dissimilarity = 53.43

	Group S2-may	Group N1-may				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Palmophyllum?	0.56	25.56	10.38	1.32	19.43	19.43

Feldmannia sp.	0.00	25.67	7.88	0.89	14.74	34.17
Cutleria multifida sporophyte	0.00	1.39	7.03	4.59	13.15	47.32
Sphacelaria cirrosa	0.61	0.00	5.69	3.67	10.64	57.97

Groups S2-may & N2-may

Average dissimilarity = 67.00

	Group S2-may	Group N2-may				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Feldmannia sp.	0.00	70.56	17.23	7.96	25.72	25.72
Palmophyllum?	0.56	13.33	8.33	1.87	12.42	38.15
Gyraudia sphacelarioides	0.00	2.11	7.01	9.80	10.47	48.61
Cutleria multifida sporophyte	0.00	1.22	6.23	6.46	9.29	57.91
Cladosiphon sp.	0.00	1.11	5.49	4.52	8.20	66.10

Groups N1-may & N2-may

Average dissimilarity = 41.83

	Group N1-may	Group N2-may				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Feldmannia sp.	25.67	70.56	8.33	1.29	19.92	19.92
Palmophyllum?	25.56	13.33	5.08	1.47	12.14	32.06
Cladosiphon sp.	0.00	1.11	4.36	3.96	10.43	42.49
Hydrolithon farinosum	0.44	0.00	3.52	6.50	8.43	50.92
Gyraudia sphacelarioides	0.22	2.11	3.31	1.45	7.92	58.84

Groups S2-may & N3-may (similar pH)

Average dissimilarity = 84.29

	Group S2-may	Group N3-may				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Feldmannia sp.	0.00	75.56	21.98	7.23	26.08	26.08
Cianobatteri	0.00	26.67	14.74	2.22	17.48	43.56
Myrionema sp.	40.56	4.11	11.46	1.70	13.60	57.16
Sphacelaria cirrosa	0.61	0.00	6.56	4.05	7.78	64.94
Titanoderma mediterraneum	0.56	0.00	6.36	5.75	7.54	72.48

Groups S3-may & N3-may

Average dissimilarity = 44.44

	Group S3-may	Group N3-may				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Cianobatteri	0.00	26.67	14.72	2.17	33.13	33.13
Myrionema sp.	10.33	4.11	5.88	0.96	13.23	46.37

Cutleria multifida sporophyte	0.00	1.11	5.60	1.31	12.61	58.97
Enteromorpha sp.	0.00	3.33	4.79	0.66	10.79	69.76
Chaetomorpha linum	5.56	0.00	4.46	0.66	10.04	79.80

Groups N1-may & N3-may

Average dissimilarity = 64.16

	Group N1-may	Group N3-may				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Cianobatteri	0.00	26.67	11.02	2.18	17.18	17.18
Feldmannia sp.	25.67	75.56	10.44	1.37	16.28	33.45
Palmophyllum?	25.56	0.00	9.63	1.25	15.01	48.46
Myrionema sp.	20.78	4.11	6.01	1.10	9.37	57.83
Hydrolithon farinosum	0.44	0.00	4.27	6.72	6.65	64.48

Groups N2-may & N3-may

Average dissimilarity = 51.50

	Group N2-may	Group N3-may				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Cianobatteri	0.00	26.67	10.03	2.29	19.47	19.47
Palmophyllum?	13.33	0.00	9.19	3.95	17.85	37.32
Myrionema sp.	38.89	4.11	7.54	1.68	14.64	51.95
Gyraudia sphacelarioides	2.11	0.11	4.93	2.16	9.57	61.52
Cladosiphon sp.	1.11	0.00	4.82	4.29	9.36	70.89

JUNE

Groups C3-june & S1-june

Average dissimilarity = 51.01

	Group C3-june	Group S1-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Myrionema sp.	10.11	0.17	6.23	2.68	12.21	12.21
Microspongium tenuissimum	28.11	10.00	6.02	1.26	11.81	24.02
Palmophyllum?	2.56	10.22	5.94	1.58	11.65	35.67
Pseudolithoderma adriaticum	4.78	0.00	4.78	1.28	9.36	45.03
Cianobatteri	0.50	0.00	3.00	1.27	5.87	50.91

Groups S1-june & S2-june

Average dissimilarity = 48.47

	Group S1-june	Group S2-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Sphacelaria cirrosa	0.06	5.11	8.06	4.00	16.64	16.64
Microspongium tenuissimum	10.00	11.83	6.14	1.32	12.67	29.31
Palmophyllum?	10.22	2.17	5.74	1.10	11.84	41.15

Titanoderma mediterraneum	2.22	4.56	4.52	1.36	9.33	50.48
Corallina sp.	0.00	0.17	4.05	8.03	8.36	58.84

Groups C3-june & S3-june

Average dissimilarity = 54.95

	Group C3-june	Group S3-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Feldmannia sp.	0.39	61.67	8.95	3.75	16.29	16.29
Titanoderma mediterraneum	8.00	0.00	6.36	5.75	11.57	27.86
Chaetomorpha linum	0.50	17.28	5.86	2.55	10.66	38.53
Hydrolithon farinosum	3.50	0.00	5.23	5.91	9.52	48.05
Pseudolithoderma adriaticum	4.78	0.00	3.90	1.27	7.09	55.14

Groups S1-june & S3-june

Average dissimilarity = 72.12

	Group S1-june	Group S3-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Feldmannia sp.	0.00	61.67	13.90	13.15	19.27	19.27
Chaetomorpha linum	0.00	17.28	10.11	10.67	14.02	33.30
Palmophyllum?	10.22	0.00	8.38	4.27	11.63	44.92
Hydrolithon farinosum	4.00	0.00	6.59	3.48	9.14	54.06
Titanoderma mediterraneum	2.22	0.00	5.80	6.02	8.04	62.10

Groups S2-june & S3-june

Average dissimilarity = 71.73

	Group S2-june	Group S3-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Feldmannia sp.	0.00	61.67	13.58	9.08	18.94	18.94
Myrionema sp.	0.00	7.78	7.78	6.02	10.85	29.78
Chaetomorpha linum	0.17	17.28	7.64	3.63	10.66	40.44
Sphacelaria cirrosa	5.11	0.00	7.27	16.53	10.13	50.57
Titanoderma mediterraneum	4.56	0.00	4.93	1.27	6.87	57.44

Groups S1-june & N1-june

Average dissimilarity = 45.31

	Group S1-june	Group N1-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Cutleria multifida sporophyte	0.00	4.11	6.98	6.65	15.41	15.41
Chaetomorpha linum	0.00	3.50	5.82	5.28	12.84	28.25
Palmophyllum?	10.22	55.00	5.04	1.83	11.11	39.37
Microspongium tenuissimum	10.00	6.83	4.37	1.52	9.64	49.01

Hincksia	0.00	1.67	3.75	1.33	8.27	57.28

Groups S2-june & N1-june (similar pH)

Average dissimilarity = 50.97

	Group S2-june	Group N1-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Palmophyllum?	2.17	55.00	8.98	1.90	17.62	17.62
Cutleria multifida sporophyte	0.00	4.11	6.82	5.90	13.39	31.01
Sphacelaria cirrosa	5.11	0.22	5.75	2.32	11.28	42.29
Hincksia	0.00	1.67	3.67	1.32	7.20	49.49
Chaetomorpha linum	0.17	3.50	3.49	1.60	6.85	56.33

Groups S2-june & N2-june

Average dissimilarity = 56.01

	Group S2-june	Group N2-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Gyraudia sphacelarioides	0.00	5.33	7.04	6.29	12.58	12.58
Palmophyllum?	2.17	29.11	7.02	1.71	12.53	25.11
Cutleria multifida sporophyte	0.00	6.06	6.96	2.94	12.42	37.53
Sphacelaria cirrosa	5.11	0.33	5.67	2.21	10.12	47.65
Feldmannia sp.	0.00	2.33	4.19	1.30	7.47	55.13

Groups N1-june & N2-june

Average dissimilarity = 32.38

	Group N1-june	Group N2-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Gyraudia sphacelarioides	0.44	5.33	3.58	1.48	11.07	11.07
Hydrolithon farinosum	2.67	0.17	3.15	2.31	9.72	20.79
Myrionema sp.	0.00	1.22	3.11	1.31	9.59	30.38
Feldmannia sp.	1.11	2.33	3.09	1.13	9.54	39.92
Hincksia	1.67	0.00	3.08	1.33	9.51	49.44

Groups S2-june & N3-june

Average dissimilarity = 76.28

	Group S2-june	Group N3-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Feldmannia sp.	0.00	82.78	12.86	10.91	16.86	16.86
Gyraudia sphacelarioides	0.00	5.89	6.58	9.15	8.63	25.48
Sphacelaria cirrosa	5.11	0.00	6.36	17.25	8.34	33.82
Cianobatteri	0.00	1.78	4.91	12.29	6.44	40.26
Titanoderma mediterraneum	4.56	0.00	4.34	1.27	5.68	45.95

Groups S3-june & N3-june

Average dissimilarity = 34.65

	Group S3-june	Group N3-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Myrionema sp.	7.78	0.00	5.77	6.51	16.66	16.66
Cladophora socialis	0.00	1.22	3.64	5.08	10.49	27.15
Cutleria multifida sporophyte	0.00	3.22	3.48	1.33	10.06	37.21
Cianobatteri	0.06	1.78	3.39	2.94	9.78	46.99
Nemacystus sp.	0.00	0.56	3.07	13.33	8.85	55.84

Groups N1-june & N3-june

Average dissimilarity = 64.64

	Group N1-june	Group N3-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Palmophyllum?	55.00	0.00	9.61	5.31	14.87	14.87
Feldmannia sp.	1.11	82.78	9.30	2.99	14.38	29.25
Hydrolithon farinosum	2.67	0.00	4.52	9.38	6.99	36.24
Cianobatteri	0.00	1.78	4.10	9.76	6.34	42.59
Titanoderma mediterraneum	1.72	0.00	4.07	9.36	6.30	48.89

Groups N2-june & N3-june

Average dissimilarity = 58.13

	Group N2-june	Group N3-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Palmophyllum?	29.11	0.00	8.21	8.18	14.12	14.12
Feldmannia sp.	2.33	82.78	7.71	2.73	13.26	27.38
Cianobatteri	0.00	1.78	4.14	24.12	7.13	34.51
Cladophora socialis	0.00	1.22	3.62	5.04	6.23	40.74
Diatomee	0.00	0.89	3.46	8.61	5.95	46.68

JULY

Groups C3-july & S1-july

Average dissimilarity = 44.38

	Group C3-july	Group S1-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Cladophora socialis	0.17	15.44	5.22	2.49	11.76	11.76
Myrionema sp.	0.00	17.22	4.89	1.30	11.02	22.78
Sphacelaria cirrosa	3.89	3.89	3.55	2.05	8.00	30.78
Pseudolithoderma adriaticum	32.22	4.56	3.30	1.76	7.44	38.22

Sphacelaria sp.	2.22	3.89	2.97	1.12	6.69	44.90

Groups S1-july & S2-july

Average dissimilarity = 45.59

	Group S1-july	Group S2-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Cladophora socialis	15.44	0.11	5.31	2.37	11.65	11.65
Palmophyllum?	8.33	26.11	4.41	1.10	9.66	21.31
Myrionema sp.	17.22	0.67	4.17	1.58	9.14	30.45
Chaetomorpha linum	0.00	3.94	3.84	2.47	8.42	38.87
Sphacelaria cirrosa	3.89	3.44	3.24	2.13	7.11	45.98

Groups C3-july & S3-july

Average dissimilarity = 63.02

	Group C3-july	Group S3-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Titanoderma mediterraneum	66.67	0.00	9.86	7.98	15.65	15.65
Hydrolithon farinosum	26.67	0.00	7.90	4.79	12.53	28.18
Feldmannia sp.	0.00	48.33	7.21	1.31	11.44	39.62
Chaetomorpha linum	0.78	23.33	4.55	1.63	7.21	46.84
Pseudolithoderma adriaticum	32.22	14.44	3.73	0.91	5.92	52.76

Groups S1-july & S3-july

Average dissimilarity = 70.69

	Group S1-july	Group S3-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Titanoderma mediterraneum	32.78	0.00	7.16	5.62	10.13	10.13
Feldmannia sp.	0.06	48.33	6.27	1.40	8.86	18.99
Chaetomorpha linum	0.00	23.33	6.06	4.29	8.57	27.57
Hydrolithon farinosum	11.56	0.00	5.82	10.19	8.24	35.80
Myrionema sp.	17.22	0.00	4.82	1.30	6.81	42.62

Groups S2-july & S3-july

Average dissimilarity = 67.85

	Group S2-july	Group S3-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Feldmannia sp.	0.00	48.33	7.03	1.31	10.35	10.35
Titanoderma mediterraneum	20.78	0.00	6.90	5.00	10.17	20.53
Palmophyllum?	26.11	0.00	5.47	1.24	8.06	28.58
Hydrolithon farinosum	6.00	0.00	4.97	3.36	7.33	35.91
Pseudolithoderma adriaticum	6.56	14.44	3.69	1.18	5.43	41.34

Groups S1-july & N1-july

Average dissimilarity = 54.88

	Group S1-july	Group N1-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Palmophyllum?	8.33	83.89	6.43	1.98	11.71	11.71
Cladophora socialis	15.44	0.11	4.62	2.74	8.42	20.13
Chaetomorpha linum	0.00	5.67	4.09	4.85	7.46	27.58
Sphacelaria cirrosa	3.89	0.00	3.80	7.61	6.93	34.52
Pseudolithoderma adriaticum	4.56	0.00	3.73	4.01	6.80	41.31

Groups S2-july & N1-july (similar pH)

Average dissimilarity = 43.81

	Group S2-july	Group N1-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Palmophyllum?	26.11	83.89	4.20	1.04	9.59	9.59
Pseudolithoderma adriaticum	6.56	0.00	3.13	1.31	7.15	16.74
Dictyota dichotoma	1.22	8.89	3.06	1.53	6.98	23.72
Sphacelaria sp.	8.33	2.78	2.55	1.87	5.83	29.55
Diatomee	0.22	3.83	2.54	1.80	5.80	35.35

Groups S2-july & N2-july

Average dissimilarity = 48.08

	Group S2-july	Group N2-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Dictyota dichotoma	1.22	28.89	4.62	2.00	9.60	9.60
Lobophora variegata	0.00	6.44	4.60	3.21	9.57	19.17
Sphacelaria sp.	8.33	0.56	3.36	1.41	6.99	26.16
Sphacelaria cirrosa	3.44	5.67	3.30	1.75	6.87	33.03
Palmophyllum?	26.11	45.56	3.23	0.83	6.72	39.75

Groups N1-july & N2-july

Average dissimilarity = 33.94

	Group N1-july	Group N2-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Sphacelaria cirrosa	0.00	5.67	4.16	16.55	12.24	12.24
Dictyota dichotoma	8.89	28.89	2.75	1.05	8.10	20.35
Sphacelaria sp.	2.78	0.56	2.43	1.67	7.15	27.49
Lobophora variegata	0.67	6.44	2.33	1.25	6.85	34.34
Myrionema sp.	0.56	2.00	1.77	1.37	5.21	39.56

Groups S2-july & N3-july (similar pH)

Average dissimilarity = 72.11

Species	Group S2-july Av.Abund	Group N3-july Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Feldmannia sp.	0.00	60.56	8.07	3.73	11.19	11.19
Titanoderma mediterraneum	20.78	0.00	6.20	5.61	8.60	19.78
Palmophyllum?	26.11	0.00	4.92	1.26	6.82	26.61
Hydrolithon farinosum	6.00	0.00	4.46	3.59	6.19	32.80
Chaetomorpha linum	3.94	34.44	3.44	1.84	4.78	37.57

Groups S3-july & N3-july

Average dissimilarity = 50.17

Species	Group S3-july Av.Abund	Group N3-july Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Pseudolithoderma adriaticum	14.44	0.00	4.25	1.29	8.47	8.47
Dictyota dichotoma	0.00	2.11	3.57	3.82	7.11	15.58
Feldmannia sp.	48.33	60.56	3.36	1.03	6.70	22.28
Lobophora variegata	0.00	7.22	3.28	1.22	6.54	28.83
Antithamnion cruciatum	0.00	1.33	3.16	4.22	6.31	35.14

Groups N1-july & N3-july

Average dissimilarity = 60.35

Species	Group N1-july Av.Abund	Group N3-july Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Palmophyllum?	83.89	0.00	8.24	13.58	13.66	13.66
Feldmannia sp.	0.44	60.56	5.69	2.63	9.44	23.09
Titanoderma mediterraneum	6.89	0.00	4.19	5.62	6.94	30.04
Gyraudia sphacelarioides	0.00	5.00	3.02	1.31	5.00	35.03
Hydrolithon farinosum	1.56	0.00	2.94	5.62	4.88	39.91

Groups N2-july & N3-july

Average dissimilarity = 61.87

Species	Group N2-july Av.Abund	Group N3-july Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Palmophyllum?	45.56	0.00	7.19	9.99	11.62	11.62
Feldmannia sp.	0.56	60.56	6.32	2.66	10.22	21.84
Sphacelaria cirrosa	5.67	0.00	4.25	16.09	6.88	28.72
Titanoderma mediterraneum	5.00	0.00	3.69	2.63	5.97	34.69
Gyraudia sphacelarioides	0.00	5.00	3.10	1.31	5.01	39.70

APPENDIX V (continued)

SIMPER ANALYSIS AMONG SECTORS DURING TIME ON COLONIZATION DATA

Groups C3-may & C3-june

Average dissimilarity = 58.91

	Group C3-may	Group C3-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Microspongium tenuissimum	0.00	28.11	11.30	3.49	19.19	19.19
Feldmannia sp.	14.78	0.39	5.96	1.64	10.13	29.31
Titanoderma mediterraneum	0.33	8.00	5.52	1.66	9.37	38.68
Myrionema sp.	63.89	10.11	5.46	3.26	9.27	47.95
Pseudolithoderma adriaticum	0.00	4.78	4.92	1.25	8.36	56.31

Groups C3-may & C3-july

Average dissimilarity = 80.93

	Group C3-may	Group C3-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Myrionema sp.	63.89	0.00	13.42	4.59	16.58	16.58
Pseudolithoderma adriaticum	0.00	32.22	11.23	3.34	13.88	30.46
Titanoderma mediterraneum	0.33	66.67	11.01	2.90	13.60	44.06
Dinoflagellate	0.00	21.94	8.78	2.45	10.85	54.92
Feldmannia sp.	14.78	0.00	6.21	1.24	7.67	62.58

Groups C3-june & C3-july

Average dissimilarity = 55.75

	Group C3-june	Group C3-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Microspongium tenuissimum	28.11	0.00	8.31	3.98	14.90	14.90
Dinoflagellate	0.00	21.94	6.87	2.62	12.32	27.22
Myrionema sp.	10.11	0.00	6.43	5.09	11.54	38.76
Pseudolithoderma adriaticum	4.78	32.22	5.08	1.44	9.11	47.87
Titanoderma mediterraneum	8.00	66.67	4.53	3.61	8.12	55.99

Groups S1-may & S1-june

Average dissimilarity = 69.24

	Group S1-may	Group S1-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Myrionema sp.	50.56	0.17	17.41	4.35	25.14	25.14
Palmophyllum?	0.00	10.22	13.27	4.15	19.16	44.31
Microspongium tenuissimum	0.00	10.00	8.72	1.19	12.59	56.90
Sphacelaria sp.	0.00	0.50	6.42	6.17	9.26	66.17
Hydrolithon farinosum	0.44	4.00	5.79	1.22	8.36	74.53

Groups S1-may & S1-july

Average dissimilarity = 69.37

Species	Group S1-may Av.Abund	Group S1-july Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Cladophora socialis	0.11	15.44	7.63	2.49	10.99	10.99
Titanoderma mediterraneum	0.39	32.78	6.77	3.85	9.77	20.76
Sphacelaria cirrosa	0.00	3.89	6.18	8.66	8.91	29.67
Pseudolithoderma adriaticum	0.00	4.56	6.05	4.35	8.72	38.39
Hydrolithon farinosum	0.44	11.56	5.65	2.60	8.15	46.54

Groups S1-june & S1-july

Average dissimilarity = 61.29

Species	Group S1-june Av.Abund	Group S1-july Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Cladophora socialis	0.00	15.44	7.77	3.67	12.68	12.68
Myrionema sp.	0.17	17.22	5.55	1.68	9.05	21.74
Pseudolithoderma adriaticum	0.00	4.56	5.40	4.26	8.81	30.54
Palmophyllum?	10.22	8.33	5.36	1.85	8.74	39.28
Sphacelaria cirrosa	0.06	3.89	4.63	3.26	7.56	46.85

Groups S2-may & S2-june

Average dissimilarity = 69.27

Species	Group S2-may Av.Abund	Group S2-june Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Myrionema sp.	40.56	0.00	18.76	6.89	27.09	27.09
Microspongium tenuissimum	0.00	11.83	12.71	4.23	18.34	45.43
Titanoderma mediterraneum	0.56	4.56	5.91	2.73	8.53	53.96
Palmophyllum?	0.56	2.17	5.40	1.17	7.79	61.75
Corallina sp.	0.00	0.17	4.76	7.08	6.88	68.63

Groups S2-may & S2-july

Average dissimilarity = 75.21

Species	Group S2-may Av.Abund	Group S2-july Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Myrionema sp.	40.56	0.67	9.85	2.64	13.09	13.09
Palmophyllum?	0.56	26.11	7.46	1.33	9.92	23.02
Chaetomorpha linum	0.00	3.94	6.06	2.37	8.05	31.07
Titanoderma mediterraneum	0.56	20.78	5.96	2.99	7.92	38.99
Sphacelaria sp.	0.00	8.33	5.92	1.25	7.88	46.87

Groups S2-june & S2-july

Average dissimilarity = 61.98

	Group S2-june	Group S2-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Microspongium tenuissimum	11.83	0.00	7.49	3.64	12.09	12.09
Palmophyllum?	2.17	26.11	5.97	1.50	9.63	21.72
Sphacelaria sp.	0.11	8.33	4.90	1.48	7.91	29.63
Cianobatteri	0.00	1.50	4.78	3.38	7.71	37.34
Titanoderma mediterraneum	4.56	20.78	4.67	1.18	7.53	44.88

Groups S3-may & S3-june

Average dissimilarity = 46.22

	Group S3-may	Group S3-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Microspongium tenuissimum	0.00	8.06	9.33	9.66	20.19	20.19
Chaetomorpha linum	5.56	17.28	8.19	1.42	17.72	37.90
Sphacelaria sp.	0.00	0.83	5.13	4.85	11.10	49.00
Gyraudia sphacelarioides	0.00	1.00	5.12	4.42	11.07	60.07
Diatomee	0.00	0.44	4.45	6.59	9.63	69.71

Groups S3-may & S3-july

Average dissimilarity = 74.74

	Group S3-may	Group S3-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Myrionema sp.	10.33	0.00	8.71	6.04	11.66	11.66
Chaetomorpha linum	5.56	23.33	7.42	1.82	9.92	21.58
Diatomee	0.00	4.83	6.81	5.39	9.12	30.70
Pseudolithoderma adriaticum	0.00	14.44	6.81	1.26	9.11	39.81
Dinoflagellate	0.00	4.50	6.72	4.12	8.99	48.80

Groups S3-june & S3-july

Average dissimilarity = 49.63

	Group S3-june	Group S3-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Microspongium tenuissimum	8.06	0.00	6.15	7.58	12.40	12.40
Myrionema sp.	7.78	0.00	5.93	5.48	11.94	24.34
Pseudolithoderma adriaticum	0.00	14.44	5.09	1.28	10.27	34.60
Osmundea truncata	0.00	1.50	3.52	4.16	7.08	41.69
Cianobatteri	0.06	2.33	3.51	2.11	7.08	48.76

Groups N1-may & N1-june

Average dissimilarity = 54.60

	Group N1-may	Group N1-june

Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Myrionema sp.	20.78	0.00	9.21	3.83	16.87	16.87
Microspongium tenuissimum	0.00	6.83	6.70	7.86	12.27	29.14
Feldmannia sp.	25.67	1.11	5.35	1.00	9.80	38.93
Palmophyllum?	25.56	55.00	4.87	0.88	8.93	47.86
Chaetomorpha linum	0.33	3.50	4.02	1.54	7.36	55.22

Groups N1-may & N1-july

Average dissimilarity = 63.89

	Group N1-may	Group N1-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Myrionema sp.	20.78	0.56	5.05	2.51	7.90	7.90
Palmophyllum?	25.56	83.89	4.78	1.09	7.48	15.38
Dictyota dichotoma	0.00	8.89	4.45	1.32	6.96	22.35
Sphacelaria sp.	0.00	2.78	4.31	3.88	6.74	29.09
Chaetomorpha linum	0.33	5.67	4.05	1.84	6.34	35.43

Groups N1-june & N1-july

Average dissimilarity = 52.92

	Group N1-june	Group N1-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Microspongium tenuissimum	6.83	0.00	4.80	7.84	9.07	9.07
Cutleria multifida sporophyte	4.11	0.00	4.50	9.02	8.51	17.57
Diatomee	0.00	3.83	4.23	6.53	7.99	25.57
Dictyota dichotoma	0.56	8.89	3.66	1.33	6.91	32.48
Dinoflagellate	0.00	1.44	3.41	6.13	6.45	38.92

Groups N2-may & N2-june

Average dissimilarity = 53.87

	Group N2-may	Group N2-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Feldmannia sp.	70.56	2.33	8.43	2.41	15.65	15.65
Myrionema sp.	38.89	1.22	7.06	3.03	13.10	28.74
Microspongium tenuissimum	0.00	9.56	7.04	6.73	13.08	41.82
Chaetomorpha linum	0.00	1.50	4.50	7.55	8.35	50.17
Cladosiphon sp.	1.11	0.00	3.86	4.17	7.17	57.34

Groups N2-may & N2-july

Average dissimilarity = 79.02

	Group N2-may	Group N2-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%

Feldmannia sp.	70.56	0.56	8.55	3.97	10.82	10.82
Dictyota dichotoma	0.00	28.89	7.63	8.91	9.66	20.48
Myrionema sp.	38.89	2.00	5.63	2.15	7.13	27.61
Sphacelaria cirrosa	0.00	5.67	5.20	23.24	6.58	34.19
Lobophora variegata	0.00	6.44	5.15	3.35	6.52	40.71

Groups N2-june & N2-july

Average dissimilarity = 61.08

	Group N2-june	Group N2-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Dictyota dichotoma	0.06	28.89	6.67	4.61	10.92	10.92
Microspongium tenuissimum	9.56	0.00	5.57	6.26	9.11	20.03
Lobophora variegata	0.00	6.44	4.96	3.37	8.12	28.16
Gyraudia sphacelarioides	5.33	0.00	4.81	6.33	7.88	36.03
Cutleria multifida sporophyte	6.06	0.00	4.73	3.07	7.75	43.78

Groups N3-may & N3-june

Average dissimilarity = 56.28

	Group N3-may	Group N3-june				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Microspongium tenuissimum	0.00	6.22	6.79	10.00	12.06	12.06
Chaetomorpha linum	0.00	5.39	6.15	3.70	10.93	23.00
Gyraudia sphacelarioides	0.11	5.89	5.68	2.92	10.09	33.09
Myrionema sp.	4.11	0.00	4.49	1.32	7.98	41.07
Cladophora socialis	0.00	1.22	4.37	4.93	7.77	48.84

Groups N3-may & N3-july

Average dissimilarity = 65.09

	Group N3-may	Group N3-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Chaetomorpha linum	0.00	34.44	9.15	9.49	14.05	14.05
Dinoflagellate	0.00	4.33	5.35	5.47	8.22	22.27
Dictyota dichotoma	0.00	2.11	4.54	3.81	6.98	29.25
Lobophora variegata	0.00	7.22	4.16	1.24	6.39	35.64
Myrionema sp.	4.11	0.00	4.04	1.32	6.21	41.84

Groups N3-june & N3-july

Average dissimilarity = 40.28

	Group N3-june	Group N3-july				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Microspongium tenuissimum	6.22	0.00	4.70	10.20	11.67	11.67

Lobophora variegata	0.00	7.22	3.22	1.22	7.99	19.66
Cutleria multifida sporophyte	3.22	8.11	3.19	1.40	7.93	27.59
Antithamnion cruciatum	0.00	1.33	3.10	4.55	7.70	35.28
Chaetomorpha linum	5.39	34.44	2.80	1.92	6.96	42.25

APPENDIX VI

LIST OF SAMPLES WITH REFERENCE NUMBER, USED FOR MOLECULAR ANALYSES AND THEIR NATIVE ENVIRONMENT (a: acidified; **-A**: not acidified).

Reference number	Morphotype	Environment
61	*D. dichotoma*	a
62	*D. dichotoma*	a
63	*D. dichotoma*	a
64	*D. dichotoma*	a
65	*D. pulchella*	a
66	*D. pulchella*	a
67	*D. pulchella*	a
68	*D. pulchella*	a
69	*D. pulchella*	a
70	*D. dichotoma* v. *intricata*	a
71	*D. dichotoma* v. *intricata*	a
72	*D. dichotoma* v. *intricata*	a
73	*D. dichotoma* v. *intricata*	a
74	*D. dichotoma* v. *intricata*	a
75	*D. dichotoma* v. *intricata*	a
76	*D. dichotoma* v. *intricata*	a
77	*D. pulchella*	**-A**
78	*D. pulchella*	**-A**
79	*D. pulchella*	**-A**
80	*D. pulchella*	**-A**
81	*D. dichotoma* v. *intricata*	**-A**
82	*D. dichotoma* v. *intricata*	**-A**
83	*D. dichotoma* v. *intricata*	**-A**
84	*D. dichotoma* v. *intricata*	**-A**
85	*D. dichotoma*	**-A**
86	*D. dichotoma*	**-A**

87	*D. dichotoma*	**-A**
88	*D. dichotoma*	**-A**

APPENDIX VII

DICTYOTA DNA EXTRACTION PROCEDURE

- Heat the water bath to 60°C
- Prepare 300 µl CTAB isolation buffer for each sample and heat in the bath
- Set up 1,5 mL tubes with tissue and grind after putting in liquid nitrogen and 200 µL not heated CTAB
- Transfer the omogenate in a 1,5 mL tube
- Add 300 µL di CTAB caldo (from the water bath at 60°) and 10 µL di Proteinasi-K (PK 20 mg/mL)
- Vortex
- Incubate at 60° for 1h (inverse after 30')
- Add 30 µL RNAase (10 µg/µL) and vortex
- Incubate at 37° for 30'
- Add 500 uL of 24:1 Chloroform:isoamyl alcohol and mix to emulsify (CI)
- Centrifuge at 14000rpm for 2'
- Transfer aqueous (upper) layer to a clean tube
- Add an higher volume of cold Isopropanol (~500 µL) than the transferred volume and mix
- Centrifuge 14000rpm for 35' at 14 °C
- Remove the upper layer (Isopropanol) and add 500µL cold Absolute Ethanol
- Centrifuge14000rpm for 5' at 14 °C
- Remove the Ethanol and leave the pellet to dry for 1h on blotting paper
- Dissolve the pellet in 10 µL ddH_2O
- Mix gently and leave at mean temperature

CTAB isolation buffer (100 ml):

* 2,0 g CTAB
* 28 mL of 5M NaCl
* 4 mL of 0,5M EDTA
* 10 mL Tris-HCl

Ringraziamenti

È bello poter vedere realizzata quella che all'inizio era solo un'Idea. Tre anni sono volati se ora ci penso…e solo adesso realizzo quanto lavoro!!

Quando si sceglie di fare qualcosa per passione il tempo passa e la fatica non si sente. Di certo ci sono stati momenti difficili ma di quelli mi è rimasto solo un vago e lontano ricordo! E questo grazie a tutte le persone che mi hanno aiutato in questo percorso di vita lavorativa e non.

Ho scritto la mia tesi di dottorato in inglese, ormai la lingua ufficiale utilizzata nel mondo scientifico, ma i ringraziamenti li faccio in italiano, "la lingua del cuore". Vorrei iniziare dalle persone più importanti della mia vita: i miei genitori, che mi hanno sostenuto moralmente e materialmente; senza di loro tutto questo non sarebbe potuto esserci. Grazie sempre..

E cosa dire di quelle persone che mi hanno seguito e guidato nella realizzazione di questo lavoro? I miei Guru che si sono avvicendati nel corso della realizzazione sperimentale. Un grazie infinito a Maria Cristina che ha lasciato libero sfogo alla mia creatività scientifica nel raggiungimento dei miei obiettivi, e che mi è stata vicina sempre. Ringraziare la mia Tutor universitaria, Carmen, è dir poco: non si è risparmiata in nulla per aiutarmi nell'analisi dei dati, nell'incoraggiarmi e sostenermi in una materia che non avevo mai affrontato prima. E per lo stesso motivo vorrei ringraziare Maurizio che mi ha introdotto nel fantastico mondo del diving-PAM, per la sua pazienza e costanza nel guidarmi nelle misure in immersione e nell'analisi dei dati. Un ringraziamento sentito va anche alla Prof. Anna De Maio che con il suo aiuto sono riuscita ad inserire persino un fuori-programma nel mio lavoro!

E come potrei dimenticare Francesco!? Da tempo desideravo avvicinarmi al mondo del microlitro! E lui me l'ha permesso armandosi di taaaanta pazienza e lasciandomi familiarizzare con pipette e PCR, che meraviglia! Per non parlare dell'aiuto immane per l'analisi dei dati…

Senza queste persone non avrei potuto realizzare neanche un decimo dei miei obiettivi!! Grazie millee!

Vi lascio con una citazione di Nelson Mandela: Un vincitore è solo un sognatore che non si è arreso!

Tutti quelli che mi conoscono mi definiscono una sognatrice…e si, in questo momento mi sento vincitrice perché con la fine del Dottorato vengono realizzati parte dei miei sogni, iniziati in una soleggiata giornata a bordo di una nave, circondata solo dall'oceano. Ero una bimba, ma il ricordo di quel momento resta sempre vivo, e poco importa se la realtà spesso si scontra col mondo dei sogni, l'importante è crederci!