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PLASTICITÉ PHÉNOTYPIQUE CHEZ LE CNIDAIRE SYMBIOTIQUE *ANEMONIA VIRIDIS*: ANALYSE DE LA RÉPONSE AU STRESS A DIFFÉRENTS NIVEAUX DE COMPLÉXITE STRUCTURALE

Phenotypic plasticity in the symbiotic cnidarian *Anemonia viridis*:
stress response at multiple levels of structural complexity

Soutenue le 12 Décembre 2016 devant le jury composé de :

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LIST OF ABBREVIATIONS

ABH: Adaptive Bleaching Hypothesis

BSA: Bovine Serum Albumin

CA: Carbonic Anhydrase

CCM: Carbon Concentrating Mechanism

DIC: Dissolved Inorganic Carbon

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

EdU: 5-Ethynyl-2'-Deoxyuridine

FP: Fluorescent Protein

GFP: Green Fluorescent Protein

GIM: Grace's Insect Medium

GMIM: Grace's Modified Insect Medium

H3P: Phophorylated Histone H3

MAA: Mycosporine-Like Amino Acid

OA: Ocean Acidification

PBS, PBT: Phosphate Buffered Saline, Phosphate Buffered Saline and Tween

PCR, RT-PCR, qRT-PCR: Polymerase Chain Reaction, Reverse transcription polymerase chain reaction, Quantitative Reverse transcription polymerase chain reaction

pHi: intracellular pH

SASW: Sterile Artificial Seawater

SCaFSW: Sterile Calcium-Free Artificial Seawater

ROS: Reactive Oxygen Species

UVR: Ultra-Violet Radiation

"*For such a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied.*" August Krogh Principle, 1929

Preamble

Biologists have always looked for an easy model organism to study biological processes and able to address fundamental questions. In general, a good animal model would have the following characteristics: (i) structural simplicity, but at the same time containing basic cellular processes that more complex organisms have; (ii) accessible for research and simple to manipulate; (iii) easy and economical to grow in the laboratory; (iv) easily amenable to genetic manipulation. Today we recognize the invaluable knowledge model organisms have brought to science. Classical model organisms, such as the fruitfly *Drosophila melanogaster*, the nematode worm *Caenorhabditis elegans*, the yeast *Saccharomyces cerevisiae* and the mouse *Mus musculus* have largely contributed to the core of biological knowledge (Bolker 2012). However, they are unable to answer all the biological questions we pose and answers are limited by their characteristics (Cook et al. 2016). The study of alternative models allowed overcoming the scientific and technical obstacles. Among them, xenopus (*Xenopus laevis*) and sea urchins eggs (e.g. *Lytechinus pictus, Arbacia punctulata, Paracentrotus lividus*) allowed breakthroughs in the identification of cell cycle regulation proteins mainly due to the facility in laboratory use, with production of high quantities of large eggs, robust zygotes and synchronous cell cycle (Siefert et al. 2015, Cormier et al. 2016, Sluder 2016). Sea slugs (*Aplysia* species) with their simple nervous system composed of a small number of large cells, allowed the discovery of the mechanisms behind learning, and short, long-term memory (Carew & Kandel 1973). Zebrafish (*Danio rerio*) has become undoubtedly the most popular fish model in many research fields, especially for developmental biology, toxicology and genetic research (Ribas & Piferrer 2014). More recently, with the development of new cellular technologies and new generation sequencing approaches (allowing the access to their complete genome), emerging model organisms arose to the rank of true alternative models (Cook et al. 2016). However, the ideal candidate species will have a significant impact because they present the traits for which the questions addressed mattered. It would be a mistake to think we could answer all the biological questions from a limited number of species.

In the present work, we chose to work on diploblastic organisms, from the phylum Cnidaria, displaying attractive properties (from ecological to cellular characteristics), which allow using them as model species for biomedical and environmental research.

1. Chapter 1 - General introduction

1.1 The Phylum Cnidaria

The Phylum Cnidaria includes sea anemones, corals, hydroids and jellyfish, in approximately 9 000 species (Technau & Steele 2011), inhabiting aquatic environments but predominantly marine habitats.

1.1.1 Biodiversity

Cnidarians have complex life cycles being present in the form of a polyp (attached to the substrate) or a medusa (mainly planktonic). When reproducing sexually, the medusa produces the gametes, which after fertilization, form a planktonic larva, planula, which then settles and metamorphoses into a polyp. The polymorphism is the criteria that allow distinguishing between the 5 classes of Cnidaria: Hydrozoa, Cubozoa, Scyphozoa, Staurozoa (all included in the subphylum Medusozoa) and Anthozoa (Fig. 1.1; Technau & Steele, 2011). Hydrozoans (hydroids) have generally both a tiny polyp and medusa stage; Cubozoans (box jellyfish) and Scyphozoans (true jellyfish) are predominantly in the medusa form; Staurozoans (stalked jellyfish) have an attached medusa and not alternate between polyps and medusa stage; Anthozoans (sea anemones and corals) are exclusively in the form of solitary or colonial polyps.

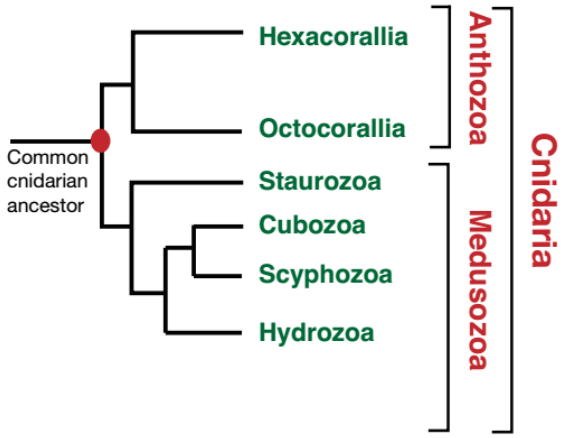

Figure 1.1 – Phylogenetic relationship between the different classes of the phylum Cnidaria (modified from Technau & Steele 2011).

1.1.2 Ecological importance

Cnidarians evolved approximately 500 million years ago (Carthwright et al. 2007) and are the simplest living metazoans. Cnidarians have a global distribution, in polar, temperate and tropical latitudes. Their range of distribution goes from shallow coastal waters to the deep sea, although they are more abundant at shallow warmer waters of tropical regions.

Tropical coral reefs are a hotspot of biodiversity with ecological and economic relevance (revised in Dubinsky 1990). Coral reefs are a source of shelter, food and a nursery ground for fish species and hundreds of other species. Also, they are an important source of food for humans and are an important economical source of tourism in these regions. Yet, the ecological importance of cnidarians does not reside only on the coral reefs. Cnidarians are carnivorous, and play a role in the food web, as predator and prey. They prey mainly on plankton but also on small crustaceans, fish larvae and are preyed upon by molluscs, fishes, crustaceans and sea turtles (Mitchell et al. 1988). Moreover, they are an important source of bioactive compounds (see segment 1.2.1).

1.1.3 General anatomy

Cnidarians are simplistic organisms with external radial symmetry and an adult dimorphism (polyp and medusa). The gastrovascular cavity has only one central opening, working both as a mouth and an anus. A crown of tentacles surrounds the mouth (Fig.1.2).

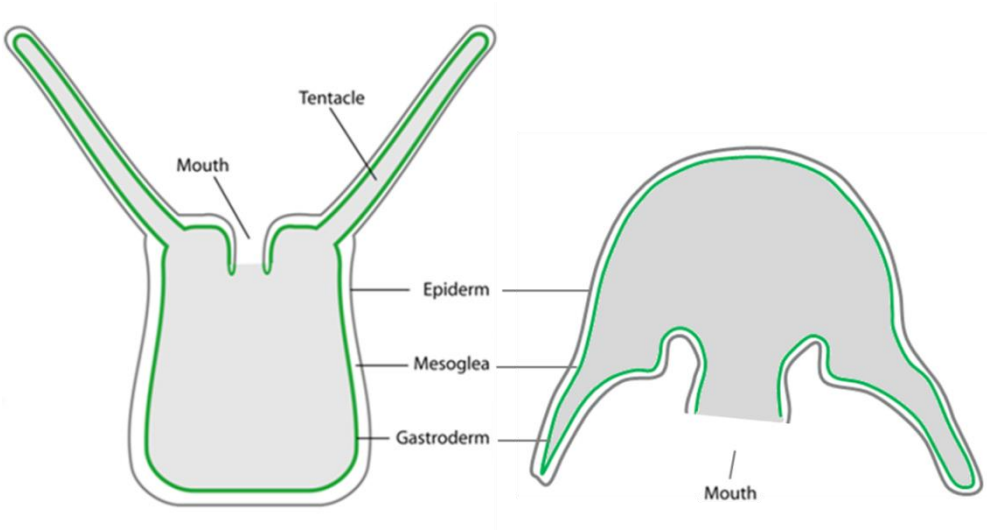

Figure 1.2 Simplified diagram of a polyp and medusa body plan, showing diploblastic tissue organization (modified from Sabourault et al. 2009).

1.1.4 Cellular anatomy

Being diploblastic animals, cnidarians are composed by two epithelial monolayers, the epiderm facing the seawater and the gastroderm facing the gastrovascular cavity, both separated by the acellular mesoglea.

Mainly located in the epiderm, cnidarians possess specialized stinging cells, cnidocytes, which are used for predation and defence. Moreover, in the epiderm we can find other cell types: (1) epithelio-muscular cells (longitudinal fibers) used for movement and contraction,

(2) interstitial cells (for tissue renewal, see below, 1.2.1), (3) mucous gland cells, which produce mucus for feeding and protection and, (4) neuronal and sensory cells (Hündgen 1984). In the gastroderm, cell types are (1) gland cells which produce digestive enzymes, (2) neuronal cells, and (3) epithelio-muscular cells (circular fibres) used for movement and phagocytosis (Technau & Steele 2011) (Fig. 1.3).

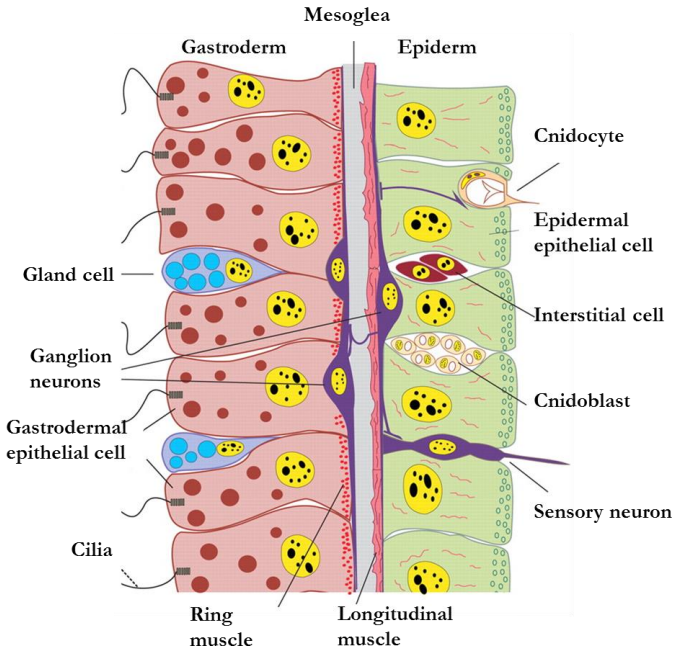

Figure 1.3 - Cross section of Hydra body plan showing the different cell types present both in the epiderm and in the gastroderm (modified from Technau & Steele 2011).

Epithelial cells of cnidarians show a high diversity of functions. Epithelial cells from the epiderm have predominantly a protective function while those from the gastroderm are responsible for food uptake and digestion. In some cnidarians, others gastrodermal cells also have a symbiotic function, hosting unicellular algae (see below segment 1.2.3).

1.2 Physiological properties of cnidarians

Cnidarians possess striking physiological properties, which make them attractive model species for biomedical and environmental researches. Indeed, cnidarians (1) express high diversity of fluorescence proteins, which allowed the development of new biotechnological tools; (2) have great longevity and high regeneration capacity, allowing studies on aging and on the processes of regeneration; and (3) some species are adapted to life in symbiosis with photosynthetic unicellular algae, making them good bioindicators of the health of ecosystems.

1.2.1 Diversity of natural fluorescence

In 1962, Shimomura and co-authors, while working on the bioluminescence of the jellyfish *Aequorea victoria* (class Hydrozoa), isolated and identified two proteins responsible for the bioluminescence, a calcium binding protein (aequorin) and a green fluorescent protein (GFP; (Shimomura et al. 1962) (Fig. 1.4).

Figure 1.4 - The jellyfish Aequorea Victoria (classe Hydrozoa) expressing the green fluorescence protein (GFP). Photo by Phil Blackburn.

It was much later that (Prasher et al. 1992) cloned the GFP gene in order to better understand the mechanisms leading to light generation. This GFP found in cnidarians is unusual and different from other natural pigments, since it does not require added substrate or co-factors other than oxygen to produce its green fluorescence (Chalfie et al. 1994, Heim et al. 1994). This constituted an advantage for the studies in living cells since until then there was no non-invasive technique available, able to maintain the integrity of tissues or cells. It was Chalfie et al. (1994) who first used GFP as a marker for gene expression in living cells of the nematode worm, *Caenorhabditis elegans*. The authors used GFP gene as a reporter gene attached to a gene of interest and monitored the GFP fluorescence as a proxy of gene expression in living cells or tissues. *A. victoria* GFP has since then become an invaluable versatile marker in biological research. It has been widely used in protein dynamics, fluorescence microscopy, cell biology and biotechnology, molecular biology, cancer research and many more (see (Zimmer 2002, 2009) for a review). Additionally, other fluorescent proteins (FP) homologous to GFP have been found in cnidarians, mostly in species from class Anthozoa (Lukyanov et al. 2005). Five colour classes are now defined thanks to their respective emission: cyan, green, 2 red classes and yellow (Remington 2011). The discovery of other FP has enlarged the applicability of GFP-

like proteins to live cell imaging, allowing the development of multicolour labelling experiments. Each FP has a particular characteristic that confers an advantage in respect to others. For instance, the red FP, which present reduced background fluorescence, is used for the localization of cells in the whole organisms (Wiedenmann et al. 2011).

Fluorescent proteins are only an example of how cnidarians can be an important source of marine natural products for the biological research. Indeed, over 3000 natural products have been identified from cnidarians, with many applications (Rocha et al. 2011). Some examples are secondary metabolites, such as terpenoids, which have an anti-inflammatory potential and diterpenoids, which have been tested as an antitumor drug (see Rocha et al. 2011 for a review). Therefore, cnidarians show a biomedical and biotechnological potential to be exploited by worldwide researchers.

1.2.2 Cnidarian longevity and regeneration

Cnidarians are amongst the longest living animals, and in some cases immortality has been hypothesized. Nevertheless, the range of life span shows a high plasticity within cnidarians. Some polyps of hydrozoans live only a few days (e.g. *Campanularia flexuosa*; (Strehler & Crowell 1961)), whereas the deep-sea corals *Gerardia* sp. and *Leiopathes* sp. are longest lived, 2742 and 4265 years, respectively (Roark et al. 2009). In addition, the hydrozoan species *Turritopsis dorhnii* (previously known as *T. nutricula*), is considered immortal, by reverse development of sexually mature medusa into clonal polyps (Piraino et al. 1996).

Most living organisms get old, in a process known as aging. Aging represents one of the biological processes from which we still lack a full answer, mainly due to the diversity of aging across the tree of life. Attempts have been made to define the mechanisms leading to aging, and (López-Otín et al. 2013) categorized nine main molecular and cellular events occurring in cells aging (cf Fig.1.5).

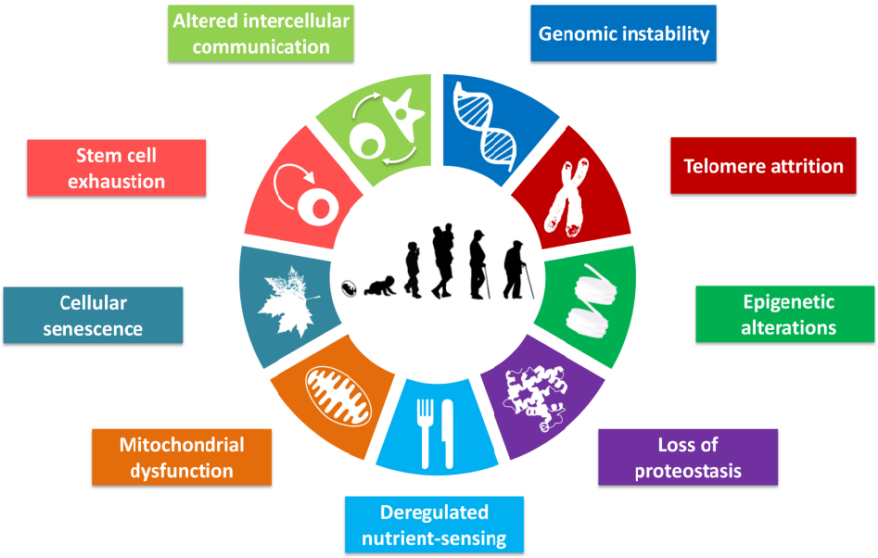

Figure 1.5 - Schematic representation of the mechanisms contributing to the aging process in mammals (López-Otín et al. 2013).

These mechanisms are involved at different levels of cellular complexity (from nucleus to the cell tissue). In the nucleus, aging process includes epigenetic alterations (DNA methylation, chromatin remodelling and transcriptional alterations), genomic instability (genomic damages) and telomere attrition (shortening of nucleoprotein structures located at the extremities of the chromosomes). At the organelle level, aging is due to the mitochondria dysfunction (reducing energy source and overproducing reactive oxygen species). At the cellular level, aging is provoked by cellular senescence (the arrest of the cell cycle, i.e. leading to cell death), stem cell exhaustion (depletion of undifferentiated cells capable of differentiation into specific cell types ensuring cell renewal) and loss of proteostasis (accumulation of protein degradation and misfolding of proteins). Finally, aging is the result of altered intercellular communication (perturbation of cell signalling, which leads to failures in the response to inflammation) (López-Otín et al. 2013).

Conversely, in cnidarians, the longevity has been suggested to be explained by some specific strategies overcoming the aging processes. Specifically, mechanisms leading longevity in cnidarians could be linked to the presence, in adult organisms, of stem cells with high and continuous cellular renewal potential. This propriety could then be responsible of two cnidarian specificities: asexual reproduction (responsible of clonal and colonial growth) and tissue regeneration (the process by which animals regrow lost body parts or entire organisms from small body fragments).

From all the above-mentioned mechanisms in cnidarians, regeneration is the most studied. Regeneration can happen in two ways, (1) morphallaxis, i.e. regeneration by reorganization

and differentiation of pre-existing cells and (2) epimorphosis, which involves cell proliferation, i.e. cell growth and division leading to an increase in the number of cells (Li et al. 2015). For a long time, the high regenerative capacity of cnidarians was attributed to the presence of stem cells, meaning that regeneration was only done by morphallaxis. In Cnidarians, stem cells were first identified in the hydrozoan genus *Hydractinia* and later on *Hydra* (Weismann, 1883). Both groups possess stem cells in the interstitial space of epithelial cells, commonly referred as i-cells (Frank et al. 2009). The totipotency associated to i-cells is responsible for the high regenerative capacity of *Hydra*, which is able to regenerate two entirely new individuals from head and foot, the head will regrow a foot, and the foot will regrow a head (reviewed in (Holstein et al. 2003b)). Recognized stem cell genes (e.g. *piwi, vasa, PL 10*) have been used to identify stemness in cnidarian tissues (e.g. Seipel et al. 2003, Siebert et al. 2014) . For instance, *piwi* is present in the all developmental stages of the hydrozoan *Podocoryne carnea* with higher expression in the egg and medusa (Seipel et al. 2013) and *vasa, piwi and PL 10* have been identified in the epiderm and gastroderm of gastrozooids of the hydrozoan species *Nanomia bijuga* (Siebert et al. 2014). Nevertheless, recent studies have also shown that other cnidarian species (e.g. the anthozoan *Nematostella vectensis*) are not able to regenerate without differentiated cell proliferation (Passamaneck & Martindale 2012, DuBuc et al. 2014), showing that regeneration by epimorphosis is also present in cnidarians.

Although regeneration has greatly contributed to our understanding on the mechanisms of longevity in cnidarians, others studies on aging processes could give us a new insight into the extreme life span of cnidarians. One of them is the dynamics of the telomere maintenance in cnidarians. One of the aging mechanisms is the telomere shortening, prevented by the presence of a telomerase. The telomerase is only expressed in germ cells, stem cells and tumours and its activity is responsible for the synthesis of new telomere repeats. In cnidarians, telomerase activity has also been identified (Traut et al. 2007, Ojimi et al. 2009, Zielke & Bodnar 2010); however telomere dynamics over time and their relation with events leading to cell cycle arrest and apoptosis has still to be determined. A relevant way to study telomere and telomerase activity related with cnidarian longevity, growth and stress response could be by the development of *in vitro* cnidarian culture cells and the analysis of the chromosome dynamics during cell divisions and time. Cell cultures would allow the study of telomerase activity within different types of cells, dividing cells and to determine extension of life span in culture.

1.2.3 Environmental adaptation

Cnidarians, as mention above, have a worldwide distribution inhabiting temperate, tropical, deep and surface waters. Colonization of different ecosystems implied adaptation to local environmental conditions, and more recently, anthropogenic threats. One of the ways through which cnidarians adapted was the establishment of mutualistic symbiosis with unicellular phototrophs, an association estimated to have been established 225 million years ago (Rosen 2000).

Symbiosis

Some cnidarians live in association with a photosynthetic dinoflagellate from the genus *Symbiodinium spp*., and form one of the most studied symbioses in the marine realm - cnidarian-dinoflagellate symbiosis. *Symbiodinium* are unicellular algae that can be found as free-living species or associated with other unicellular organisms (e.g. dinoflagellate, foraminifera) but also cnidarians, molluscs (e.g. giant clams) and sponges (Trench 1993).

The symbiosis between *Symbiodinium* and Anthozoa – the class of cnidarians comprising sea anemones and corals - is one of the most ecologically significant symbioses, due to its role in the formation of tropical coral reefs, and the great biodiversity herein present (Dubinsky 1990). It is a mutualistic endosymbiotic intracellular association, where the symbiont (*Symbiodinium*) is located intracellularly in the gastrodermal cells of the host cnidarian, enveloped by a symbiosome membrane that separates the symbiont from the host cytoplasm (Fig. 1.6).

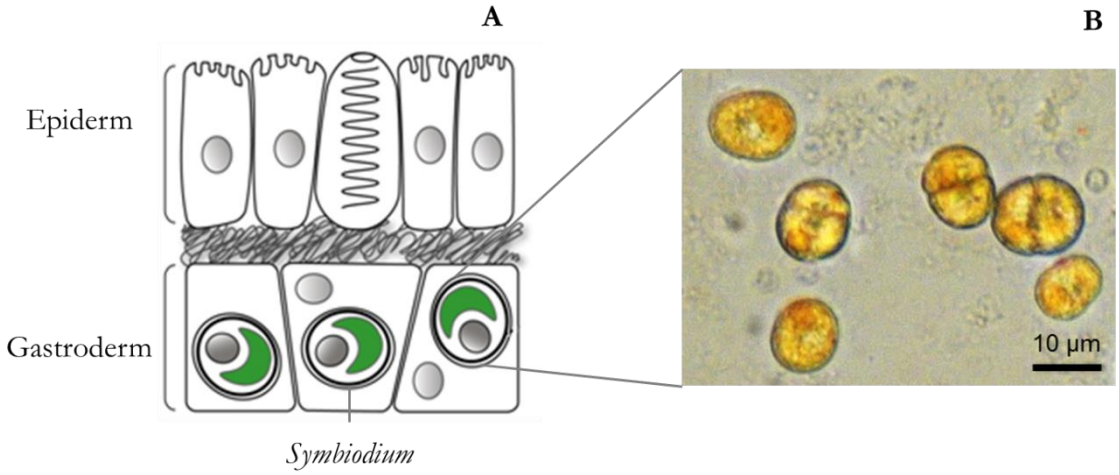

Figure 1.6 - Schematic representation of the diploblastic organization of a polyp from a symbiotic cnidarian.

(A) Location of the symbiont *Symbiodinium* in the gastroderm tissue (modified from Sabourault et al. 2009). (B) S*ymbiodinium* cells in division isolated from the sea anemone *Anemonia viridis*.

The genus *Symbiodinium* is divided into different phylogenetic groups, referred as clades (9 clades; A-I). Within each clade, there is additional genetic diversity which supports the distinction of strains or sub-types (e.g D1, G2; (Coffroth & Santos 2005), (Pochon et al. 2014). Their genetic diversity could be linked with the capacity to inhabit multiple environments, and the specificity of tolerance to environmental changes (e.g. (Brading et al. 2011, Oakley et al. 2014).

Symbiodinium are acquired by vertical or horizontal transmission. The former implies the maternal transmission of symbionts directly by asexual or sexual reproduction through incorporation of symbionts into the released eggs. The horizontal transmission involves the release of symbiont-free eggs and the acquisition of the symbiont by phagocytosis, is done externally from the environment, at each generation (Smith and Douglas, 1987). The mechanisms of symbiont recognition have not yet been determined but it is suggested to involve the recognition of specific glycoproteins, more precisely the binding of symbiont glycans by host lectins (Vidal-Dupiol et al. 2009).

- Benefits of this symbiosis

The success of this symbiosis is dependent on the mutual exchanges promoted by both partners. While in the host, *Symbiodinium* continue to photosynthesize and transfer until 90% of the organic carbon produced during photosynthesis, contributing to host metabolism, reproduction, growth and calcification (Fig.1.7) (Furla et al. 2005, Davy et al. 2012).

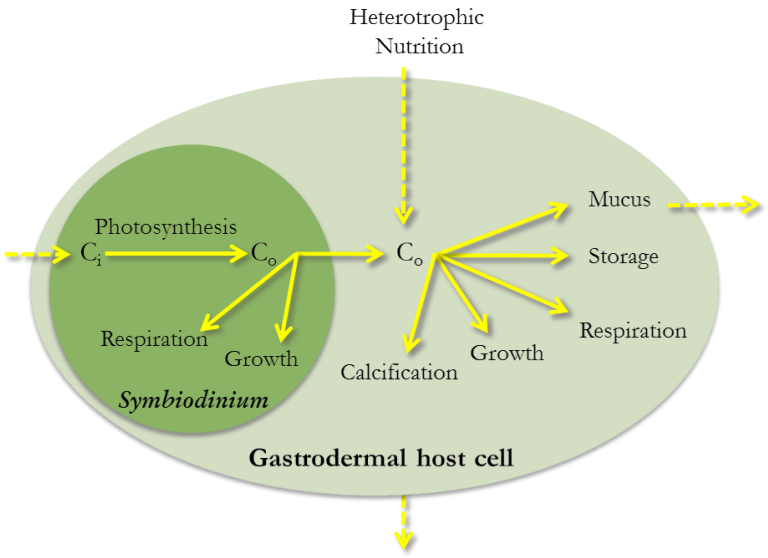

Figure 1.7 - Organic carbon supply and use in a symbiotic cnidarian.
C_i: inorganic carbon (carbon dioxide, bicarbonate); C_o: organic carbon (sugars, lipids, amino acid) **(Casado-Amezúa et al. 2014)**.

In this way, the host, although capable of heterotrophic nutrition, can satisfy its metabolic needs by autotrophy. From all the photosynthate products, glycerol, glucose and lipids are the most translocated (Trench 1971, Sutton & Hoegh-Guldberg 1990, Burriesci et al. 2012). In opposite direction, the host will contribute to symbiont photosynthesis with essential nutrients derived from its metabolism or from the external medium (such as nitrogen, phosphorus, inorganic carbon) (Muscatine 1990). This association constitutes a new biological entity, with original metabolic properties, the holobiont, which is notably responsible of the colonization of nutrient-poor environments.

- Adaptations to symbiosis

Life in symbiosis between an animal host and a free-living algal species can be both beneficial as risky. In order to establish this successful mutualistic relationship, cnidarians evolved and acquired several mechanisms which allowed adaptation to the constrains imposed by the symbiont. For example, the animal host is constricted to inhabit shallow areas in the euphotic zone where light exposure is enough to fulfil photosynthetic requirements of the *Symbiodinium*; implying nevertheless a major exposure to UV radiations (UVR). Potential photo-damage is prevented by the presence in the holobiont of mycosporine-like amino acids (MAAs, a family of aromatic amino acids), as they absorb UVR and dissipate it as heat (Shick & Dunlap 2002). Although MAAs are more concentrated in the host tissues, it is not clear who is responsible for the biosynthesis of the MAAs, symbionts or host (Shick & Dunlap 2002, Roth 2014). However, recent studies on the coral *Acropora digitifera* showed that the animal genome possesses the genes necessary for their biosynthesis (Shinzato et al. 2011). Another constraint that cnidarian hosts have to overcome is the circadian variations of intracellular O_2 concentrations. Daily, the symbiotic cnidarians fluctuate from hyperoxia state (i.e. high [O_2]) at day-time due to symbiont photosynthesis, to hypoxia (i.e. low [O_2]) during night-time, due to the host and symbiont respiration (Richier et al. 2003). During photosynthesis, the high concentrations of generated O_2 lead to the production of reactive oxygen species (ROS; (Dykens et al. 1992). Consequently, the host evolved high diversity of anti-oxidant defences to overcome the increased production of ROS, such as superoxide dismutases, catalases, peroxidases which neutralize oxidant species, reducing its toxicity (Furla et al. 2011, Pey et al. 2016). Finally, for photosynthesis to take place, CO_2 most arrive continuously to the symbionts. Due to the intracellular location of the symbionts in the most inner layer of tissue, the gastroderm,

access to inorganic carbon is dependent on the host. The host has then selected mechanisms of inorganic carbon absorption, further described in chapter 2 (Furla et al. 2005).

Therefore, the cnidarians have evolved a great flexibility in functions to adapt themselves to the continuous constrains imposed by the presence of the intracellular symbiont and to ensure the success and the stability of the symbiosis.

Local environmental responses

Cnidarians are not restricted to stable environments with a limited range of conditions. During evolution they were able to exploit different ecological niches, from fresh to marine waters, such as shallow waters in tropical reefs and open ocean where abiotic conditions (e.g. temperature, nutrient availability, pH, light intensity and water movement) differ substantially. For instance, the range of light exposure goes from full sunlight in shallow waters (e.g. cnidarians in tropical coral reefs), to darkness in the deep-sea (cold-water corals). That capacity suggests a high level of phenotypic plasticity, a mechanism by which a same genotype can produce different phenotypes influenced by the environment. It can reflect changes in morphology, physiology, life history and behaviour of a genotype (Pigliucci 2001, 2005).

One proof of this high phenotypic plasticity is the morphological changes observed in many symbiotic corals species due to abiotic factors, such as light and water movement (Todd 2008). For example, the coral *Porites sillimaniani*, which is an explanate (flat) coral, was observed to form branches when exposed to high light conditions, while it remained flat at low light (Muko et al. 2000). Another example is the coral *Stylophora pistallata,* which presents a gradual change of the colony morphs with the depth (Fig. 1.8; Furla & Allemand 2009). This morphological plasticity ensures the efficiency of light exposure for the symbiont photosynthesis.

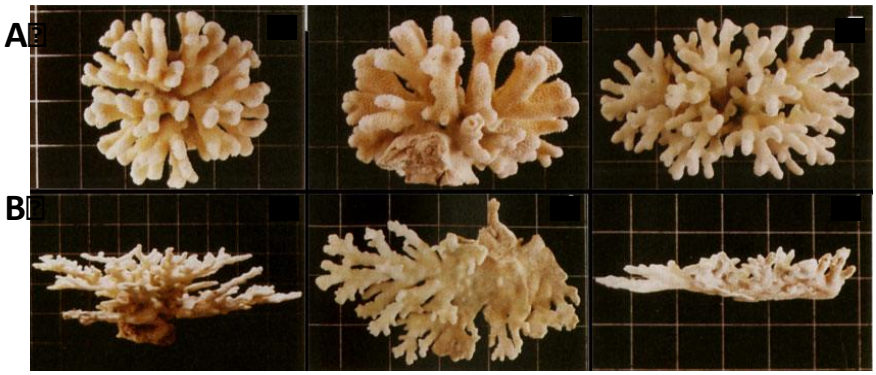

Figure 1.8 - Example of Stylophora pistilata adapted ecomorphs (Modified from Gattuso, PhD Thesis, 1987).

(A) Shallow ecomorphs. (B) Deep-sea ecomorphs.

Moreover, cnidarians are adapted not only to different habitat constrains but also to fluctuating environments. In the current scenario of environmental changes, due to natural or anthropogenic threats (e.g. pollution, sedimentation, ocean acidification, rise of sea water temperature), this capacity is called into question. Again, cnidarian phenotypic plasticity could be involved through acclimation process, with a short-term reversible response, conferring resilience to a particular stressor. For instance, Mitchelmore et al. (2003) have shown that in the sea anemone *Anthopleura elegantissima* following an induced stress of cadmium, capacity to acclimate to these toxic conditions was possible due to the presence of higher levels of symbiont metal-binding antioxidant glutathione.

With the increasing rise in seawater temperature, we are seeing a recurrent phenomenon in coral reefs known as bleaching. Bleaching is the loss of the symbionts and/or loss of photosynthetic pigments, leading to the disruption of cnidarian-dinoflagellate symbiosis and to high mortality rates (Fig. 1.9a; Hoegh-Guldberg 1999). Bleaching phenomenon is mainly considered as a pathogenic state induced by a former dysfunctioning of the photosynthetic complex of the symbiont following cellular damages to symbiont and host cells (Fig. 1.9b).

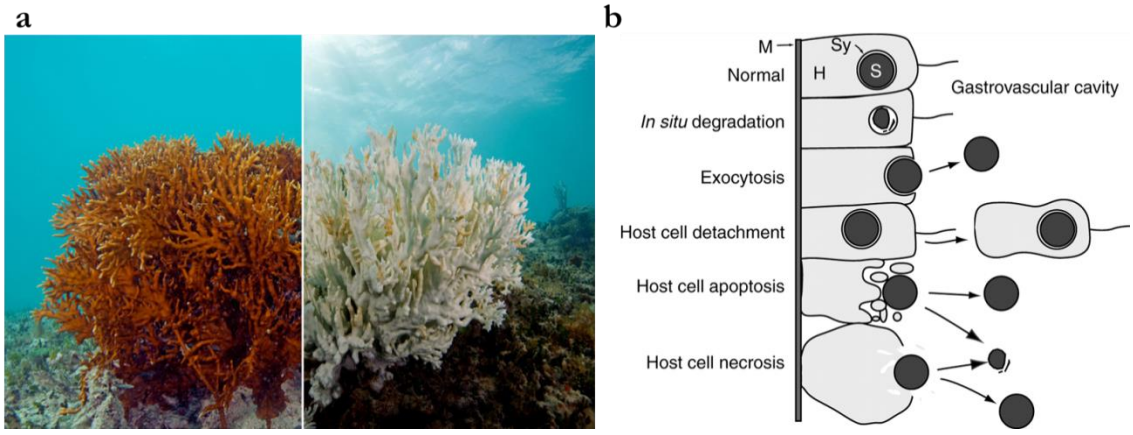

Figure 1.9 - Bleaching phenomenon in cnidarian-dinoflagellate symbiosis.
(a) On the left a healthy fire coral with its symbionts and on the right a bleached fire coral, where the whitish coral results from the loss of symbionts, therefore the pigments responsible for the colour (source*: XL Catlin Seaview Survey)*. (b) Cellular mechanisms leading to the loss of symbiont in cnidarians. Five mechanisms are *in situ* degradation, exocytosis of the symbiont to the gastrovascular cavity, host cell detachment with the symbiont into the gastrovascular cavity, formation of apoptosis body leaving the host cell and finally host cell necrosis. H-normal host cell, Sy-symbiosome, S-*Symbiodinium*, M-mesoglea (source: Weis 2008).

However, bleaching is hypothesized as one adaptive strategy to changing environmental conditions: the adaptive bleaching hypothesis (ABH) (Buddemeier et al. 2004, Fautin &

Buddemeier 2004). According to the ABH, bleaching represents a strategy of host to switch from a less favourable symbiont partner to a more favourable one, in a way that symbionts shape the host phenotype. In other words, colonies of corals can be inhabited by different sub-types of *Symbiodinium* (e.g. Chen et al. 2005). Each of these sub-types can have environmental optima; therefore, during a stress event, the most sensitive species to that particular stress can trigger cellular mechanisms leading to the loss of that specific sub-type, favouring growth of another sub-type. For example, Brading et al. (2011) showed that *Symbiodinium* sub-types are differentially sensible to increase pCO_2, clade A1 and B1 are not affected, and clades A13 and A2 were favoured by this increase. Therefore, dynamics changes in *Symbiodinium* could be an important strategy to respond to fluctuating environmental conditions.

Nowadays, as environmental stressors become more and more recurrent, and are expected to increase in intensity (Hoegh-Guldberg et al. 2007, Doney et al. 2009), it is fundamental to understand the limit of the extended cnidarian phenotypes allowing them to acclimate and/or adapt to predicted future conditions.

1.3 Our cnidarian study model: the sea anemone *Anemonia viridis*

The snakelocks sea anemone *Anemonia viridis* (Forskål, 1775) (class Anthozoa) (Fig. 1.10) was adopted as our model species.

Figure 1.10 - The snakelocks anemone *Anemonia viridis*. Photo by Alexis Pey.

It is a temperate species found abundant in shallow waters (up to 20 m) of the Mediterranean, Eastern Atlantic, English Channel and North Sea. It is a non-calcifying

symbiotic cnidarian that leaves in association with the dinoflagellate *Symbiodinium* temperate A clade (Casado-Amezúa et al. 2014). Reproduction in this species is done asexually (by fission) or sexually, and the symbionts are transmitted vertically.

The use of *A. viridis* as model species presents several advantages: (1) long and non-retractable tentacles gives access to large amounts of tissue; (2) *A. viridis* has a high tentacle regenerative capacity, (3) facility to obtain aposymbiotic (deprived of symbionts) individuals in laboratory, (4) easy dissociation into epiderm, gastroderm and symbionts, and (5) the absence of a calcium carbonate skeleton favours tissue dissociation. Besides, in the last years, numbers of studies on *A. viridis* have increased knowledge on its physiology (Furla et al. 1998, 2000, Laurent et al. 2014), biochemistry (e.g. Richier et al. 2003), genetics (e.g. Ganot et al. 2011), cellular biology (Barnay-Verdier et al. 2013) and stress response (Richier et al. 2006, Moya et al. 2012, Suggett et al. 2012, Jarrold et al. 2013, Borell et al. 2014, Horwitz et al. 2015). Evidence from these studies suggests an extended plasticity of *A. viridis,* at different levels of biological complexity (from whole organism to the cell), as mechanism enabling its success under environmental changes and then justifying the use of *A. viridis* as a model for our studies.

1.4 Thesis objectives

In this PhD thesis we wanted to investigate the extent of the phenotypic plasticity of *A. viridis* to environmental stresses at different temporal scales (i.e. short and long term stress exposures) and at multiple levels of structural complexity (i.e. whole organism and isolated cells).

Specific goals of this thesis were:

(1) *in vivo*, identification of the mechanisms behind the physiological plasticity that enable non-calcifying photosynthetic anthozoans to survive and thrive under future ocean acidification (OA) conditions and assess the plasticity under synergetic effects (OA and increased temperature) (**Chapter 2**).

(2) *in vitro*, the development of animal primary cell culture as model to determine plasticity to environmental stresses at the cellular level (**Chapter 3**).

These chapters must be seen as two pieces of a puzzle that in the discussion session (**Chapter 4**) are combined to draw a comprehensive conclusion concerning the phenotypic plasticity of cnidarians.

2. Chapter 2 – *In vivo* studies of phenotypic plasticity

2.1 Introduction

2.1.1 Stress response: general context

Every organism, in the course of its life cycle, faces a change in its natural environment (e.g. arrival of a new predator, abiotic changes). The capacity of an organism genotype to change its phenotype (physiology, behaviour, morphology, development) in response to environmental changes is commonly referred to as phenotypic plasticity. The responses of the organism to a changing environment can then happen in one of four ways: (1) migration to a more favourable environment, (2) adaptation via genetic selection (adaptive phenotypic plasticity), (3) acclimatization via phenotypic plasticity (quick response) and (4) extinction (Hoffman & Parsons 1991, Foo & Byrne 2016). Adaptive phenotypic plasticity corresponds to a modification of the genome leading to a long-term phenotypic change. It is advantageous since it potentiates tolerance to multiple environments by increased fitness (Pigliucci 2001, Ghalambor et al. 2007). Acclimatization represents a short-term plastic change (without genomic modifications), where a single genotype produces a range of phenotypes as a result of an environmental perturbation (Foo & Byrne 2016).

Nowadays, symbiotic cnidarians are exposed to numerous threats (e.g. increased sea water temperature, ocean acidification, pollution, sedimentation, pathogens) that have the potential to disrupt the stability of the symbiotic relationship, possibly causing the breakdown of this symbiosis and ultimately the death of both partners (Hoegh-Guldberg et al. 2007). An environmental perturbation inducing a stress state corresponds to a change for which values fall outside the optimum response range of each species. Here, we are interested in assessing phenotypic plasticity (through adaptation or acclimatization) in a symbiotic cnidarian dealing with global change. Currently, the Intergovernmental Panel on Climate Change (IPCC 2013) described the environmental stressors involved in the global change. Among them, ocean acidification (OA) and global warming are the two major factors affecting marine biodiversity. In that context, we investigated OA and/or ocean warming on a symbiotic cnidarian by understanding the role of the phenotypic plasticity.

The model species of choice, *Anemonia viridis* has the great advantage of inhabiting natural acidified sites, Vulcano Island, South of Italy. In that particular site, submarine gas vents release CO_2 creating a natural pCO_2 gradient suitable for long-term exposure studies on phenotypic plasticity in response to OA. This allows us to compare phenotypic plasticity of *A. viridis*, with short-term high pCO_2 laboratory controlled exposure.

2.1.2 Ocean acidification

Since the Industrial Revolution, human activity had a huge impact on the excessive amounts of CO_2 being released to the atmosphere. Since the oceans act as a sink of atmospheric CO_2, accumulation of CO_2 is also happening in the oceans. Estimates are that the oceans absorbed up to one-third of atmospheric CO_2 released due to human activities (Hoegh-Guldberg & Bruno 2010). When CO_2 reaches the ocean, it reacts with water forming carbonic acid (H_2CO_3), which dissociates into bicarbonate (HCO_3^-) and protons (H^+), decreasing pH of the seawater while increasing the acidity of oceans, in a process known as OA (Fig. 2.1) (Caldeira & Wickett 2005, Doney et al. 2009). The term OA does not imply that the ocean will become acidic (pH < 7). Instead, pH is expected to decrease from current levels of 8.08 to 7.6-7.7 by 2100 (IPCC, 2013).

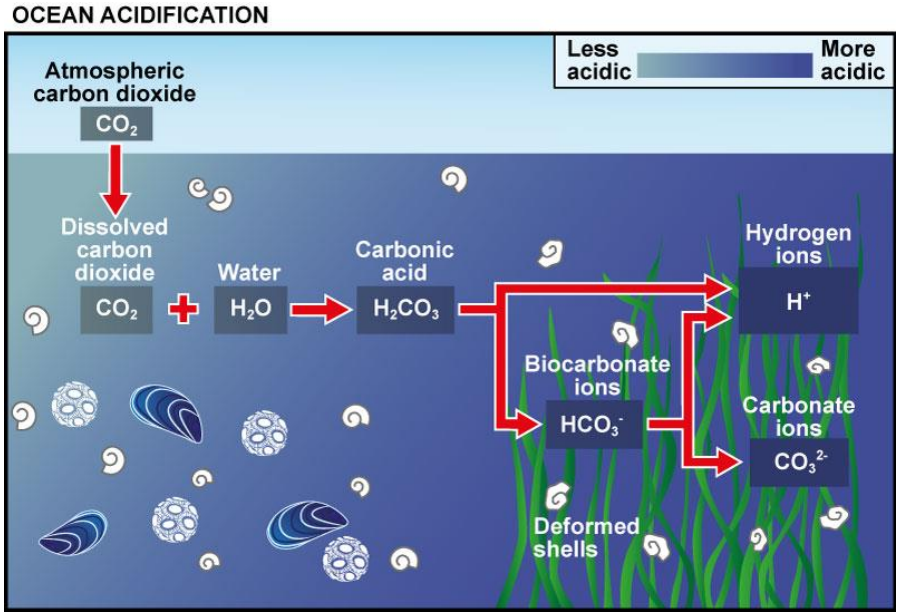

Figure 2.1 - Chemical reactions during absorption of atmospheric carbon dioxide in the seawater.
The increase in dissolved carbon dioxide in the seawater will increase the amount of hydrogen ions, in a process known as OA (source: University of Maryland).

In the current seawater-carbonate chemistry scenario, dissolved inorganic carbon (DIC) concentration is uneven, with bicarbonate (HCO_3^-) present in higher concentration (ca. 2.1 mM), representing ~ 90% of total DIC while CO_2 is present in low concentration (ca. 12 μM), approximately 1%; carbonate ion (CO_3^{2-}) accounts for the other 9% (Doney et al. 2009). However, under OA, i.e. lower pH, the chemical equilibrium of the seawater will change, with the increase of HCO_3^- and CO_2 and the decrease in CO_3^{2-} concentration (Fig. 2.2) (Fabry et al. 2008).

Changes in the chemical composition of seawater may impact the marine organisms by modifying the biological processes linked to inorganic carbon availability (i.e. calcification and carbon-concentrating mechanisms, cf below).

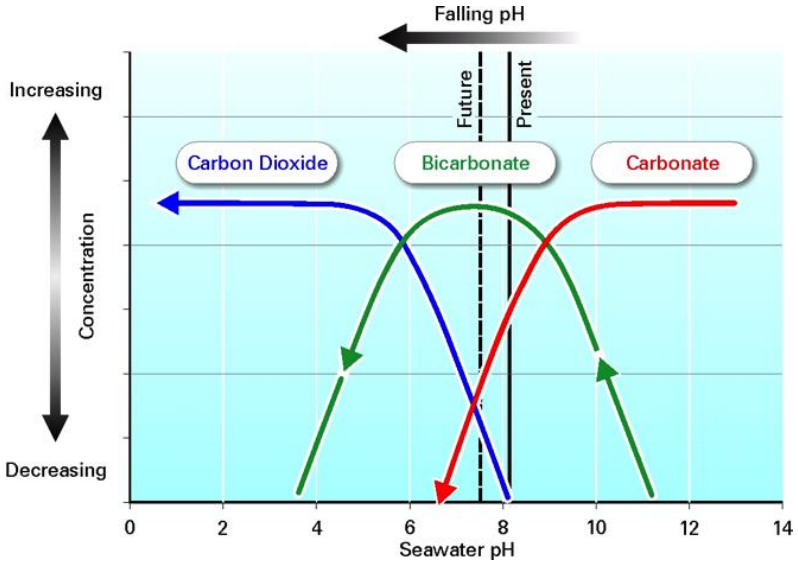

Figure 2.2 - Chemical equilibrium of dissolved inorganic carbon of seawater in a closed system (adapted from Holmen 1992).

2.2 Impact of ocean acidification on marine organisms

Marine organisms, from algae to fishes, show different sensitivities to OA, and studies predict that there will be winners and losers under future elevated CO_2 conditions (Table 1; Ries et al. 2009, Fabricius et al. 2011). Therefore, it is correct to assume that OA will shape marine communities. Indeed, OA may negatively impact a wide range of species across various phyla, especially calcifying species from plankton to corals. Conversely, some non-calcifying species are able to cope with OA, in natural or experimental conditions, with potential benefits on productivity (Doney et al. 2009, Fabricius et al. 2011). However, most of the studies on the effect of OA in marine organisms have been focused on the ecological impact rather than the mechanisms that trigger the OA response.

In marine biota unicellular eukaryotes and cnidarians are two major representative groups for studies of OA impact on calcifying and non-calcifying organisms.

Table 1. Effects of OA in marine calcifiers and non-calcifiers .
Table was built from meta-analysis of Kroeker et al. 2013 and supplemented with data for sea anemones from independent studies (Suggett et al. 2012, Towanda & Thuesen, 2012, Jarrold et al. 2013). n.d. – non-determined by insufficient number of studies; no effect – no significant changes; - decrease; + increase (simplified from Kroeker et al. 2013).

	Taxa	Parameter	Effect
CALCIFIERS	Calcifying algae	Survival	n.d
		Calcification	No effect
		Growth	n.d
		Photosynthesis	-
		Abundance	-
	Corals	Survival	No effect
		Calcification	-
		Growth	No effect
		Photosynthesis	No effect
		Abundance	-
	Coccolithophores	Survival	n.d
		Calcification	-
		Growth	No effect
		Photosynthesis	No effect
		Abundance	No effect
	Molluscs	Survival	-
		Calcification	-
		Growth	-
		Photosynthesis	-
		Abundance	No effect
	Echinoderms	Survival	No effect
		Calcification	No effect
		Growth	-
		Photosynthesis	-
		Abundance	No effect
NON-CALCIFIERS	Fish	Survival	n.d
		Growth	No effect
		Abundance	n.d
	Fleshy algae	Survival	n.d
		Growth	+
		Photosynthesis	No effect
		Abundance	No effect
	Seagrass	Survival	n.d
		Growth	n.d
		Photosynthesis	No effect
		Abundance	n.d
	Sea anemones	Survival	n.d
		Growth	+
		Photosynthesis	+/no effect
		Abundance	+

2.2.1 Impact of ocean acidification on calcification

Bio-calcification is the process by which calcifying organisms form a calcium carbonate ($CaCO_3$) skeleton, by the combination of calcium ion (Ca^{2+}) with CO_3^2. Current changes in seawater chemistry, with increased CO_2, HCO_3^-, H^+ and the decrease of CO_3^{2-}, may have an impact in the formation of a skeleton by calcifying species. Briefly, when the CO_2 reaches the seawater, it will react with water and form H_2CO_3, which will then dissociate into H^+

and HCO_3^-. The increased concentration of H^+ will then bind with CO_3^{2-}, outcompeting the association of Ca^{2+} from the surrounding water and CO_3^{2-}, thus reducing the availability of ion carbonate in the water. An excessive environmental acidification could even act on biological processes of calcification if the organism overcomes its capacity to maintain pH homeostasis (intracellular and/or in calcification site). Therefore, shell-forming marine organisms may see their skeleton reduced.

Effect on calcification of free-living unicellular eukaryotes

Coccolithophores and foraminifera are two of the most studied calcareous unicellular eukaryotes, and are responsible for the majority of pelagic $CaCO_3$. While studies have shown multiple responses to calcification in coccolithophores (Meyer & Riebesell 2015), with reduced calcification (e.g. 25% to 66% decrease; Fig. 2.3; Riebesell et al. 2000, Sciandra et al. 2003), increased calcification (doubling values of calcification; Iglesias-Rodriguez et al. 2008) or no significant changes in calcification (Langer et al. 2006, Oviedo et al. 2016), impact of increased pCO_2 in foraminifera seems to be always deleterious (e.g (Spero et al. 1997, Bijma et al. 1999, Uthicke et al. 2013). In fact, studies on volcanic CO_2 seeps, where concentrations of CO_2 range from present day to those expected for 2100, have shown a decrease in foraminifer's densities and diversity, as it happened before in the Paleocene, where there was a decrease in calcification, hypothesizing that this group can be menaced due to OA (Uthicke et al. 2013, Stillman & Paganini 2015).

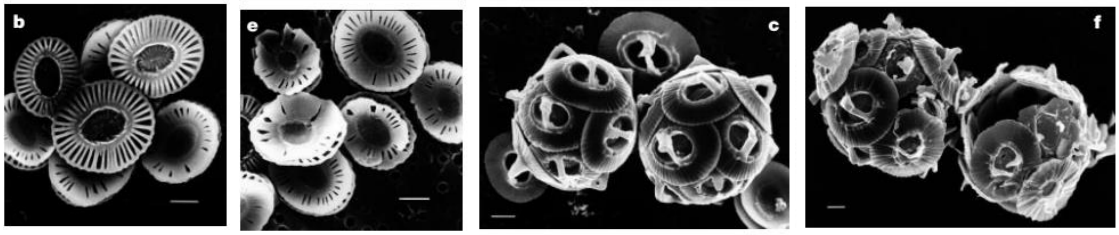

Figure 2.3 - Scanning electron microscopy photographs of coccolithophores under OA.
(b, e) *Emiliania huxleyi* and (c, f) *Gephyrocapsa oceanica*. Coccolith structure and degree of calcification under control pCO_2 levels of about 300 ppmv (b, c) and high pCO_2 levels, 780-850 ppmv (source: Riebesell et al. 2000).

Effect on calcification of corals

Corals are one of the highest producers of $CaCO_3$ in the oceans. Therefore, the predicted decrease in carbonate ion concentration will significantly reduce the capacity of corals to build their calcium carbonate skeleton (e.g. Gattuso et al. 1998, Hoegh-Guldberg et al. 2007). For instance, Kroeker et al. 2013 showed that on average, OA have a deleterious

effect of 32% on coral calcification. However, the impacts on coral calcification seem to be species specific, as not all corals seem to be impacted by OA future scenarios (Reynaud et al. 2003, Doney et al. 2009). A few studies have shown that cold-water corals (e.g. Maier et al. 2013, Rodolfo-Metalpa et al. 2015) and diverse coral communities naturally exposed to OA (Shamberger et al. 2014) seem to have a greater capacity to cope with changes in DIC chemistry.

2.2.2 Ocean acidification and carbon-concentrating mechanisms

Microalgae are the most important primary producers in the ocean. They absorb inorganic carbon and fix CO_2 into organic compounds through Rubisco (ribulose bisphosphate carboxylase/oxygenase), an enzyme with affinity not only to CO_2 but also to O_2. To overcome this limitation they possess carbon-concentrating mechanisms (CCM) that efficiently exploit HCO_3^- pool (largest oceanic DIC pool) in the seawater, and convert it in CO_2 in order to increase the concentration of available for the active site of the Rubisco (Giordano et al. 2005). An additional component of CCM is carbonic anhydrase (CA) an enzyme responsible for the acceleration of the rather slow inter-conversion between carbon species.

Symbiotic cnidarians have inside their host cells dinoflagellates from the genus *Symbiodinium*. While, free living *Symbiodinium* have direct access to DIC from the seawater, once inside host cells, several membranes form a barrier to the diffusion of DIC. One of the crucial contributions of the animal host to the symbiont is then the provision of DIC allowing the process of photosynthesis to proceed. There are two possible sources of DIC to the symbiont, (1) animal host's metabolism, i.e. respiration and (2) seawater. Animal host respiration rates are generally lower in respect to the net photosynthetic rates (Furla et al. 2005), indicating the presence of an external source of inorganic carbon. In addition, *Symbiodinium* Rubisco has low affinities to CO_2 therefore efficient allocation of CO_2 to the site of fixation is crucial (Rowan et al. 1996). CO_2 diffuses passively through host membranes but the low concentration in the seawater makes it insufficient to optimal photosynthesis, making HCO_3^- the major DIC source for carbon fixation. However, host lipid membranes are impermeable to HCO_3^- and therefore it must be actively transported through host membranes.

Similarly to microalgae, symbiotic cnidarians possess CCM that efficiently exploits HCO_3^- pool in the seawater and increases the concentration of CO_2 reaching Rubisco (Allemand et al. 1998). Bicarbonate is actively transported by the presence of an H^+-ATPase pump located in the epidermal layer, which by the secretion of H^+ leads to the acidification of the

external medium, and consequent protonation of HCO_3^- to carbonic acid (H_2CO_3) (Furla et al. 2000, Bertucci et al. 2010). A membrane-bond carbonic anhydrase (CA) is responsible for the reversible conversion of HCO_3^- to CO_2, which can then freely diffuse into host cells cytoplasm. Once in the cytoplasm, CO_2 is trapped and converted to HCO_3^- by another CA, in agreement to intracellular pH (Venn et al. 2009). The transport of HCO_3^- to the gastrodermal cells may involve the presence of bicarbonate active transporters (Zoccola et al. 2015). Once HCO_3^- finally reaches the gastrodermal cells, a CA located close to the symbiont membrane protonates HCO_3^- into CO_2, and this is made available to photosynthesis (Fig. 2.4) (Furla et al. 2000a).

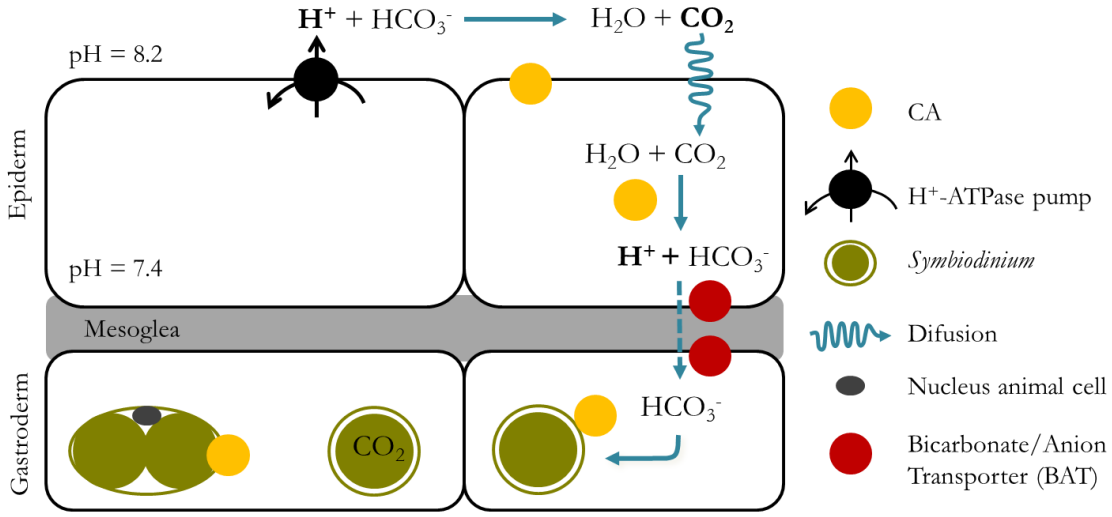

Figure 2.4 - Model of dissolved inorganic carbon absorption in non-calcifying symbiotic cnidarians (modified from Furla et al. 2000 and Zoccola et al. 2015).
CA = carbonic anhydrase

Key enzymes involved in the CCM are the carbonic anhydrases. They are present in almost all organisms (Eubacteria, Archaea and Eukaryota) and comprise six distinct classes: α-CAs, β-CAs, γ-CAs, δ-CAs, ζ-CAs and η-CAs (Le Goff et al. 2016). They are located in the cytosol, mitochondria, and membrane or can be secreted. In cnidarians, CA have been molecularly identified and divided according with their phylogeny into three distinct groups, secreted or membrane-bound, cytosolic and mitochondrial proteins and carbonic anhydrase related proteins (Bertucci et al. 2013). The α-CA are present in all metazoans and in cnidarians their role has been mainly investigated in biocalcification and photosynthesis, both processes related with the inorganic carbon uptake (Bertucci et al. 2013, (Le Roy et al. 2014). For instance, the STPCA (*Stylophora pistillata* CA), located in the calicoblastic epiderm (site of calcification), is directly linked to calcification, while the two CA transcripts identified in *A. viridis* (*AvCa2m*, *AvCa2c*; Ganot et al. 2011) are upregulated

in symbiotic (*vs.* aposymbiotic) specimens. *AvCa2m* is expressed in the gastrodermal tissues where symbionts are located, and the cytoplasmic *AvCa2c* is equally distributed in both epiderm and gastroderm. Both CAs are involved in inorganic carbon exchanges in membranes (*AvCa2m*) and in cytosol (*AvCa2c*) (Ganot et al. 2011). Additionally, the recent analysis performed in the laboratory of the whole *A. viridis* transcriptome has allowed the identification of two other complete α-CA: *AvCa2mE* (with specific epidermal expression pattern and membrane-bound sequence characteristics) and *AvCas* (with no specific tissue expression pattern and cytosolic sequence characteristics) (cf Annexe 1).

Effect on CCM of unicellular eukaryotes

Studies up-to-date show very different responses of microalgae species to high pCO_2, with results being species specific and related to the efficiency of CCM's (e.g. Salih et al. 2011; Johnson et al. 2013; (Gao & Campbell 2014). For instance, species that have no HCO_3^- uptake ability (inefficient CCM) and are currently CO_2-limited could directly benefit from this increase in CO_2. These species are physiological identified as "CO_2-users" (Fig 2.5B). Species with efficient CCMs, "HCO_3^--users", are under-saturated at current carbon chemistry and could any way benefit from future predicted CO_2 increase (Fig 2.5A).

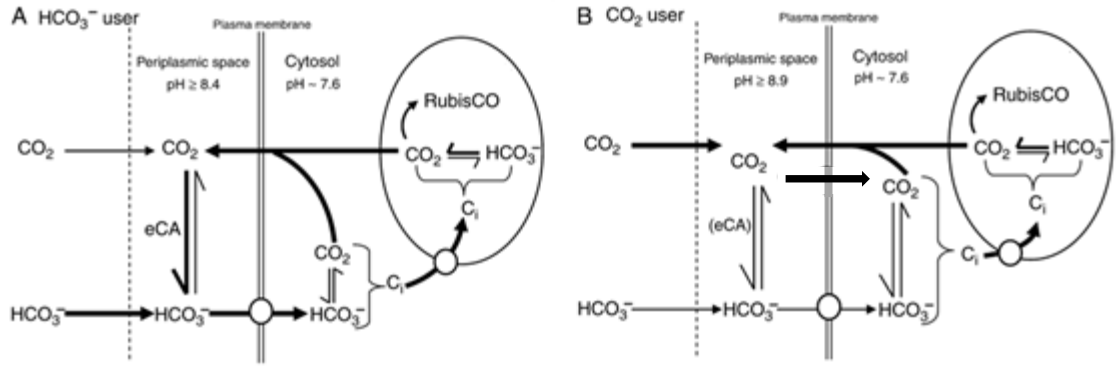

Figure 2.5 - Model of CCM in diatoms.
(A) in "HCO_3-users", the DIC necessary for photosynthesis (Ci) comes mainly from HCO_3^- transporters at the plasma membrane. In addition, CO_2 leaking out of the cell would be converted by an external CA (eCA) to HCO_3^- and subsequently removed through HCO_3^- transporters. (B) in "CO_2-users", CO_2 is the favourite DIC source used for the photosynthesis coming from external seawater and CO_2 leaking out the cell (from (Trimborn et al. 2008).

The increase of passive CO_2 diffusion and the concomitant down-regulation of active uptake of HCO_3^- allow energy save, favouring allocation to growth and reproduction (Wu et al. 2008, Rost et al. 2008). Indeed, (Hopkinson et al. 2011)) showed that a doubling increase in CO_2 concentration could result in a 20% save in CCM active carbon transport mechanisms in several diatom species. In addition, other studies on diatoms species

(*Thalassiosira weissflogii*, *Phaeodactylum tricornutum* and *Nitzchia navis-varingica*) have shown a down regulation of CCM accompanied by a decrease in external CA activities (Burkhardt et al. 2001, Trimborn et al. 2008) following exposure to elevated pCO_2.

Dinoflagellates are another group of phytoplankton which has an additional challenge in DIC absorption since those algae have the form II Rubisco, with much lower affinity to CO_2 than the form I in other microalgae (Whitney et al. 1995). At current carbon chemistry, dinoflagellates are under-saturated on CO_2 and once again the role of CCM is fundamental (Leggat et al. 1999). However, with the expected increase in pCO_2, diffusion of CO_2 will also increase. Brading et al. (2011) observed different responses to increase CO_2 in different phylotypes of dinoflagellates from the genus *Symbiodinium*: (1) no phenotypic effect, (2) increased photosynthesis but not growth, and (3) increased growth without change in photosynthesis. These differences were probably the result of different carbon species preference and efficiency on CCM modulation in the different phylotypes; the phylotypes with absence of response are strictly "HCO_3^--users", therefore increase in CO_2 did not bring higher benefits. Those phylotypes using preferentially CO_2 have seen their photosynthesis and by direct consequent their growth stimulated. In between, some phylotypes present the plasticity to modulate CCM to adjust to increase CO_2, with notably down-regulation of CA (Van de Waal et al. 2013).

Effect on CCM of non-calcifying symbiotic cnidarians

Recent studies on non-calcifying symbiotic cnidarians have shown that some species can thrive under future elevated pCO_2 conditions (e.g. (Towanda & Thuesen 2012, Suggett et al. 2012b, Jarrold et al. 2013a). Studies on the response of non-calcifying symbiotic cnidarians to future predicted pCO_2 have been done following two strategies: (1) through a prolonged exposure in the field, or (2) through a short-term acclimation in laboratory. *In situ* studies use CO_2 vents as a natural laboratory to investigate the response of marine organisms to OA. Briefly, CO_2 vents produce a natural gradient of pH, with a carbon chemistry environment that can be used as an experimental site to assess the impact of future predicted pCO_2 levels (Hall-Spencer et al. 2008). A few CO_2 vents have been identified, characterized and validated for *in situ* studies: in the Mediterranean Sea (Ischia and Vulcano Island, Italy), in Papua New Guinea (Dobu, Esa'Ala and Upa Upasina) and in the Atlantic Sea (La Palma Island). Each of these vents occurs in different habitats, allowing studies on different communities, macroalgae and coralligenous in the Mediterranean, shallow coral reefs in Papua New Guinea. These areas are considered important to study acclimatization to OA and understand the impact on ecosystems. A

number of studies in different CO_2 vents have shown major shifts in benthic ecosystems: (1) from hard to soft corals (Inoue et al. 2013), (2) from corals to macroalgae dominance (Fig. 2.6A; (Enochs et al. 2015). In contrast, others investigations have revealed putatively tolerant species to high pCO_2 (Johnson et al. 2012, Uthicke et al. 2016), specially non-calcifying species (Suggett et al. 2012; Horwitz et al. 2015). Particularly, studies on *A. viridis* in Vulcano Island CO_2 vents report the capacity of this species to thrive under future predicted pCO_2 levels; with increased sea anemone abundance, enhanced photosynthetic productivity, increased fitness and shift for a higher autotrophic/hererotrophic input (Fig. 2.6B; Suggett et al. 2012, Borell et al. 2014, Horwtiz et al. 2015). Therefore, these studies suggest the potential for adaptation of this species to long-term chronic exposure to high pCO_2.

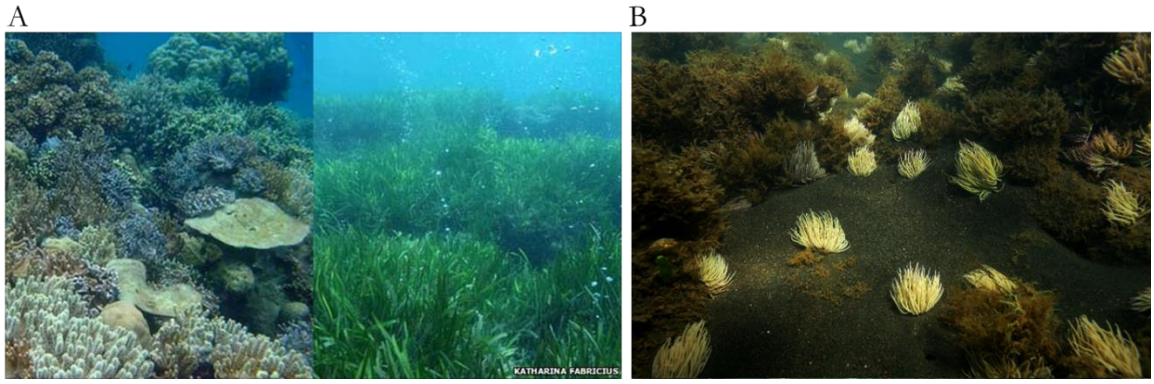

Figure 2.6 - CO_2 vents naturally bubbling CO_2 from seafloor acidify water.
(A) Papua New Guinea CO_2 vents. In the left image we can see a higher dominance of coral species (in normal pH site), clearly contrasting with the seagrass dominance on the right (in acidified site) (source: Katharina Fabricius); (B) Vulcano CO_2 vents, with dominance of non-calcifying species (source: Riccardo Rodolfo-Metalpa).

The second strategy to understand impact of OA to non-calcifying organisms is through short-term, rapid perturbation experiments (few weeks). Short-term laboratory experiments show similar results to *in situ* studies with general benefits in the physiology performance of these organisms, with a general trend of increased photosynthetic efficiency and productivity, carbon translocation to the host and fitness (e.g. (Jarrold et al. 2013a, Gibbin & Davy 2014, Hoadley, Pettay, et al. 2015). Both approaches have shown the outcomes of exposure to high pCO_2, a crucial step to understand the consequences to marine ecosystems, showing the capacity of non-calcifying cnidarians to cope with future predicted increases in pCO_2. More importantly, *in situ* and laboratory studies suggest the existence of high levels of phenotypic plasticity that allow maintenance and/or increase in the physiological traits of these species. However, it is fundamental to determine the mechanisms behind this capacity to thrive under future OA scenarios, and define if the

response of these organisms is the result of an adaptive phenotypic plasticity, leading to genetic changes, therefore acclimatization or an intermittent response, with no consequence for the evolution of these organisms.

2.3 Role of phenotypic plasticity in the response to OA

Future predicted CO_2 levels induce a plastic response to non-calcifying organisms, which are therefore capable to thrive and survive under future scenarios. However, it is fundamental to understand the mechanisms behind this plasticity and the consequences at the level of physiological traits.

Therefore, the first question addressed in this PhD was:

Q1: Does the increase in *p*CO_2 concentrations affect DIC uptake mechanisms in the sea anemone, *Anemonia viridis*? Are they able to acclimate/adapt after short- and/or long-term exposure, and by which mechanisms?

PAPER 1

Resilience to ocean acidification: decreased carbonic anhydrase activity in sea anemones under high pCO_2 conditions

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Resilience to ocean acidification: decreased carbonic anhydrase activity in sea anemones under high pCO_2 conditions

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ABSTRACT: Non-calcifying photosynthetic anthozoans have emerged as a group that may thrive under high pCO_2 conditions *via* increased productivity. However, the physiological mechanisms underlying this potential success are unclear. Here we investigated the impact of high pCO_2 on the dissolved inorganic carbon (DIC) use, in the temperate sea anemone *Anemonia viridis*. We assessed the impacts of high pCO_2 long-term exposure, i.e. *in situ* natural CO_2 vents (Vulcano) sampling, and short-term exposure, i.e. during a three-week controlled laboratory experiment. We focused on photo-physiological parameters (net photosynthesis rates, chlorophyll *a* content and *Symbiodinium* density) and on carbonic anhydrase (CA) activity, an enzyme involved in the energy demanding process of DIC absorption process. Long-term exposure to high pCO_2 did not have an impact on *Symbiodinium* density and chlorophyll *a* content. Contrary, short-term exposure to high pCO_2 induced a significant reduction in *Symbiodinium* density, which together with unchanged net photosynthesis, resulted in the increase of *Symbiodinium* productivity *per* cell. Finally, in both *in situ* long-term and laboratory short-term exposure to high pCO_2 we observed a significant decrease in anemones' CA activity, suggesting a change in DIC use (i.e., from a HCO_3^- to a CO_2 user). This change could enable a shift in the energy budget that may increase the ability of non-calcifying photosynthetic anthozoans to cope with ocean acidification.

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Abstract

Non-calcifying photosynthetic anthozoans have emerged as a group that may thrive under high pCO_2 conditions *via* increased productivity. However, the physiological mechanisms underlying this potential success are unclear. Here we investigated the impact of high pCO_2 on the dissolved inorganic carbon (DIC) use, in the temperate sea anemone *Anemonia viridis*. We assessed the impacts of high pCO_2 long-term exposure, i.e. *in situ* natural CO_2 vents (Vulcano) sampling, and short-term exposure, i.e. during a three-week controlled laboratory experiment. We focused on photo-physiological parameters (net photosynthesis rates, chlorophyll *a* content and *Symbiodinium* density) and on carbonic anhydrase (CA) activity, an enzyme involved in the energy demanding process of DIC absorption process. Long-term exposure to high pCO_2 did not have an impact on *Symbiodinium* density and chlorophyll *a* content. Contrary, short-term exposure to high pCO_2 induced a significant reduction in *Symbiodinium* density, which together with unchanged net photosynthesis, resulted in the increase of *Symbiodinium* productivity *per* cell. Finally, in both *in situ* long-term and laboratory short-term exposure to high pCO_2 we observed a significant decrease in anemones' CA activity, suggesting a change in DIC use (i.e. from a HCO_3^- to a CO_2 user). This change could enable a shift in the energy budget that may increase the ability of non-calcifying photosynthetic anthozoans to cope with ocean acidification.

Introduction

Increasing anthropogenic CO_2 emissions are causing a reduction in ocean pH and shift in the relative proportion of dissolved inorganic carbon (DIC) species, a process called ocean acidification (OA; (Doney et al. 2009). Under future lower pH conditions the concentration of bicarbonate ions (HCO_3^-, most abundant DIC species) and dissolved CO_2 will be significantly higher, while the concentration of carbonate ions (CO_3^{2-}) will be lower (Doney et al. 2009). Reduced ocean pH and CO_3^{2-} are expected to negatively impact a wide range of calcifying species (Hofmann et al. 2010). In contrast, non-calcifying photosynthetic species may benefit from the increase in $[CO_2]$ associated with OA, through increased levels of productivity and growth (Koch et al. 2013).

Symbioses between non-calcifying anthozoans and dinoflagellates of the genus *Symbiodinium* are widespread in the marine environment, and play key ecological and biogeochemical roles (Muller-Parker & Davy 2001). These symbioses are mutualistic, thus providing advantages for both partners. The host supplies the *Symbiodinium* with mainly HCO_3^- as metabolic substrate and in return uses the photosynthate products to support its

own metabolism, growth and reproduction (Furla et al. 2005). To take advantage of the large HCO_3^- pool available at current ocean pH non-calcifying photosynthetic anthozoans, like many marine photosynthetic organisms, have evolved energy consuming carbon concentrating mechanisms (CCMs) (Giordano et al. 2005b). Initial DIC uptake by the host involves the secretion of H^+ by an H^+-ATPase, which acidifies the external medium at the level of the boundary layer, resulting in the protonation of HCO_3^- to carbonic acid (Furla et al. 2000b). DIC incorporation is then facilitated by a key CCM enzyme, carbonic anhydrase (CA). CA accelerates the conversion of carbonic acid into CO_2, which then diffuses into the host (Furla et al. 2000b, 2005, Bertucci et al. 2013). A number of studies have recently demonstrated that *Symbiodinium* productivity in non-calcifying anthozoans is enhanced under high pCO_2 (Towanda & Thuesen 2012, Suggett et al. 2012b, Jarrold et al. 2013a, 2014), suggesting that the DIC uptake mechanism is somehow affected. Increased photosynthetic activity is also closely linked with the ability of the host cells to maintain intracellular pH under acidified conditions (Gibbin et al. 2014). How future OA scenarios will affect the host' physiological mechanisms facilitating the process of photosynthesis is much less known.

Here we investigated how the symbiotic sea anemone, *Anemonia viridis* (Forskål, 1775), regulates its DIC use under high pCO_2 conditions. We tested the photo-physiology (net photosynthetic rates, chlorophyll *a* content and *Symbiodinium* density) and CA activity of *A. viridis* following exposures to present and future predicted CO_2 conditions *in situ* and in the laboratory. To assess the responses to long-term exposure to OA, we collected specimens of *A. viridis* living near natural CO_2 vents found at the volcanic island of Vulcano (Italy). Here, sessile benthic organisms are continuously exposed to high pCO_2 levels, similar to the ones expected for 2100 (Boatta et al. 2013). In addition, to investigate the responses of the symbiotic system under controlled conditions, we carried out a three-week laboratory experiment exposing anemones collected from the intertidal zone on the SW Coast of the UK. It was hypothesised that the increase in seawater pCO_2 would cause a decrease in CA activity, as anemones would need to rely less on HCO_3^- as their primary DIC source.

Materials and Methods

Long-term in situ exposure to high pCO_2

Individuals of *Anemonia viridis* were collected, from a maximum depth of 2.0 m, in May 2011 at Vulcano island (38º25'N, 14º57'E), NE coast of Sicily (Italy). We selected two sites, each one representing a different environmental condition (Table 2), according to (Suggett

et al. 2012b). One site at ambient pCO_2 (482 µatm, control pCO_2, n = 12), 300 m away from the vents and a second one, closer to the vents, at high and fluctuating pCO_2 (1461 µatm, high pCO_2, n=11). Samples were frozen and kept at -80 ºC.

Table 2. Seawater physico-chemical parameters measured at Vulcano (*in situ*) at both control and high pCO_2 sites (n=7) and during the 21 days of laboratory experiment: pH (NBS scale), temperature, total alkalinity (TA), carbon dioxide partial pressure (pCO_2), bicarbonate and carbonate ion concentration ([HCO_3^-] and [CO_3^{2-}], respectively), and aragonite saturation states (Ωara).
Data are expressed as means (min – max).

	Long-term *in situ*		Laboratory	
	Control	High pCO_2	Control	High pCO_2
pH	8.13 (8.35 - 8.06)	7.71 (8.24 - 6.80)	8.01 (7.99 – 8.07)	7.59 (7.56 – 7.67)
Temp (°C)	21.0	21.0	15.59 (15.1 – 16.2)	16.40 (15.9 – 16.6)
TA (µequiv kg^{-1})	2513.50	2535.36	2301.8 (2275.1 – 2340.9)	2276.1 (2201.1 - 2299.8)
pCO_2 (µatm)	482 (257 - 584)	1461 (358-12839)	520.5 (472.41 – 580.1)	1841.4 (1404.0 – 1955.1)
[HCO_3^-] (µmol kg^{-1})	2015 (1782-2076)	2319 (1926-2319)	1920.6 (1887.5 – 1944.0)	2141.7 (2041.0 – 2168.9)
[CO_3^{2-}] (µmol kg^{-1})	201 (177-290)	88 (11-248)	154.7 (139.9 – 164.6)	54.5 (51.7 – 64.5)
Ω_{ara}	3.1 (2.7-4.5)	1.3 (0.2-3.8)	2.4 (2.2 – 2.6)	0.8 (0.8 – 1.0)

Laboratory exposure to high pCO_2

On the 12th April 2014, 24 *A. viridis* individuals were collected from the intertidal zone of Burgh Island, Bigbury, UK (50°16'N and 3° 54' W). Anemones were maintained individually during four weeks in small pond plant baskets, to allow adjusting to laboratory conditions (T = 15.0 ± 0.5 °C, salinity = 37 ± 2 ‰, pH = 8.10 ± 0.05 (NBS scale) and under 200 µmol photons m^{-2} s^{-1} using LED strips (Reef White Aquabeam 600 Ultra Strips, Tropical Marine Centre, Bristol, UK) on a 14 h: 10 h (L: D) photoperiod. Sea anemones were fed *ad libitum* once a week on frozen shrimp.

Laboratory experimental design

Twelve sea anemones were exposed for three weeks to either control (450 µatm) or high (2,000 µatm) pCO_2 conditions. The high pCO_2 condition was chosen because previous experiments, both *in situ* (Suggett et al. 2012b) and laboratory (Jarrold et al. 2013a), have

shown that *A. viridis* is able to tolerate it. Photo-physiological parameters and CA activity were measured at the beginning of the experiment (day 0) and then again after 5 and 21 d of exposure. To ensure that feeding did not influence measurements, feeding was always carried out after our measurements.

Laboratory experimental setup

Re-circulating seawater CO_2 system was used to monitored and provide seawater at control or high pCO_2 conditions (for details see supplementary material). During the experiment, pH, temperature, salinity, total alkalinity and carbonate system parameters were measured and calculated in each experimental tray as detailed by (Jarrold et al. 2013a). Values for all seawater parameters are presented in Table 2.

Determination of photosynthetic rates

Photosynthetic rates were measured on two tentacles detached from each individual as the rate of net O_2 production, using closed system respirometry, and at the incubation light intensity of ~200 μmol photons m^{-2} s^{-1}. Tentacles were placed on the bottom of 5 mL chamber, with filtered (0.22 μm) autoclaved seawater at the corresponding pCO_2 treatment for 1 h, and a small magnetic flea to ensure water mixing. Oxygen levels in the chambers were first measured after 20 min of stabilisation, using a calibrated optical oxygen meter (OxySense 5250i, OxySense, Dallas, USA) and again after 60 min. All photosynthetic rate measurements were carried out in a controlled temperature room, at 15 ± 0.6 °C. Oxygenconsumption (MO_2) was calculated as the change in pO_2 h^{-1} from the linear least-squares regression of pO_2 (mbar) plotted against time (min). This was multiplied by the solubility coefficient for oxygen, adjusted for salinity and temperature, and the volume of water within each respirometer. MO_2 values were expressed as mmol O_2 min^{-1} g^{-1} protein.

Determination of chlorophyll *a* content, *Symbiodinium* density and protein content

Chlorophyll *a* was extracted from two tentacles, in cold absolute ethanol at 4 ºC in the dark, for 24 hours according to (Ritchie 2006). Chlorophyll *a* concentration was determined using a spectrophotometer reader (SAFAS Xenius XM, Monaco), calculated following the equation parameters of (Ritchie 2006)) and standardised *per Symbiodinium* cell numbers.

Symbiodinium density was determined according to (Zamoum & Furla 2012). Chlorophyll-free tentacles were immersed in NaOH 1M, incubated at 37 ºC for 30-45 min, to dissolve all animal tissue. To determine *Symbiodinium* density, 3 replicates *per* sample were counted

using a Neubauer haemocytometer. The remaining extract was used to determine protein content from which we normalised *Symbiodinium* density.

Determination of carbonic anhydrase activity

CA activity was measured using animal extracts. Animal cytosoluble proteins were extracted from four tentacles in ice-cold extraction medium (50 mM potassium phosphate, pH 7.8 and protease inhibitor cocktail P-8340, Sigma), and kept at 4 °C throughout the extraction procedure. Animal CA activity was determined according to (Weis et al. 1989). Briefly, animal CA activity was measured using CO_2 as a substrate and following the reduction of pH due to the hydration of CO_2 into HCO_3^- and H^+. Assays were performed by adding Veronal buffer (25 mM Na Veronal, 5 mM EDTA, 5 mM DTT and 10 mM $MgSO_4$; pH 8.2) into a measuring chamber and adding 100 μg proteins of animal extracts. The CA activity was normalized *per* animal protein content.

Statistical analyses

For the *in situ* experiment, we used Mann-Whitney U Tests to compare all measured parameters in anemones' tissues between control and high pCO_2 sites. For the laboratory experiment, the comparison of all measured parameters (except chlorophyll *a*) between control and high pCO_2 conditions was investigated with repeated measures ANOVA since the same individuals were sampled along the three weeks. Post-hoc tests were performed with Tukey's HSD tests. When assumptions failed, appropriate transformations were used. For chlorophyll *a*, no transformation could follow the assumptions and the non-parametric Friedman test was used. All statistical tests were carried out using STATISTICA 10.0.

Results

Long-term in situ exposure to high pCO_2

Symbiodinium density was not affected by exposure to high pCO_2. No differences between control and high pCO_2 sites were found (U = 143, p = 0.707; Fig. 2.7a). The same result was observed for chlorophyll *a* concentration, where values of chlorophyll *a* were similar for control and high pCO_2 sites (U = 156, p = 0.751; Fig. 2.7b).

Anemones CA activity was approximately 30 % lower in anemones exposed to higher pCO_2 conditions (Fig. 2.7c), this difference being statistically significant (U = 81, p = 0.042).

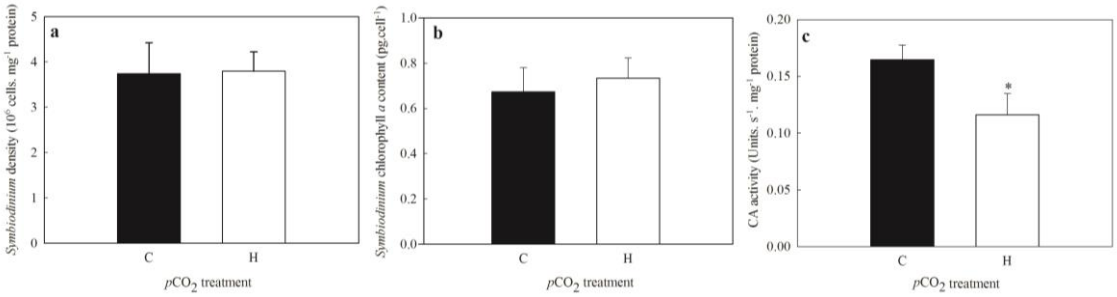

Figure 2.7 - The effect of *in situ* long-term exposure to 'control' (C, black) and 'high' (H, white) pCO_2 conditions on the mean *Symbiodinium* density (a) mean *Symbiodinium* chlorophyll *a* content (b), and mean carbonic anhydrase (CA) activity (c) in *A.viridis* at the Vulcano CO_2 vent.

* $p < 0.05$. Histograms represent mean data with error bars, n=12.

Short-term laboratory exposure to high pCO_2

Incubation during 5 and 21 d at high pCO_2 conditions resulted in a significant reduction of *Symbiodinium* density ($F_{2,44} = 16.015$, $p = 0.00001$; 0 vs. 5 d, $p = 0.003$; 0 vs. 21 d, $p = 0.008$; Fig. 2.8a). Conversely, concentration of chlorophyll *a* was not affected by exposure to high pCO_2, with values being unchanged during the 21 d of exposure (χ^2 (2) = 0.750, $p = 0.687$; Fig. 2.8b).

Net photosynthetic rates on individuals incubated during 5 and 21 d at high pCO_2 ($p = 0.6677$) were not affected by pCO_2 exposure ($F_{2,38} = 0.684$, $p = 0.510$; Fig. 2.8c).

Anemones CA activity decreased during the first 5 d of exposure to high pCO_2, but it was only significantly strongly down-regulated after 21 d of exposure ($F_{6,38} = 3.975$, $p = 0.003$; 0 vs. 21 d, $p = 0.0003$). Indeed, we found that CA activity decreased by 32% after 1 week of exposure to high pCO_2 and by 78% at the end of the 21 d experiment (Fig. 2.8d).

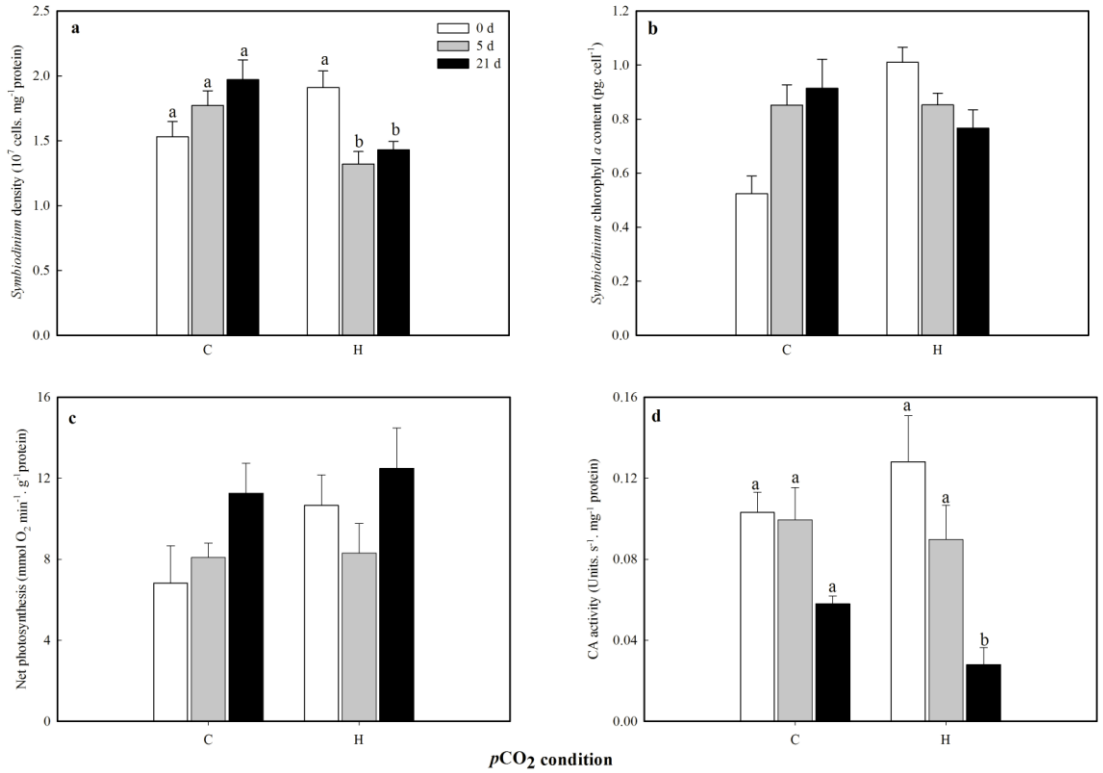

Figure 2.8 - The effect of laboratory short-term exposure to control (C) and high (H) pCO_2 on the mean *Symbiodinium* density (a) mean *Symbiodinium* chlorophyll *a* content (b), on the net photosynthesis rates (c) and on the CA activity (d) in *A.viridis*.

Data are shown at the beginning of the exposure (0 d, white bars), after 5 d (grey bars) and after 21 d (black bars). Histograms represent mean data with error bars, n=12. Means with different letters differ significantly from 0 d ($p < 0.05$).

Discussion

Contrary to hard corals, symbiotic sea anemones may thrive under future high pCO_2 levels ((Towanda & Thuesen 2012, Suggett et al. 2012b, Inoue et al. 2013, Gibbin & Davy 2014). To understand the mechanisms that underpin symbiotic sea anemones' ability to be successful under high pCO_2 conditions, we performed both an *in situ* (long-term) and a laboratory (short-term) exposure experiment. In particular, we wanted to define how future OA conditions would affect the use of DIC by the sea anemone, *A. viridis*.

Long-term in situ exposure to high pCO_2

Several studies have demonstrated the capacity of *A. viridis* to colonize the pCO_2 rich environment found near the CO_2 vents around Vulcano Island (Suggett et al. 2012b, Borell et al. 2014, Horwitz et al. 2015a). Even without significant difference in *Symbiodinium* density between *A. viridis* from the control and high pCO_2 sites (Borell et al. 2014, Horwitz et al. 2015a); and this study), increases in photosynthetic productivity in high pCO_2 sites

with an enhanced autotrophic/heterotrophic ratio have been observed (Suggett et al. 2012b, Horwitz et al. 2015a). However, an increase in *Symbiodinium* density at high pCO_2 sites compared with control sites was reported by Suggett et al. (2012). This result was potentially related with the normalization procedure used: surface area instead of milligrams of proteins an issue already raised by Horwitz et al. (2015).

One of the hypotheses proposed to explain the increase of productiveness is the modification of DIC use. In a "normal" pCO_2 environment, HCO_3^- is the preferential DIC species removed from sea water (Al-Moghrabi et al. 1996, Bénazet-Tambutté et al. 1996). In a high pCO_2 environment, however, CO_2 could replace HCO_3^- as the main carbon source for photosynthesis leading to a decrease in energy investment for arbonconcentrating mechanisms (CCMs). If the proportion of HCO_3^- uptake decreases favouring the less costly diffusion of CO_2, the role of CA could then be reduced. Indeed, we found a significant 30% decrease in total animal CA activity of *A. viridis* specimens from the high pCO_2 sites. A significant down-regulation in CA activity under OA conditions was also observed in the calcifying coral *Acropora millepora* (Moya et al. 2012b). Taking into account the role of CA in pH homeostasis, this CA reduction could reflect an adaptive mechanism that enable the anemone to deal with low pH conditions. This result is in agreement with the behaviour of free-living unicellular eukaryotic organisms, which modify the relative contributions of CO_2 and HCO_3^- uptake as a function of environmental pH, and become CO_2 users at low pH as demonstrated in diatoms (Wu et al. 2015) and coccolithophorids (Kottmeier et al. 2014); (Lohbeck et al. 2014a). Reduction of CCMs energy demands under high pCO_2 conditions could result in increased energy availability to other functions (i.e. reserve production or reproduction). Indeed, (Suggett et al. 2012b) reported an increase in the abundance of *A. viridis* around Vulcano at the high pCO_2 sites. Similarly, high pCO_2 exposure coincided with significantly greater asexual budding rates in the sea anemone *Exaiptasia pallida* (Hoadley, Rollison, et al. 2015).

Laboratory exposure

In this study we further explored the ability of *A. viridis* to tolerate OA by investigating its phenotypic plasticity response under controlled laboratory conditions. We exposed *A. viridis* specimens from the British Channel, which are genetically distant from those from the Vulcano population (D. Forcioli, *pers. comm.*), to high pCO_2 conditions for three weeks. We measured the same photosynthetic rate with 23% less *Symbiodinium*, resulting in increased productivity *per Symbiodinium* at high pCO_2, which was also observed in Vulcano

anemones (Horwitz et al. 2015). These results are in agreement with previous short-term experiments performed on other symbiotic cnidarians, which show that exposure to OA conditions induces a decrease in *Symbiodinium* density concomitantly with an increase of photosynthetic activity of the symbiotic cnidarian (Langdon & Atkinson 2005, Krief et al. 2010).

The depletion of *Symbiodinium* load in *A. viridis* could be linked to a shuffling of intra-individual *Symbiodinium* populations causing a modification of *Symbiodinium* productivity under high pCO_2 conditions. (Brading et al. 2011) showed that the response of several *Symbiodinium* tropical strains to high pCO_2 levels was phylotype-specific, going from unaffected to highly sensitive strains with changes in growth rate and photosynthetic capacity. In temperate symbiotic cnidarians, most of the *Symbiodinium* populations belong to phylotype 'temperate clade A', which nevertheless show high intra-clade variability (Forcioli et al. 2011). Unfortunately, no data is available concerning different physiological properties or pCO_2–specific-sensitivity of these intra-clade populations. Future works will need to address this important aspect of the biology of the *A. viridis-Symbiodinium* system, in order to more deeply understand the contribution of the resilience property of the symbiont.

Symbiodinium are thought to be CO_2-limited at current ocean pH conditions (Davy & Cook 2001, Verde & McCloskey 2007, Jarrold et al. 2013a), despite the presence of hosts CCM. Consequently, our observed increase in *Symbiodinium* productivity suggests that the DIC uptake mechanism is in some way affected. Indeed, similarly to the *in situ* experiment we report a decline of anemone CA activities after 21 d, which corroborates the hypothesis of a similar change in DIC use in non-pre-conditioned organisms (compared to Vulcano) and on the short-term scale. Host CA activities remained however unchanged after the first week of exposure. A similar result was observed in *E. pallida*, where no significant difference was detected between normal and high pCO_2 treatments (400-1000 ppm), within seven days, (Siddiqui & Bielmyer-Fraser 2015), demonstrating that shifting DIC use is not immediate.

The similarity of responses observed in our *in situ* and laboratory experiments suggest that the physiological plasticity observed in DIC use is an intrinsic property of *A.viridis* that does not appear to be the result of selection *in situ* at the Vulcano CO_2 vents. However, we cannot completely discard the idea that specimens of *A. viridis* from the UK population may have evolved the capacity to tolerate pH and pCO_2 fluctuations as a consequence of

inhabiting intertidal environments. The physiological plasticity we observed could also be linked to circadian intracellular pH variation (from pH 7.4 at night to 8.9 at day time; Furla et al. 2000) experienced by anemones during photosynthetic activity of the *Symbiodinium*. This confers symbiotic cnidarians a high buffering capacity (Laurent et al. 2014, Gibbin et al. 2014), which could increase resilience to OA.

In conclusion, this study showed that being able to shift the utilisation of different inorganic carbon species toward energetically most favourable ones (i.e. from HCO_3^- to CO_2) is a key feature enabling non-calcifying photosynthetic anthozoans to tolerate and thrive under long-term exposure to high pCO_2 levels, but also to allow rapid (3 weeks, from this study) acclimation to future predicted CO_2 conditions.

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Supplementary materials

Seawater chemistry for long-term in situ exposure

During the one week fieldwork, seawater pH (NBS scale) was measured each day (n=7) using a pH-meter and an electrode (Metrohm pH mobile). Seawater samples were filtered with a Whatman GF/F, treated with 0.05 ml of 50 % $HgCl_2$ (Merck, Analar) and stored in the dark at 4°C pending analysis. Three replicate were analysed at 25°C. Titration of TA standards provided by A.G. Dickson was within 0.5 μmol kg^{-1} of the nominal value. The other parameters of the carbonate system (pCO_2, CO_3^{2-}, HCO_3^-, and Ω_{ara}) were calculated from pH, mean TA, temperature, pressure and mean salinity using the free-access CO_2SYS (Pierrot et al. 2006) package. Data were in the range reported by Suggett et al (2012).

Experimental CO_2 re-circulating seawater system

Briefly, the system comprised of two large holding trays (vol. 300 L; one *per* pCO_2 treatment). The trays fed into one sump in which sea water was filtered, heavily aerated, and recirculated, *via* a submersible pump (1262; EHEIM GmbH and Co. KG, Deizisau,

Germany). The experimental design involved a certain level of pseudoreplication since one common sump supplied the two trays. However, this allowed the standardization of sea water quality before conducting experimental exposures. CO_2-enriched air was supplied to the corresponding holding tray *via* two large air stones and monitored using a CO_2 analyzer (280; LI-COR, Lincoln, NE, USA). Each holding tray contained two 900 L h^{-1} circulation pumps (Koralianano 900, Hydor, Sacramento, USA) to provide the anemones with ample flow rate. Temperature of the experimental system was maintained at 15 °C *via* the use of chiller units (L-500, Boyu, Raoping Guangdong, China). Finally, each tray contained 12 small baskets (L = 10 cm x W = 10 cm x H = 11 cm) to house anemones individually, and which were illuminated by three LED light strips (Reef White Aquabeam 600 Ultra Strips, Tropical Marine Centre, Bristol, UK). Approximately 10 % of the sea water in the system was replaced weekly, and deionized water was added as needed to maintain stable salinity levels.

Effect of repeated cutting of tentacles

In order to determine whether the repeated cutting of tentacles throughout the experiment had a significant effect on anemones performance we kept another two sets of eight anemones incubated at control conditions that were only sampled once, one set on day 5 and the other on day 21. We performed a Mann-Whitney U Tests to compare the different responses (Table S1).

Table S1. Comparison of the responses of anemones in control condition with tentacles removed at each time point versus anemones from which we remove tentacles only on a specific sampling point.

Trait	Time point (d)	Control	Time point 5d	Time point 21 d
Symbiodinium density	5 d	1.77±0.15	1.57±0.126	-
(10^7 cells. mg^{-1} protein)	21 d	1.97±0.153	-	1.81±0.06
Symbiodinium chlorophyll *a* content	5 d	0.851±0.08	0.918±0.094	-
($pg.cell^{-1}$)	21 d	0.914±0.107	-	1.091±0.092
Net Photosynthesis	5 d	8.087±0.701	8.39±1.318	-
(mmol O_2 min^{-1}. g^{-1} protein)	21 d	11.26±1.48	-	15.82±5.476
CA activity	5 d	0.09±0.01	0.07±0.01	-
(Units s^{-1}. mg^{-1} protein)	21 d	0.093±0.02	-	0.083±0.001

2.4 Impact of multiple stressors in cnidarians

Rising CO_2 emissions to the atmosphere are responsible for OA as well as global warming. The oceans uptake 30% of the atmospheric CO_2, yet its buffering capacities endure also the absorption of heat energy, leading to the rise of sea water temperature (Hoegh-Guldberg & Bruno 2010). In the last century, seawater temperature has increased by 0.4 – 0.8 ºC and is expected to increase from 1.2-3.2 ºC by 2100 (IPCC 2013; (Gattuso et al. 2015).

Increased seawater temperature has the potential to disrupt symbiosis in symbiotic cnidarians, leading to the loss of *Symbiodinium* or the decrease in photosynthetic pigments *per* cell as part of a stress response, a process commonly referred as bleaching (Lesser 2011). A change in the average temperature as low as 2-3 ºC is enough to induce damage, and the consequent loss of symbionts (Lesser 2004). Bleaching events have increased in recent years, rising the potential to long-term damage and mortality in corals and sea anemones, leading to the decline of coral reef ecosystems (Hoegh-Guldberg 2014).

OA and seawater-increased temperatures are two of the main threats to marine organisms. However, even if these two stressors will likely be combined in future climate change, the synergistic or antagonistic effects of these two factors are poorly known (Hoegh-Guldberg et al. 2007, 2014). The multiple effects of OA and increased seawater temperature are expected to negatively impact marine organisms (Hoegh-Guldberg et al. 2007). Studies of interaction between high pCO_2 and increased temperature on calcification, metabolism and coral mortality foresee a poor future for coral reefs, driving the ecosystems towards non-calcifying species (Hoegh-Guldberg et al. 2007, (Anthony et al. 2008). For instance, changes in OA and increased seawater temperature had an effect on the calcification rates (i.e. reduction by 50 % in the coral *Stylophora pistillata*; Reynaud et al. 2003) and also on metabolism in cold-water corals (Gori et al. 2016). While several studies on the impact of combined increased temperature and acidification clearly show a negative trend for symbiotic calcifiers, only few investigations have been carried out on non-calcifying species. The non-calcifying symbiotic sea anemone, *A. viridis*, inhabits rock pools in the intertidal zone, being exposed frequently to short-term environmental fluctuations (e.g. light intensity and temperature). *A. viridis* has demonstrated tolerance in response to OA, with increased abundance and improvement of physiological performance, showing clear signs of plasticity under future predicted pCO_2 scenarios (Suggett et al. 2012, Jarrold et al. 2013; Ventura et al. 2016). However, an increase in seawater temperature induces bleaching events in this species, due to oxidative stress and apoptotic events, yet without increased mortality (Richier et al. 2006, Moya et al. 2012). Thus, these two stressors independently

induce two different responses in this species. In this study we were interested in assessing the impact of the combined effects of high pCO_2 and increased temperature in *A. viridis* and evaluate the mechanisms behind a putative plasticity to climate change.

Q2: Does the combined effect of multiple stressors, increased temperature and high pCO_2 affect DIC uptake mechanisms in the sea anemone, *Anemonia viridis*?

Material and Methods

To investigate the effects of short-term exposure to high pCO_2 and increased temperature on the carbon uptake mechanism of *A. viridis* two levels of pCO_2 (cf. Ventura et al. 2016) and temperature were chosen. The two selected temperatures represented current average summer seawater temperatures in the South Coast of England (15 °C), and a +5 °C increase (20 °C) based on prediction for the year 2100 (IPCC, 2013). Anemones were exposed for 21 days to one of four treatments: "control" (15 °C + pCO_2 450 μatm), "high pCO_2" (15 °C + pCO_2 2000 μatm), "elevated temperature" (20 °C + pCO_2 450 μatm), or "combined" (20 °C + pCO_2 2000 μatm; Table 3) in a NERC-funded Experimental Gas and Temperature Manipulation Mesocosm (cf. Ventura et al. 2016). Seawater temperature was increased to 20 °C in the elevated temperature and combined treatment using aquarium heaters (EHEIM Jager GmbH and Co. KG, Stuttgart, Germany) placed into the corresponding header tanks (50 W) and holding trays (300 W). Measurements of physiological parameters (*Symbiodinium* density, chlorophyll *a* and net photosynthesis) and CA activity were taken before the beginning of exposure (0 d), after 5 d and in the end of the exposure (21 d). For measurement details please refer to Ventura et al. (2016).

Table 3. Summary of aquarium set-up treatments.
Data presented as mean ± SE, n=12.

	Control	High pCO_2	Elevated Temperature	Combined
pCO_2 (μatm)	500	2000	500	2000
Temperature (ºC)	15	15	20	20

Results

Mean *Symbiodinium* density in *A. viridis* samples ranged from 2.0×10^7 mg^{-1} protein at 0 d to 1.24×10^7 mg^{-1} protein after 21 d of exposure to elevated temperature, reflecting a significant decrease of 34 % (Fig. 2.9a; $F_{(6, 88)} = 15.091$; $p = 0.00012$). Similarly, the combined (20 °C and pCO_2 2000 μatm) treatment induced a loss of symbionts after 21 d of

exposure, from 1.54 × 10^7 mg^{-1} protein at 0 d to 1.04 × 10^7 mg^{-1} protein at 21 d (Fig. 2.9a; $F_{(6, 88)}$ = 15.091; p = 0.002).

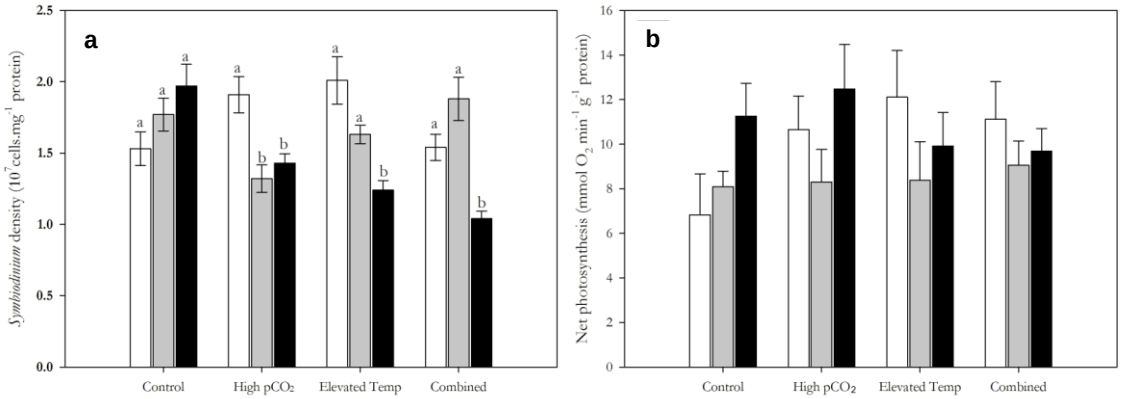

Figure 2.9 - The effect of laboratory short-term exposure to control, high *p*CO_2, elevated temperature and combined treatment on (a) the mean *Symbiodinium* density, (b) on the net photosynthetic rates.
Data are shown at the beginning of the exposure (0 d, white bars), after 5 d (grey bars) and after 21 d (black bars). Means with different letters differ significantly from 0 d (p < 0.05). Mean data with error bars, n = 12.

Mean sea anemone net photosynthetic rate was not affect by exposure to elevated temperature, neither to combined stressors during the 21 d of exposure (Fig. 2.9b; $F_{(3, 41)}$ = 0.57562, p = 0.63431). Similarly, mean *Symbiodinium* chlorophyll *a* content was not affect by both exposure to elevated temperature and combined treatments during the 21 d of exposure ($\chi^2(2)$ = 0.291667, p = 0.86430).

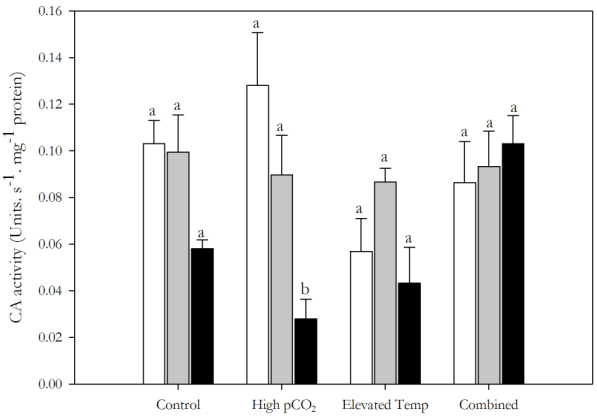

Figure 2.10 - The effect of laboratory short-term exposure to control, high *p*CO_2, elevated temperature and combined treatment on the carbonic anhydrase activity in *A.viridis*.
Data are shown at the beginning of the exposure (0 d, white bars), after 5 d (grey bars) and after 21 d (black bars). Means with different letters differ significantly from 0 d (p < 0.05). Mean data with error bars, n = 12.

In contrast with pCO_2 treatment, showing a significant decrease after 21 d mean anemones CA activity was not affected by exposure to elevated temperature neither combined treatments after 21 d of exposure (Fig. 2.10; $F_{(6,38)} = 3.9755$, $p = 0.622803$).

Discussion

Overall, this study shows that when global change stressors are combined, increased temperature override high pCO_2 effects on physiology and DIC absorption mechanisms in *A. viridis*.

A. viridis bleached under elevated temperature alone, after an exposure of 21 d contrasting with the earlier symbiont loss at 5 d after an exposure to high pCO_2. Sensitivity to thermal stress was previously observed in *A. viridis* with bleaching events being caused by an increase in oxidative stress after a thermal stress (+8 and +10 ºC; Richier et al. 2006, Moya et al. 2012). Moya et al. (2012) saw that the major thermal stress in gene expression occurs immediately after 24 hours exposure followed by a return to baseline gene expression after 2 days of exposure. However, in these two studies, induced bleaching was caused by a +10 ºC thermal stress, whereas in this study we induced a rather lower thermal stress (+5 ºC). This suggest that a +5 ºC temperature stress in short-term (5 d) probably does not induce immediate oxidative stress and only a prolonged exposure (21 d) results in oxidative stress finally triggering the expulsion of symbionts. Similarly to high pCO_2, elevated temperature did not induced change in net photosynthetic rates, which together with loss of *Symbiodinium* reflects a higher productivity *per Symbiodinium* cell under elevated temperature.

Up until now, the effects of temperature in the components of CCM, and specifically on CA activity are rather unclear (Giordano et al. 2005). An increase in temperature would generate a reduced solubility of CO_2, therefore, an increased need for CCM, and consequently to CA activity (Beardall et al. 1998). However, studies in the Antarctic have shown that despite the low temperatures/higher solubility of CO_2, microalgae possess CCM activity (Mitchell & Beardall 1996). In our study, elevated temperature alone did not induce change in CA activity during the 21 d of exposure. This result is in accordance with (Graham et al. 2015)), which reported no effect of increased temperature in host CA activity in the zoanthid, *Zoanthus sp.* In addition, our results suggest that the down-regulation of CA genes under thermal stress measured by Moya et al. (2012) is a transient and gene specific transcriptomic state, which not affects the global CA activity in the host tissue. We could also suggest that the lower temperature and range stress, +5 °C, 15° to

20°C in our study compared to +10 °C, 18°C to 28°C in Moya et al. (2012), did not inhibit enzymatic activity, since we did not reach the hysteresis point. The temperature applied could then still be in the range of optimum temperature for enzymatic processes.

In our study, the synergistic effect of elevated temperature and high pCO_2 on *Symbiodinium* density, net photosynthesis and carbonic anhydrase activity, shows a predominant effect of elevated temperature over high pCO_2, with temperature somehow mitigating the more marked effects of pCO_2. The combined effect of elevated and high pCO_2 did not induce change in CA activity. This result is in accordance with results observed in *Palythoa* sp. (*Symbiodinium* clade C), where the combination of elevated temperature and high pCO_2, contrarily to what was expected, did not change CA activity. One possible explanation is that the thermal stress, affected lipid membrane permeability of *A. viridis*, reducing the passive diffusion of CO_2, even under a high pCO_2 environment (Raven & Geider 1988). Indeed, the macroalga *Laminaria saccharnia*, a "CO_2-user" species (totally depending on CO_2 diffusion) presents a better rate of photosynthesis at lower temperatures (i.e. higher CO_2 solubility) (Raven & Geider, 1988). Another explanation comes from the absence of temperature effect on symbiont photosynthesis (present study). The absence of photosynthetic inhibition has been demonstrated to be linked to an increase in CCM performance in free-living and freshly isolated Symbiodinium (Oakley et al. 2014). Therefore, in *A. viridis* at elevated temperature and high pCO_2, active absorption of DIC (through bicarbonate pool) is maintained and CA activity remains constant.

In conclusion, high pCO_2 and elevated temperature did not have an additive effect, with a predominance of the temperature response. At short term, the maintenance of exclusive HCO_3^- absorption under high pCO_2 and temperature increase is not detrimental. Nevertheless, we could postulate that, at long term, the absence of CCM regulation might be costly and affect the fitness of non-calcifying species, which up-until now where seen to be able to cope with the future predicted climate change.

2.5 Conclusions

In this chapter, we investigated the phenotypic plasticity of the sea anemone *A. viridis* under short and long-term exposures to high pCO_2 and multiple stressors, combined effects of elevated temperature and high pCO_2. We were interested in deciphering the mechanisms of DIC uptake, with high relevance for a comprehensive understanding of physiological changes leading to plasticity.

Under a high pCO_2 scenario, individuals from both laboratory short-term experiments and *in situ* chronic exposure change their DIC preferential species, from HCO_3^- to CO_2. The

capacity to change DIC species is a trait that allows *A. viridis* to survive and thrive under future pCO_2 scenarios. Evidence that, during both short- and long-term exposures to high pCO_2 and in two genetically different and poorly connected *A. viridis* populations (Plymouth *vs* Vulcano), the pattern of change is the same suggests an inherent phenotypic plasticity of *A. viridis* not specifically selected in the Vulcano population.

To further understand the biochemical mechanisms involved in the CA regulation we measured the expression levels (by qRT-PCR) of 4 *A. viridis* α-CA transcripts on Vulcano samples, and we have demonstrated an absence of gene expression change (Figure 2.11).

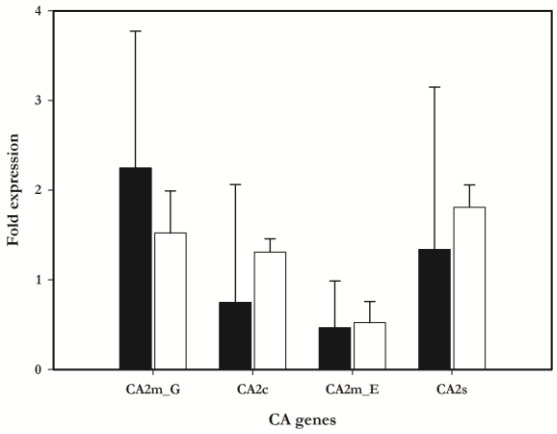

Figure 2.11 - Variation of gene expression of carbonic anhydrase genes after *in situ* long-term exposure to high pCO_2 in Vulcano CO_2 vents.

Real-time quantitative PCR quantifications (± SE) for 4 α-CA transcripts**.** cDNAs were prepared using SuperScriptII reverse transcriptase. Transcript-level quantification was performed using the SYBR green fluorescence method and a Light Cycler 480. Each sample was run in triplicate**.** Two control genes were chosen based on previous results**:** RCC2 (Regulator of Chromosome Condensation protein 2) and COP-γ (Coatomer subunit gamma). A reliable normalization factor was calculated based on the expression level of the most stable control gene. Expression levels of target genes were normalized using the normalization factor and the results given as expression relative to the control gene value as calibrator. The significance of the results was Mann-Whitney U Tests to compare CA gene expression between control and high pCO_2 sites. The results were considered statistically significant when $p < 0.05$. Black bars – control pCO_2, white bars – high pCO_2 bars; n=4.

However, these are preliminary results and an exhaustive analysis of the entire repertoire of CA genes could definitively consider the involvement of transcriptional regulations. Finally, post-translational modifications (i.e. phosphorylation or glycosylation) could explain the decrease in CA activity under high pCO_2 exposure.

3. Chapter 3 – Development of a new *in vitro* cellular tool for the study of cnidarian phenotypic plasticity

3.1 Cnidarian cellular phenotypic plasticity

In the last chapter we have shown the existence of a phenotypic plasticity in response to environmental stressors at the level of the organism. In this chapter, we propose to develop a new tool to assess cnidarian plasticity at the cellular level.

During the establishment of life in symbiosis, symbiotic cnidarians had to adjust several aspects of their cellular biology. The first evidence is the entry of *Symbiodinium* into host cell cytoplasm, which triggers a series of cellular reactions. The engulfment of the symbiont leads to adjustments in cellular architecture, such as host cytoskeleton remodelling (Fig. 3.1).

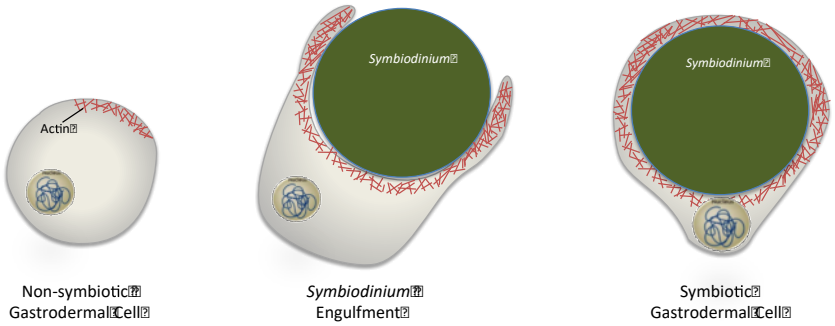

Figure 3.1 - Schematic representation of the actin cytoskeleton reorganization during symbiosis establishment (source: Paola Furla).

This cellular adjustment allows the host cell, with an average size between 2-5 μm, to alter its cellular volume and architecture to incorporate a *Symbiodinium* cell with the double its size (10 μm). Once incorporated, the space left in the host cytoplasm is minimal, being sometimes hard to identify host outer layer. Additionally, symbiotic cell-specific density contained inside a host cell, is on average 1 to 2 symbionts *per* cell but up to 12 symbionts have been found in some exceptional cases (Fig. 3.2; (Muscatine et al. 1998).

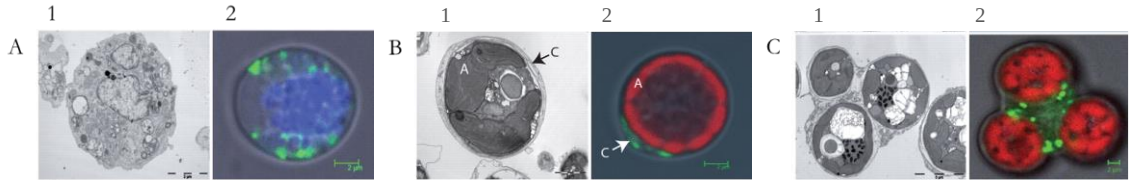

Figure 3.2 - Gastrodermal cells of *A. viridis* with and without *Symbiodinium*.
(A) Gastrodermal cell without any symbiont, (B) with one symbiont and (C) three symbionts. Panel 1 – Transmission electron microscopy image; panel 2 – confocal images. A – Intracellular alga, C – gastrodermal cell cytoplasm. Red colour – autofluorescence of *Symbiodinium* cell chlorophyll pigment; blue colour – nucleus stained with Hoechst 33342 and green colour - mitochondria stained with Rhodamine 123 (modified from **(Venn et al. 2009)**.

The second evidence is the arrest of the phagocytosis, avoiding the intracellular digestion of the algae in the symbiosome, a membrane derived from the host plasma membrane during phagocytosis. The symbiosome is perceived as an arrested phagosome, which avoids fusion of the phagosome with the lysosome, thus allowing persistence of symbionts inside host cells. Mechanisms that allow this arrest have not been fully determined yet, but some studies give us clues on how this process happens. (Mohamed et al. 2016)) observed the up-regulation of genes encoding proteins, Rab superfamily, involved in vesicle trafficking and membrane fusion, after infection of the larvae of the coral *Acropora digitifera* by *Symbiodinium*. Previous studies on the sea anemone *Aiptasia pulchella* have also identified Rab proteins as key actors of symbiosis establishment. More specifically, in this species the recruitment of specific Rab proteins is necessary for stabilization following invasion of the symbiont and to avoid maturation of the phagosome (Chen et al. 2004, Hong et al. 2009).

Environmental stressors are also able to trigger a plastic response at cellular level, which will lead to a series of cellular processes, causing symbiosis dysfunction and breakdown. Symbiont loss following a stress event is thought to happen through: (1) digestion of symbionts, with host actively digesting the symbiont *via* Rab proteins, leading to lysosome maturation and fusion with symbiosomes, or the symbiont is actually dying and degrading; (2) exocytosis by active expulsion of symbionts by host cell, (3) release of host-cell containing the symbionts, (4) apoptotic-host cell death or (5) necrosis of host cells (Fig 1.9; Weis 2008). Initiation of apoptosis of host cells could be an innate immune response of the host that detects increased oxidative stress of symbiont cells and targets host cells containing symbionts to be deleted, therefore mitigating damage of reactive oxygen species (ROS) to the host and maintaining tissue homeostasis (Dunn et al. 2004, Weis, 2008). Finally, necrosis is an uncontrolled cell death event, assumed to happen under extreme

stress conditions opposite to moderate stress where controlled apoptotic events are more frequent ((Gates et al. 1992, Dunn et al. 2004, Weis 2008).

Despite the great advances in the study of symbiosis-induced phenotypic plasticity, cellular and molecular aspects remain unanswered, as stressed recently by several authors (Weis et al. 2008, Weis & Allemand 2009, Davy et al. 2012). Specifically, fundamental aspects of cellular mechanisms: (1) the recognition of the symbiont, (2) the regulation of symbiont cell division within the host and in parallel with host cell division, (3) the inter-partner signalling for molecular communication and (4) the processes conducting to symbiosis breakdown (bleaching). The answer to these questions requires developing a simplified biological model from cnidarian organisms: the *in vitro* cell culture. Many efforts were done during the last century to develop and to establish cell cultures from multicellular organisms. All efforts on culture cell establishment have then increased the experimental possibilities in a laboratory context, opening the door to the development of new methodologies and giving access to a series of classical and emerging model organisms.

3.2 Vertebrate cell culture

3.2.1 Origin and contribution of vertebrate cell cultures

The beginning of cell culture, i.e. culturing of cells derived from tissues under controlled conditions, was in 1885, when Wilhelm Roux maintained embryonic chick cells in a saline culture. Yet, the development of cell culture methodology is attributed to (Harrison et al. 1907), who cultivated frog nerve cells in a "hanging drop" (inverted drop) method. Since then, and especially from the second half of the 20th century, knowledge on cell cultures has progress exponentially with the development of chemical culture medium (with physicochemical conditions similar to those in the animal), use of antibiotics to reduce contamination and sub-culturing of cells. Development of cell culture was done with the aim of understanding cell behaviour and functions. Specifically, mammalian cell cultures have contributed in the area of cancer research (radiation and drugs), virology (vaccine production), genetic engineering (production of viruses to use in vaccine production), toxicity (effect of drugs on cells), aging, and tissue engineering (Eibl et al. 2009).

3.2.2 Diversity of vertebrate cell culture methodologies

Primary and secondary cell cultures

Cell cultures start in one of two ways, by tissue dissociation using enzymatic or mechanistic action from living material (single cells), or from tissue explants (Fig. 3.3a). Every new culture begins as a primary culture, a culture characterized by finite life span *in vitro*, with slow cell proliferation, which has undergone very few cell doubling, and with the advantage of having representativeness of the tissue of origin. These cultures are mainly used in studies of physiology, cellular metabolism, and genetics (Fig. 3.3b). A secondary cell culture, i.e. cell line of immortalized cells with the capacity to proliferate indefinitely, can be obtained from tumour cells or from stem cells. The oldest human cell line is the HeLa cell line, which had origin on a cervical cancer from Henrietta Lacks, and one of the first applications was to develop polio vaccine (Masters 2002).

Cells can be of two types, (1) anchorage independent, i.e. suspension cells and (2) anchorage dependent, i.e. adherent cells. The former proliferate in a suspension culture while the latest proliferate in a monolayer attached to the culture plate or vessel, and are limited by the surface area (Fig. 3.3c).

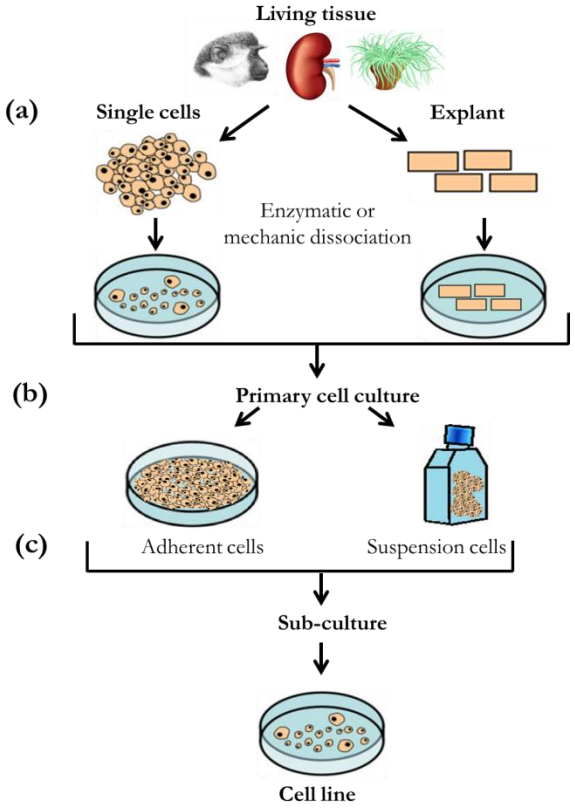

Figure 3.3 - Schematic representation for the establishment of cell culture (adapted from Rinkevich 2011).

Stem cells methodology

It was in the 1950s that human stem cells were identified in the bone marrow and their potential role in regeneration was acknowledged. Since then, this field of research has largely increased. Stem cells are undifferentiated cells with the capacity to differentiate into all types of cells and capable of self-renewal. When they divide, they can remain in an undifferentiated state or differentiate into a new cell with a specific function (e.g. nerve cell, muscle cell; (Watt & Hogan 2000). They can be from embryo origin (embryonic stem cells) or from the adult (adult or somatic stem cells). Embryonic stem cells are important in the development of a new organism since they can differentiate into all cell types of the body (pluripotent cells; (Martello & Smith 2014). Adult stem cells are fundamental to repair damage on tissues following injury, are usually located in the tissue or organ to which they can differentiate into and are limited in the number of types of cells they can be differentiated (multipotent cells) (Wilson & Trumpp 2006). While developing stem cell cultures, it is crucial to understand the signals that will contribute to maintain stem cells in an undifferentiated state in culture and those capable of trigger differentiation. For instance, adding certain proteins or specific genes can help mimic *in vivo* microenvironment, inducing differentiation. Several methodologies are used to the development of embryonic or adult stem cell culture, protocols include: coating of culture dish with mouse fibroblast feeder layer (used to nourish cells), feeder-free cultures using Matrigel or extracellular matrix proteins (Evans 2011, Villa-Diaz et al. 2013). Additionally, an old technique of cell culture, hanging-drop technique, has been used in stem cells cultures, when a 3D structure is required (Fig. 3.4; e.g. (Banerjee & Bhonde 2006).

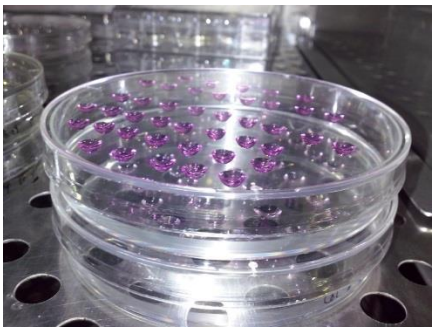

Figure 3.4 - Hanging drop culture allowing maintenance of a 3D structure.

3.3 Invertebrate cell cultures

3.3.1 Terrestrial cell cultures

Terrestrial invertebrate cell cultures have been established mainly from insects and ticks (Rinkevich 1999), and currently more than 500 cell lines are available, from different species and tissues (Lynn, 2007). Contributing to this success was the development of an exclusive growth medium, Grace's insect cell culture medium (GIM), richer in nutrients (amino acids and vitamins) when compared with vertebrate medium (Grace 1962, Smagghe et al. 2009). Insect cell lines have greatly contributed to increase knowledge on insect physiology, pathology and many aspects of insect biology; but also in studies of virology, immunology and toxicology (Smagghe et al. 2009). Therefore, the field of insect cell culture shows a huge potential and could still largely contribute to the production of human vaccines and gene delivery vectors, production of new insecticides, contributing also to agricultural science.

3.3.2 Marine cell cultures

Despite the great knowledge from mammalian and terrestrial invertebrate cell culture, the establishment of marine invertebrate cell culture has been proven difficult. Nevertheless, in the recent years many progresses in this field have been made. Attempts were focused mainly in six phyla (Porifera, Cnidaria, Mollusca, Crustacea, Echinodermata and Urochordata). In sponges (Phylum Porifera), the cell dissociation of tissues has been well established (Pomponi 2006), and some primary cell cultures have been developed (e.g. (Zhang et al. 2004, Sun et al. 2007). Even though, Sun et al. (2007) developed a dissociation protocol favouring archaeocytes in sponges (pluripotent stem-like cells in sponges) for primary cell cultures, continuous cultures have not yet been established. Indeed, the absence of cell proliferation have led researchers to analyse the cell cycle at the moment of the cell dissociation and observed that lack of proliferation in culture is due to large portion of cells in an apoptotic state (Schippers et al. 2011). In Molluscs, a series of tissues (as the haemolymph, gill, digestive gland and also embryo or larval stages) is suitable for the development of primary cell cultures (e.g. (Cao et al. 2003, van der Merwe et al. 2010). Mostly, mollusc cultures have been used for studies on toxicity due to the commercial value of some species (mussels, oysters, clams; (Domart-Coulon et al. 2000, Yoshino et al. 2013a). Yet, there has been a disinvestment in developing mollusc cell cultures favouring the studies on sponges and cnidarians (Rinkevich 2005).

3.4 Cnidarian cell culture development

3.4.1 State of the art

Cnidarian extraordinary properties (e.g. high regeneration, great longevity, symbiotic interactions) make them attractive to study fundamental cellular processes, and have been the baseline to the development of new fields in biology, such as the ecological evolutionary developmental biology (Eco-Evo-Devo). Additionally, their diploblastic structure and the limited diversity in cell types constitute an advantage for the development of cell cultures and raise them to the rank of emergent *in vitro* model.

The first steps to the development of cnidarian cell culture were made when (Gates & Muscatine 1992) used an enzymatic treatment to dissociate soft-bodied anthozoans, and a chemical treatment (low extracellular calcium protocol) to dissociate coral cells. Using both treatments the authors obtained single gastrodermal cells with their symbiont. Later, using 10 taxa of colonial marine cnidarians, (Frank et al. 1994)) observed that viability of cell culture was dependent on the type of tissue dissociation: mechanical, chemical or spontaneous dissociation approach (i.e. explant methodology). Studies progressed with the research focusing mainly in cell dissociation protocols, specific culture media and cell substrate-adhesion to find the optimum culture conditions (Frank et al. 1994, (Odintsova et al. 1994, Frank & Rinkevich 1999, Schmid et al. 1999, Domart-Coulon et al. 2004, Khalesi 2008, Barnay-Verdier et al. 2013). Despite all the efforts, the results seem to be species specific and no ubiquitous cell culture protocol is available. Most studies have preferentially used cnidarian species, with a calcium carbonate skeleton (e.g. Frank et al. 1994, (Domart-Coulon et al. 2001, 2004, Lecointe et al. 2013, Huete-Stauffer et al. 2015), instead of non-calcifying species (Frank & Rinkevich, 1999, Barnay-Verdier et al. 2013, (Rabinowitz et al. 2016). Apical branch tips of corals are promising since they have rapid growth (Domart-Coulon et al. 2004); however tissue dissociation would be harder on corals than on non-calcifying species, where dissociation and separation of tissues is favoured by the absence of a skeleton. In fact, primary cell cultures were mostly *in vitro* 3D cultures, such as tissue balls (Domart-Coulon et al. 2004, (Nesa & Hidaka 2009), Lecointe et al. 2013) or 2D monolayer transformed in 3D structure (Rabinowitz et al. 2016), than single cell cultures or cell aggregates (Barnay-Verdier et al. 2013, Huete-Stauffer et al. 2015). Despite the effort in recent years, none of the studies demonstrated that cnidarian cells are able to survive and proliferate *in vitro* for long periods, in order to establish a cell line.

The main challenges that cnidarian cell culture research faces are (1) contamination, (2) lack of knowledge on nutritional requirements (Rinkevich 1999, 2005), and (3) difficulties in cell

characterization *in vitro* (Puverel et al. 2005). Cell cultures contamination is especially due to the presence of marine microorganisms (protists, bacteria, fungi, viruses) against whom efficient treatment is not yet available. The insufficient knowledge on marine organisms' cellular biology poses another problem: we still lack good information concerning the nutritional and chemical requirements for *in vitro* cell growth and proliferation. This is normally solved by an optimized culture medium containing the appropriate osmolarity (1100 mosm), a basic pH (pH 8) and enrichment on growth factors. Growth factors are specific key molecules (e.g. insulin, heparin) for *in vitro* cell proliferation. In vertebrate cell culture, importance of growth factors has been highlighted but no data are yet available for cnidarians. The investment in new fields, such as proteomics, could therefore, determine specific molecules capable of working as growth factors in cnidarians.

Regardless of the difficulties in establishing cnidarian cell culture, some studies indirectly contributed to the development of cnidarian cell cultures by the validation of experimental tools and by the acquisition of new knowledges on cnidarian cell proliferation or regeneration. Important methodological validations are the estimation of cnidarian cells in proliferation by *in vivo* and *in vitro* immuno-detections of phosphorylated histone H3 (H3P; M-phase; Rabinowitz et al. 2016), and of EdU (S-phase; Fig. 3.5; (Passamaneck & Martindale 2012, Fransolet et al. 2013, 2014, Lecointe et al. 2013, 2016).

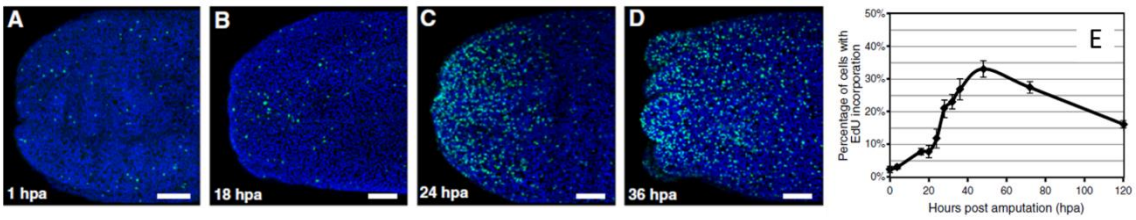

Figure 3.5 - Cell proliferation during oral regeneration of the sea anemone *Nematostella vectensis*.

(A-D) Nuclei of proliferating cells stained with EdU (green) and all nuclei stained with Hoescht (blue). (E) Percentage of cells in proliferation in the epiderm; hpa = hours post amputation (Passamaneck and Martindale, 2012).

In complement, recent efforts on deciphering the regeneration process in cnidarians have highlighted that the activation of cell proliferation would be the determinant factor separating wound healing from regeneration. More precisely, regeneration of the sea anemone *Nematostella vectensis* involves a two-step process. During the first hours after amputation, the regeneration is dependent on massive *de novo* transcription and is independent from cell proliferation, while latter steps are dependent on cell proliferation (DuBuc et al. 2014, Amiel et al. 2015).

3.4.2 *Anemonia viridis* cell culture establishment

For the establishment of the cnidarian cell culture, we chose *Anemonia viridis* since it possesses long and non-retractable tentacles with a high regeneration capacity; therefore we can collect a great quantity of biological material with a sustained potential of survival *in vitro*. Moreover, it is a non-calcifying species and thus easy to isolate the different tissue layers, epiderm and gastroderm. Additionally, recently transcriptomic approaches led to the identification of specific tissue marker genes, from which the expression allows distinguish cells from the epiderm and gastroderm (Ganot et al. 2011, Moya et al. 2012). These data guarantees the possibility to characterize cultivated cells *in vitro*, by allowing *in vitro* molecular determination of tissue layers. Also, as mentioned before in chapter 2, cellular processes involved in hyper-thermal stress response have been determined, specifically, oxidative stress and apoptosis leading to bleaching events (Richier et al. 2006). We can therefore validate the use of *in vitro A. viridis* cell cultures correlating the cellular sensitivity with the organism response under hyper-thermal stress.

More importantly, Barnay-Verdier et al. (2013) have already established a baseline protocol for the development of *A. viridis* cell culture. Using an enzymatic dissociation protocol from tentacles in regeneration, authors established a primary cell culture lasting 4 weeks, with the homogenization of the culture into aggregates of small cells (3-5 μm) happening after 18 days in culture. Authors validated the primary cell culture through specific molecular markers (*A. viridis* genes), confirming that cells in culture were definitely from cnidarian origin.

3.5 Characterization and validation of cnidarian primary cell culture

The use of cnidarian primary cell cultures could help us determine the cellular processes involved in symbiosis establishment and those leading to stress response. In this chapter, the questions we therefore proposed to answer were:

Q3: What is the tissue origin of primary cell cultures of *A. viridis* and what it is their capacity to survive, grow and proliferate?

Q4: Could we envisage using primary cell culture to assess cellular plasticity in response to exogenous stress (e.g. temperature)?

New perspectives from gastrodermal cnidarian primary cell cultures

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Paper to be submitted to ***Cell Tissue Research***

Abstract

Cnidarian primary cell cultures represent a new biological model relevant to investigate molecular and cellular processes involved in the response to environmental stressors. Full characterization of primary cell cultures from tentacles of the sea anemone, *Anemonia viridis*, has been carried out in this study. During maintenance and propagation of 31-day cultures, we determined (1) cell viability and cell growth rate, (2) cell proliferation by phosphorylated histone H3 (H3P) immunostaining and (3) the molecular expression of tissue specific markers, using two harvesting protocols (suspension vs. total cells). Cells were viable throughout the 31-day culture and capable of growth. Also, we observed during the first 17 days in culture cells in mitosis, (in proliferative state). Molecular analyses demonstrated the establishment of *A.viridis* differentiated cultured cells from gastrodermal origin, without evidence of pluripotent cells. Additionally, we measured response of primary culture cells to hyperthermal stress (25 and 28ºC) during 24 hours and 7 days. We observed loss of cell viability at 28ºC immediately after 24 hours of stress and at the end of 7 days stress (20%). The present study establishes a baseline for *in vitro* studies of marine invertebrates. Moreover, it demonstrates that primary cell cultures of *A. viridis* can be used to assess stress response mechanisms at cellular level. The presence of cells from gastrodermal origin opens the perspective for studies of *in vitro* symbiosis.

Keywords: Invertebrate cell culture, sea anemones, cell viability, hypertermal stress.

Introduction

Marine invertebrate cell cultures represent a new and relevant biological model for environmental and/or biomedical progresses. However, so far, marine invertebrate cell cultures are still challenging. Efforts have increased considerably in the last decade (see (Cai & Zhang 2014) for a review) to solve the main challenges - large contaminations and lack of *in vitro* lifestyle knowledge (Rinkevich 1999). This has led to the use of different strategies concerning cell dissociation, culture medium and culture maintenance reviewed in

(Rinkevich 2005). While reports are encouraging (e.g. see (Yoshino et al. 2013b, Barnay-Verdier et al. 2013, Mercurio et al. 2014, Huete-Stauffer et al. 2015), advances seem to be species specific and/or dependent on cell type target specificity, which makes difficult the establishment of standard protocols.

Among marine invertebrates, cnidarians are simplistic organisms with two cells layers, epiderm and gastroderm, and constitute a very interesting model to studies on biomedical and environmental fields. Indeed, they are a promising source of bioactive molecules which could be used against different pathologies (bacterial infections, cancer) (Rocha et al. 2011), have great regenerative capacities (Holstein et al. 2003a) and are long lived (Roark et al. 2009). Also, a great amount of cnidarian species is symbiotic with unicellular photosynthetic algae from the genus *Symbiodinium*. Therefore, they are frequently used as bio-indicators of global changes, since environmental perturbations such as global warming, can induce the breakdown of this symbiosis, a phenomenon commonly known as bleaching (Lesser et al. 1990, Brown 1997, Hoegh-Guldberg 1999b). Symbiotic cnidarians are a well-studied group, but for many cellular and molecular processes we still lack a deep understanding of host tissues that lead to the maintenance and collapse of symbiosis (Weis 2008). Although the development of cnidarian cell cultures should allow advances on evolutionary and functional biology no cnidarian cell line is currently available. Anthozoans (e.g. corals, sea anemones) are the class of cnidarians most studied for cell culture (e.g. Domart-Coulon et al. 2004, Khalesi 2008, Lecointe et al. 2013). Within anthozoans, the actinarians, soft-bodies cnidarians, are an interesting model due to the easy maintenance in the laboratory and long and non-retractable tentacles with a great regenerative capacity. Recently, Barnay-Verdier et al. (2013) established a primary cell culture of the sea anemone, *Anemonia viridis*, and gave a new step in cnidarian cell culture.

The aims of this study were to (1) optimize the protocol by improving cell survival and cell maintenance of primary cell cultures of the sea anemone, *A. viridis*, (2) to fully characterize the primary cell cultures at cellular and molecular levels during a 31-day culture, and (3) to apply this *in vitro* cellular model as a potential tool to evaluate the cellular stress response. Characterization of cultivated cells was performed by determining cell viability, cell growth rate and cell proliferation and by analysing the epithelial tissue origin of the cultivated cells. We chose a hyperthermal stress, known to induce symbiosis breakdown in *A. viridis*, to test the response of animal primary cell cultures to environmental stressors.

Materials and Methods

Biological Material

Four individuals of *Anemonia viridis* (Forskål 1775) were collected in shallow waters of Lido Bay at Villefranche sur Mer, France (43°41'21.68"N, 7°19'13.31"E). Animals were maintained in closed-circuit seawater aquaria at 18.0 ± 0.5 °C with water renewal every week. A metal halide lamp (HQI-TS OSRAM) provided light at a constant saturating irradiance of 200 μmol. m^{-2}. s^{-1} (measured using a special sensor QSL-100, Biospherical Instruments Inc.) on a 12h:12h (light:dark) photoperiod. Sea anemones were fed once a week with crude shrimps.

Dissection and dissociation protocol

The protocol adopted was an optimization of previous protocol developed by Barnay-Verdier et al. (2013). 15-20 tentacles' tips of *A.viridis* were cut, and let regenerate until tip closure. To take advantage from tissue in regeneration, 3 days later, a second cut of tentacles was collected in sterile calcium-free artificial seawater (SCaFSW; Domart-Coulon et al. 2004). Tentacles were then incubated for 20 minutes in 1 mM Glycine (Sigma-Aldrich) in sterile artificial seawater (SASW) to allow cnidocyst discharge (Morabito et al. 2014), slightly dried in paper, and rinsed twice for 2 minutes with SASW enriched with 1% Antibiotic antimycotic solution (10,000 units penicillin, 10 mg/ml Streptomycin and 25μg/ml Amphotericin B; Sigma-Aldrich) and MycoKill AB® (1X in final medium; PAA), under constant agitation. Tentacles were then longitudinally opened, cut in small pieces of 4 mm^2 and placed in a culture dish. Cell dissociation was performed in 0.15% collagenase type I (Sigma-Aldrich) in SASW, for 30 min at room temperature under agitation. Cell pellets were collected after centrifugation at 200 g for 5 min re-suspended in 1 mL culture medium and seeded in 4 wells of a 12-well tissue culture plate (Corning). All steps were performed in a sterile laminar-flow hood. The cell dissociations were performed independently from four *A. viridis* individuals.

Media and Culture maintenance

Cells were cultured in the dark at 20.0 ± 0.5 °C in an incubator at atmospheric pCO_2. Grace's Insect Medium supplemented (GIM; Gibco), enriched with 23.9 $g.l^{-1}$ NaCl (Grace's Modified Insect Medium, GMIM) to fulfil salinity requirements of marine animal cells, was used. The final culture medium consisted of 20% GMIM, 5% foetal bovine serum (PAA/GE Healthcare), 1% Kanamycin (100 μg/ml, Sigma-Aldrich), 1% Amphotericin B

(2.5 µg/ml; Interchim); 1% Antibiotic antimycotic solution (Sigma-Aldrich), 1% L-glutamate (Sigma-Aldrich) and 71% of SASW.

Culture medium was replaced after 3 days and then weekly until 31 days of culture. Before each medium replacement, cells were observed under light and phase contrast on a Zeiss Axio Vert inverted microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). During each weekly passage, cell harvesting was performed following two different procedures allowing collection of suspension cells or total cells (suspension and adherent cells). Suspension cells were harvested directly by serial dilutions of cell culture suspensions. Total cells were obtained by harvesting both suspension and adherent cells present in the same well. After suspension cells recovering, adherent cells were harvested by splitting with trypsin-EDTA (Sigma-Aldrich), all cells were finally pooled. In both procedures, collected cells were then been reseeded at 150.000 cells/well.

Cell viability assessment and cell growth rate of primary cell cultures

From 3 to 31 days, a sub-sample of 100 µl *per* well was harvested weekly to assess cell viability and growth rate. Cell viability was determined with Evans blue vital stain method. Cells were incubated in 1:1 ratio with 0.05% Evans blue (Sigma-Aldrich) solution during 10 minutes. An aliquot of 40 µl was placed in a Neubauer improved haemocytometer. Viable and dead animal cells and also *Symbiodinium* algae were counted. Cell viability was determined as the percentage of viable animal cells relative to total animal cells.

From 3 to 31 days, cell growth rate *per* week was determined as follows:

[Eq.1] Growth rate = (Viable cells $_{(d+7)}$ – Viable cells $_{(d)}$) / (Viable cells $_{(d)}$), d=days.

Immunolabeling of primary cell cultures

To detect cells in division, cells were immunolabeled during the first two weeks of culture (10 and 17 days) with anti-H3P antibody, a mitotic marker (M-Phase). At each time point of the kinetics, cells were harvested, rinsed with PBS 0.6M, and fixed during 30 min at room temperature in 4% paraformaldehyde in PBS 0.6M. Cells were permeabilized in PBT 0.2% (PBS containing 0.2% Triton X-100), during 15 min at room temperature, and then blocked with PBS-3% BSA for 30 min at room temperature. Then cells we incubated with Anti-H3P (1:200, mouse anti-H3P, Molecular probes) during 1 h at room temperature. After incubation and washes in PBS, cells were incubated with Alexa-labelled secondary antibody (1:200, goat anti-mouse, Molecular probes) for 30 min at room temperature. For DNA staining cells were incubated with 5 µg/ml of Hoechst 33342 (Molecular Probes).

Finally cells were mounted in a glass microscopic slide with 80% glycerol. Fluorescence images were acquired using a Zeiss Axio Observer microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Images were analysed with the open-source FIJI software. Briefly, Hoeschst-stained nuclei and H3P-stained mitosis cells were identified by using the function "analyse particles", using particle size from 2-5 microns. Each cell in the differential interference contrast image was scored manually. Each image was then overlapped to account for cells in mitosis.

Molecular analyses

Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from primary cultured cells at each time of the kinetics between 10 to 31 days. Harvested cells were centrifuged at 200 g for 5 minutes. Extraction continued following manufacturer instructions from QiAMP DNA Mini Kit protocol (QIAGEN). 50 to 100 ng of extracted genomic DNA were used for PCR amplification. PCR were performed using primers specifically designed against the Niemann Pick type C1 gene (*AvNpc1;* Ganot et al. 2011; cf Table S2). PCR positive controls corresponded to amplification of genomic DNA extracted from *A. viridis* whole tentacles. Each sample was run using the following PCR parameters: 94ºC for 2 min, followed by 40 cycles of amplification at 94ºC for 30 s, 63ºC for 30 s and 15 s at 72 ºC. PCR products were electrophoretically analysed on 2% agarose gel stained with gelRed (Interchim).

RNA extraction and RT-PCR

Total RNA was extracted from primary cultured cells at each time point of the kinetics between 10 to 31 d. Removed cells were centrifuged at 211 g for 5 minutes at 4°C. Cell pellets were then incubated in 500 µl Trizol Reagent (Invitrogen). Extraction continued following manufacturer's protocol. RNA pellet was finally re-suspended in 20 µl of RNAse-free water. Total RNA was then treated with DNase I (Sigma-Aldrich) to eliminate any potential genomic DNA still present in samples. RNA was quantified on a 150ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA).

One µg of RNA samples was reverse transcribed using Oligo (dT) primer and SuperScript II (Invitrogen). A volume of 3 µl of cDNA was used for PCR amplification using primers specifically designed against transcripts identified from *A. viridis* transcriptome (Sabourault et al. 2009; Pey et al. 2017; cf Table S2). Among them, we used three transcripts (*AvCa2mG*, *AvCa2mE*, *AvGfp)* with a tissue specific expression (epiderm or gastroderm;

Ganot et al., 2011; Table S2, Fig. S1) and three transcripts of pluripotency marker genes (*AvPiwi*, *AvVasa*, *AvPl10*) identified by cnidarian homology (Putman et al. 2007, Leclère et al. 2012). PCR positive controls corresponded to amplification of cDNA produced from RNA extracted from *A. viridis* whole tentacles. PCR products were analysed in 2% agarose gel electrophoresis stained with gelRed (Interchim). Unpublished *A. viridis* sequences are in submission to the NCBI GenBank database.

In vitro thermal stress

To test the response of primary culture cells to thermal stress, 10-day old total cells seeded in 12-well plate, were exposed to 3 different temperatures: 20 ºC (control), 25 °C and 28 °C during 24 hours or 7 days. At each time point, we accounted for cell viability.

Statistical analysis

Comparison of cell viability and proliferation between suspension cells and total cells was performed using repeated measures ANOVA due to dependency of samples (i.e. the same cells were evaluated during the 31-days of experiment), as time (days) as within-subject effect. Data in percentage was arcsine transformed. When assumptions failed, appropriate transformations were used. For growth rate, no transformations could follow the assumptions and the non-parametric Friedman test was used. The effect of temperature and exposure time on cell viability was analysed with a two-way ANOVA, with temperature and exposure time has fixed factors. Post-hoc test to assess among group differences was performed with Fisher test. All statistical analysis was computed using STATISTICA 10.0.

Results

Morphological analysis of primary cell culture

During the first 3 days of the culture we observed an heterogeneous culture, with mixed morphological cell types including cnidocytes, *Symbiodinium* cells (about 10 μm in diameter), and small rounded animal cells (about 3 μm in diameter) (Fig. 3.6a). All cell types present in the culture were in suspension.

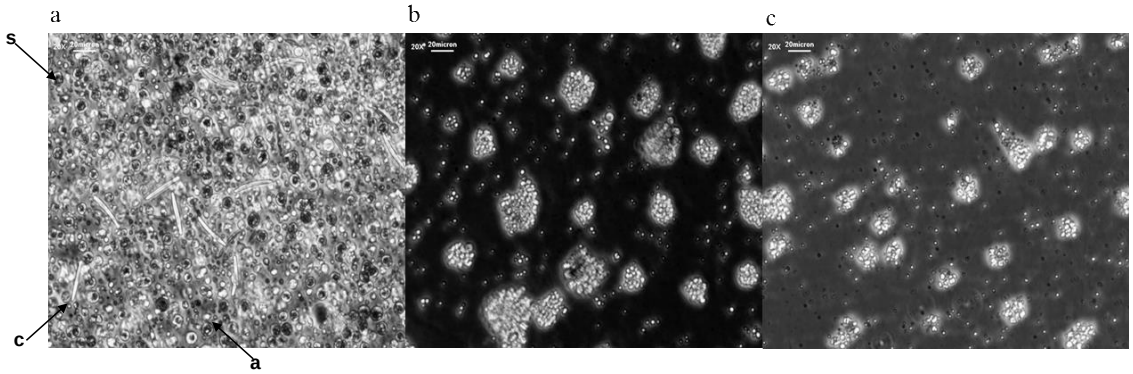

Figure 3.6 - Observation of culture cells from regenerating tentacles of *A. viridis*.
(a) Heterogeneous culture after 3 d, (b) formation of cell aggregates at 10 d and (c) cell after 31 d in culture. a – small rounded animal cells, s – *Symbiodinium* cells, c – cnidocysts. Observations were done on an inverted optic microscope, with x20 objective.

After 10-day in culture, we observed a complete change in primary cell culture architecture with the formation of cell aggregates (~20 cells *per* aggregate) (Fig. 3.6b). Most of cell aggregates were adherent and regularly dispersed along the tissue culture plate and preserved until the 31-day culture (Fig. 3.6c). Another part of cell aggregates was formed in suspension. The formation of primary cell aggregates coincides with the homogenization of cell culture, i.e. disappearance of *Symbiodinium* cells and a major enrichment in animal cells (Fig.3.6b and Fig. 3.7). Same animal cell culture enrichment and architecture has been observed through the two cell harvesting procedures (suspension and total cells) and during the 31 days of culture (Fig. 3.7).

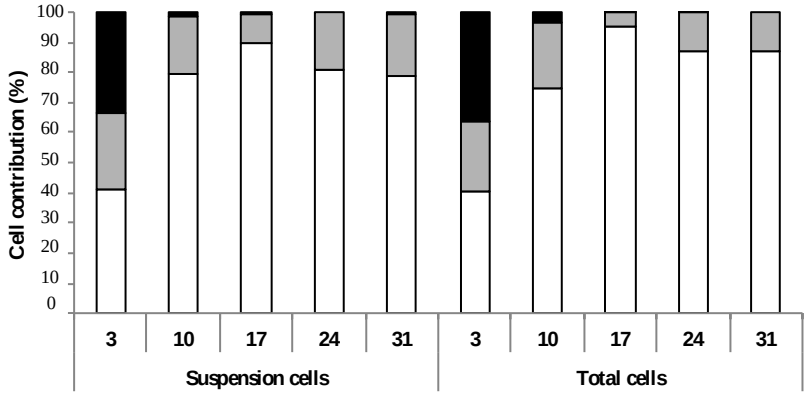

Figure 3.7 - Cell contribution in primary cell cultures along the 31 days of culture.
White bars – viable animal cells, grey bars – dead animal cells, black bars – *Symbiodinium* cells. Mean data expressed as percentage, n = 16.

Viability assessment and cell growth rate

Cell viability analysis confirmed the healthy state of primary animal cell cultures. Suspension cells displayed equivalent cell viability when compared with total cells ($F_{(1,3)}$ = 0.63488; p = 0.48380). Throughout the culture, suspension cells exhibited high and constant cell viability from 3 up to 24 days in culture (78.5%; Fig. 3.8a), with maximum reached at 31 days in culture (84%; $F_{(4, 12)}$ = 14.076, p = 0.014764). Total cells exhibited constant cell viability during the first 10 days (around 66%) followed by a significant increase from 17 to 31 days (around 89%; $F_{(4, 12)}$ = 14.076, 10 d *vs.* 17 d, p = 0.006244; 10 d *vs.* 24 d, p = 0.014886; 10 d *vs.* 31 d, p = 0.012676; Fig. 3.8a).

Although cell growth rate measurements seem to show a differential trend between suspension and total cells during the culture period, no significant difference was found (χ^2 (3) = 5.13333, p = 0.16229). On average, suspension cells showed a 5-fold increase, while total cells showed a 9-fold increase during the 31 days in culture (Fig. 3.8b).

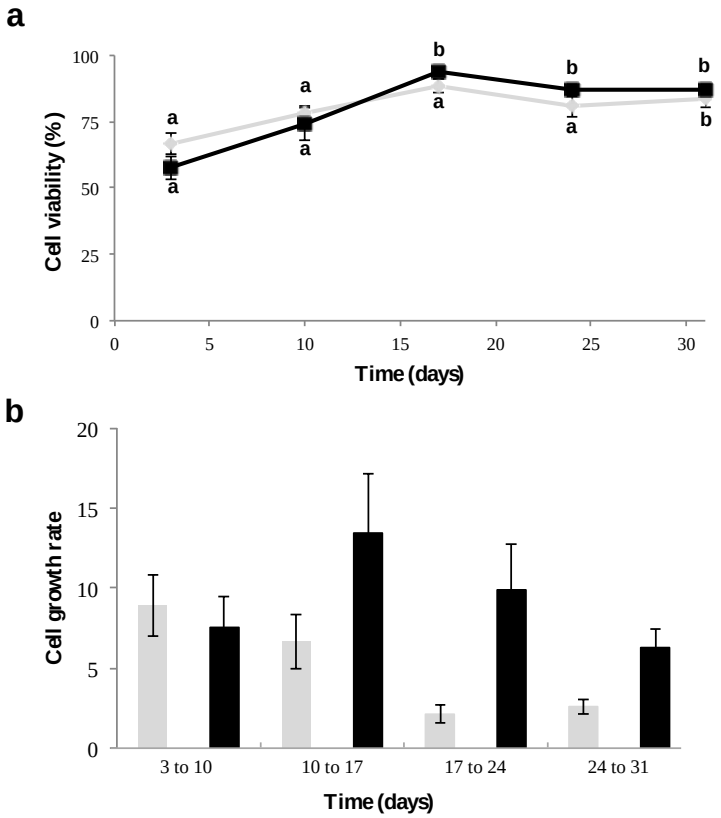

Figure 3.8 - Determination of (a) cell viability and (b) cell growth rate of suspension and total cells of *A. viridis* primary cell cultures along the 31 days of culture.
Grey dots and bars – suspension cells; black squares and bars – total cells. Mean data with error bars, n=16. No significant difference between the suspension and total cells. Means with different letters differ significantly from 3 d within each type of cell cultures.

Cell proliferation

We measured phosphorylated histone H3 (H3P) during the first 17 days in culture, for both suspension and total cells, showing cell-cycle activity on both cell-harvesting procedures (Fig. 3.9). For suspension and total cells, the cell proliferation rates were 15 % and 31 % respectively. However, no statistically difference on cell proliferation was measured between the two protocols nor between 10 d and 17d ($F(1,4) = 2.1079$, $p = 0.22017$).

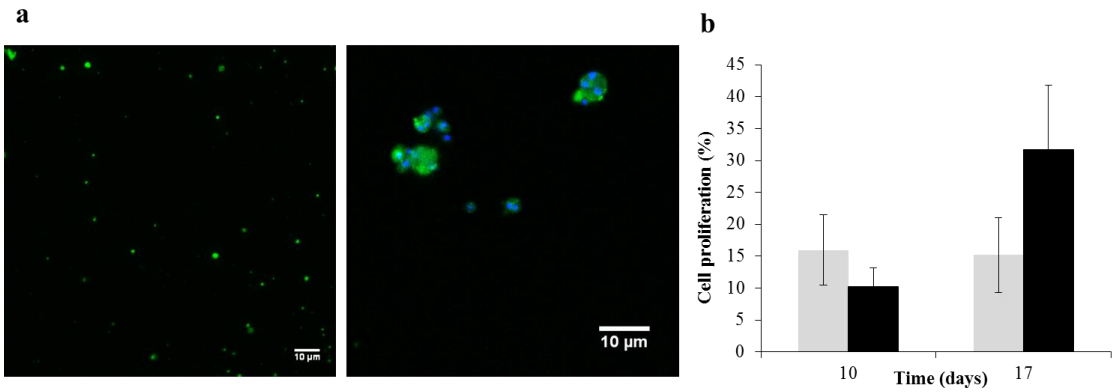

Figure 3.9 - Cell proliferation of *A. viridis* primary cells during the first two weeks of culture.
(a) H3P-labeled nuclei of suspension cells at 10 d. On the left, a representative image of nuclei immunolabeled with H3P at M-phase (in green). On the right, a representative image of Hoescht 33342 (in blue) and H3P (in green) nuclei co-staining. (b) Quantification of cell proliferation during the first two weeks of culture obtaining through 3 images (10 d and 17 d). Data are expressed as the percentage of nuclei immunolabeled with H3P related to total nuclei. Grey bars – suspension cells, black bars – total cells. Mean data with error bars, n=3.

Determination of epithelial tissue origin

To determine the animal origin of cultivated cells during the culture, we performed genomic DNA amplification with specific *AvNpc1 A. viridis* gene. During the culture period from 17 d to 31 d the *AvNpc1* gene was amplified from genomic DNA samples extracted from both cell-harvesting procedures (Fig. 3.10a).

To determine the cell tissue origin of animal cultivated cells, we performed RT-PCR to detect transcripts of genes known to exhibit a tissue-specific (i.e gastroderm or epiderm) expression in *A. viridis* tentacle (Ganot et al. 2011; Fig. S1). Analysis of the transcripts showed the expression of gene with gastroderm-specific expression (*AvCa2mG*) in both types of harvested cells at 17 d and 31 d (Fig. 3.10b). In the opposite, no transcript of genes with epiderm-specific expression, *AvCa2mE* and *AvGfp*, was ever detected, (Fig. 5b). In addition, no expression of three *A. viridis* pluripotency markers tested, *AvPiwi, AvPl10, AvVasa*, was observed during the culture period for both types of cultivated cells (data not shown).

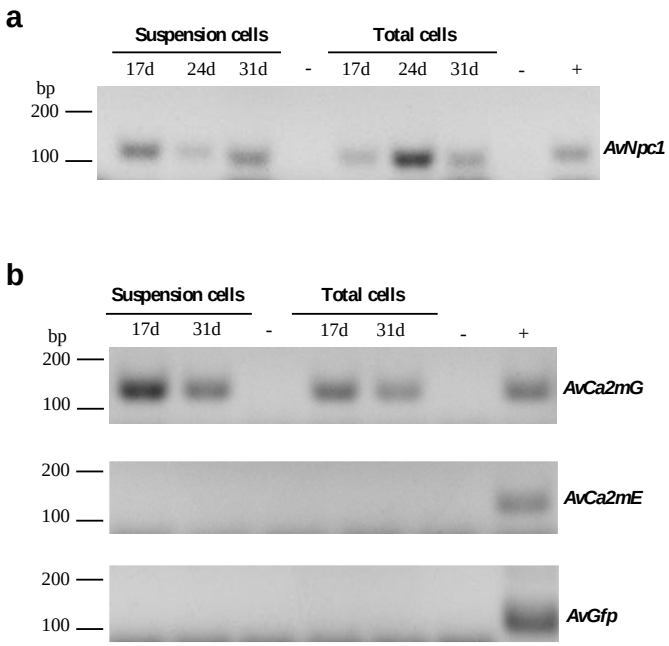

Figure 3.10 - Determination of epithelial tissue origin of *A. viridis* primary cell cultures.
(a) Amplification of *A. viridis* specific gene. PCR was performed using specific primers of *A.viridis Npc1* gene. Genomic DNA extracted from suspension and total cells at 17, 24 and 31 d of culture were used as templates. Genomic DNA from *A. viridis* tentacle was used as animal control (lane +); (-) corresponds to no genomic DNA template. (b) Amplification of *A.viridis* genes with tissue specific expression. RT-PCR amplifications were performed using primers of *AvCa2mG*, *AvCa2mE* and *AvGfp* genes. cDNA extracted from suspension and total cells at 17 and 31 d of culture were used as templates. cDNA from *A. viridis* tentacle was used as animal control (lane +); (-) corresponds to no cDNA template.

Response to thermal stress

We tested the response of total cells to hyperthermal stress between 10 to 17 d aged cell cultures, and we observed an interaction effect of temperature and exposure time, as well as exposure time alone. As shown in figure 3.11, cell viability remained constant throughout the 7 days of stress for control temperature and 25°C condition ($p > 0.05$). However, for 28°C stress condition, cell viability dropped 18% after 24 hours of exposure ($F_{(2, 33)} = 7.8711$, $p = 0.000157$) and up to 20 % at 7 days ($F_{(2, 33)} = 7.8711$, $p = 0.000684$). Cell viability was also significantly different between control temperature and 28ºC after 24 hours ($F_{(4,33)} = 2.8541$, $p = 0.007974$) and at the end of exposure time ($F_{(4,33)} = 2.8541$, $p = 0.0018704$), at both time points cell viability at 28ºC was lower (Fig. 3.11).

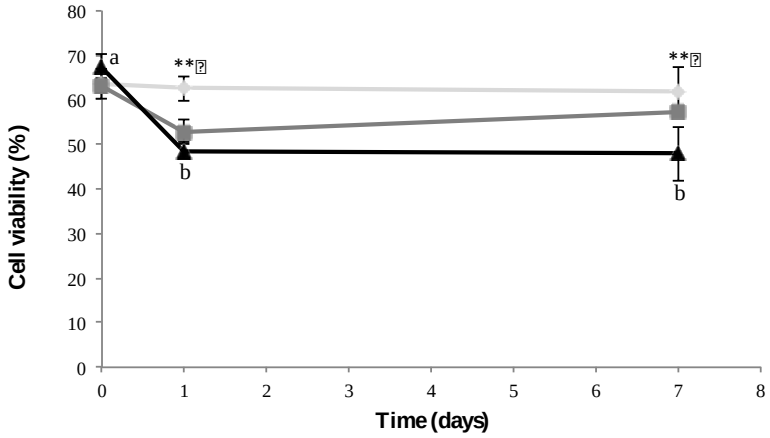

Figure 3.11 - Assessment of cell viability in total cells of *A. viridis* primary cell cultures in response to thermal stress.
Data are expressed as the percentage of viable cells. Light grey dots – 20°C (control), dark grey dots – 25°C, black dots – 28°C. Mean data with error bars, n = 8. ** significant differences between control and 28°C ($p < 0.01$). Means with different letters differ significantly from 0 d (28°C, $p < 0.01$).

Discussion

Development of cnidarian primary cell culture would allow progress on the environmental and biomedical fields. In the present work, we optimized the protocol of primary cell culture of *Anemonia viridis* previously established by Barnay-Verdier et al. (2013) and, thanks to molecular markers, characterized the culture cells as from gastrodermal origin. We then assessed the response of gastrodermal primary cell culture to induced thermal stress. We hereby propose *in vitro* primary cell cultures of *A. viridis* as a new model to study molecular and cellular processes involved in the establishment and maintenance of cnidarian-dinoflagellate symbiosis and to environmental stressors potentially disturbing this symbiosis.

Primary cell culture protocol optimization

Several studies have stressed the challenging barriers to the development of primary cell culture from marine invertebrates: the lack of knowledge on the nutritional requirements for marine invertebrate cells *in vitro* and the many unknown contaminants that can overcome the cells of interest (Rinkevich 2011, Cai & Zhang 2014). However, there are other challenges when dealing with marine invertebrates. Cnidarians possess stinging cells, cnidocytes that contain a cnidocyst that when stimulated, for instance by a prey, discharge and release a venom (Tardent 1995). (Morabito et al. 2014)) showed that incubation of tentacles of *Pelagia noctiluca* in glycine coupled with mechanical stimulation increases cnidocyst discharge. In the present study, incubation of tentacles of *A. viridis* in glycine

previous to cell dissociation decreases the cnidocyste numbers (data not shown) and the culture heterogeneity. Another factor contributing to optimize the *in vitro* culture is the choice of cell culture medium. In the present study, the use of GMIM, an invertebrate cell culture medium used in insect cell cultures and already successfully used in marine organisms (Hurton et al. 2005, Khalesi 2008), rich in nutrients and amino acids, resulted in high cell viability along the 31 d of culture. In fact, the use of GMIM seems to accelerate the formation of cell aggregates (10 d, in this study) contrary to the use of vertebrate medium (Dulbecco's modified eagle medium; 18 d, (Barnay-Verdier et al. 2013). Therefore, using GMIM as culture medium allowed us to use primary cells in earlier passages, reducing putative modifications of somatic differentiation *in vitro*.

Characterization of A.viridis primary cell culture

(Cai & Zhang 2014)) in their review stressed the importance of selecting tissues where the capacity of growth is still high. In this study we used regenerating tentacles of *A.viridis* as a tissue source in order to select cells with growth and proliferation capacity, as well as pluripotent cells capable of differentiation in epithelial cells. We observed that both suspension and total cells are capable of growth during the 31 d culture, without any appearance difference in growth. Phosphorylated histone H3 cell staining allowed the measurement of cell proliferation during the first 17 days of culture, with around 23 % of cells in the M-phase. Cell proliferation observed in this study was much higher that on tissue balls of the scleractinian coral *Pocillopora damicornis*, with only 0.2% of cells marked as in proliferation (Lecointe et al. 2013). Additionally, using BrdU marker, a thymidine analog that is incorporated during DNA synthesis, Huete-Stauffer et al. (2015) observed in aggregates of the gorgonian *Eunicella singularis*, a much similar percentage of cells in proliferation (approximately 32%). In our study, 10-day cell culture formed aggregates with high viability, growth and proliferation, suggesting that cell-cell interaction mechanisms were maintained active in culture. Appearance of cell aggregates also coincided with the reduction of *Symbiodinium* in culture. Absence of light for photosynthesis and choice of GMIM culture medium seem to be crucial to reduce and eliminate *Symbiodinium* cells in culture, potentially favouring the success of animal cell establishment. We then suggest the use of 10 d aged cells as the best time point for further cell manipulation.

Amplification of *A.viridis* specific gene (*AvNpc1*) from genomic DNA extracted samples between 17 to 31 d confirms the cnidarian signature of cultivated cells. Through the 31 d of culture, cultivated cnidarian cells are differentiated and belong to gastrodermal tissue as

shown by the expression of the specific gastrodermal gene, *AvCa2mG*, and the absence of expression of specific epidermal genes, *AvCa2mE* and *AvGfp* and of pluripotency marker genes, *AvVasa, AvPiwi* and *AvPl10*. The selection of gastrodermal cells could result from: (1) higher efficiency on gastrodermal dissociation with the selected protocol, and/or (2) a gastrodermal cell enrichment during the regeneration process. Epidermal monolayer from *A. viridis* tentacle is tightly structured compared with the gastroderm, which is a soft tissue. Consequently, collagenase treatment could have been more efficient on gastroderm, releasing more gastrodermal cells to the culture medium. In addition, the gastroderm in sea anemones has been shown to act as a driving force for tissue regeneration (DuBuc et al. 2014, Amiel et al. 2015). During our protocol, by choosing to extract cells from tentacles in regeneration, we potentially favoured gastrodermal cell enrichment. In complement, as we did not observe expression of pluripotency marker genes in cultivated cells, we suggest that *A. viridis* tentacle regeneration process does not preferentially involved proliferation of pluripotent cells.

One of the fundamental aims in the development of cell cultures is the access to the cellular level of knowledge. In order to validate the primo cell culture of *A. viridis* gastrodermal cells, we tested their response to an exogenous stress known to affect animal physiology and gastrodermal epithelial tissue integrity (Richier et al. 2005, 2006, Moya et al. 2012). Previous works on whole organism have measured an impact of an increase of +8°C not only on the symbiotic, apoptotic and redox states (Richier et al. 2005, 2006), but also on transcriptional level of gastrodermal stress marker genes (Moya et al. 2012). In our *in vitro* study, we observed a decrease in the cell viability at 28°C confirming the sensitivity of gastrodermal cnidarian cells to hyperthermal stress. However, we should highlight that 48% of cultured cells were still viable after 7 days of hyperthermal exposure, suggesting that a fraction of the cell population was able to cope with a temperature increase. This result could be linked to the previous electronic microscopic observations of health state of epithelial tissues of *Aiptasia sp.* submitted to hyperthermal stress (Dunn et al. 2002). These authors showed that only a proportion of gastrodermal cells were in necrotic/apoptotic state. Further investigations should then define the mechanisms involved in the cell death and evaluate the cell proliferation rate of the survival cells under hyperthermal stress exposure.

In conclusion, the optimized protocol developed in this study allowed us to extract and successfully culture *in vitro* gastrodermal cells from regenerative tentacles of *A. viridis*. The establishment and characterization of gastrodermal primary cell cultures of *A. viridis* opens

the door to studies on molecular and cellular responses that are inherent to the animal host. In fact, as mentioned in Weis (2008), there is a gap of knowledge in the study of cnidarian-dinoflagellate symbiosis as we lack understanding of host cell response. Using gastrodermal primary cell cultures of *A. viridis*, we can foresee studies of symbiosis *in vitro* in order to understand the cellular mechanisms of host-symbiont recognition, control and breakdown.

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Supplementary materials

Table S2. Primer sequences used for PCR and RT-PCR

Genes	Primer sequences (5'-3') Forward	Reverse	Tm (° C)	Reference
AvNpc1	GCCTGCTGTCAAGGTGTTCTC	TGCGGTTACTTTCCTGTCGTC	63	Ganot et al. 2011
AvCa2mG	CTTTGGCGGCATTTCACTTG	GTGATTGGTTGGAGCCATCG	58	Ganot et al. 2011
AvCa2mE	CTATACGAGGTTGGCGACGA	TCAGTGGTGTTTGGAAGAAGTG	58	in submission
AvGfp	GCAGAAGGGAAAGGCAATCC	GGAGAAGCAAAGCGAAAGGATG	60	Ganot et al. 2011
AvPiwi	TCAACCCAACCAGCCTACT	GGGAAACGTCAGCACAGAGT	60	in submission
AvVasa	GTCGCTGTCCAGTCCTCAT	TTGCCCTTGTTCCCTATTCTT	56	in submission
AvPl10	AAGCAGCTGGATGACTACGT	GAGCGTTCCTGACCATCTCT	58	in submission

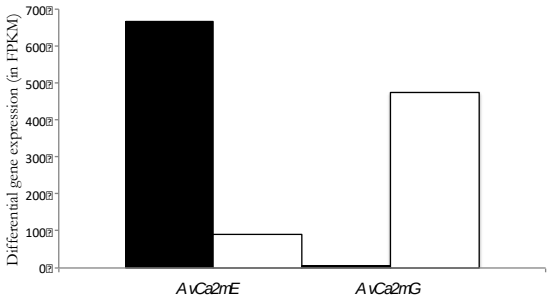

Figure S1 - Relative differential expression of *AvCa2mE* and *AvCa2mG* genes in epiderm (black bar) and in gastroderm (white bar).
Differential gene expression is given in FPKM (fragments *per* kilobase of exon *per* million fragments mapped).

3.6 Further research on diversification of cell culture

Previous results have shown that we established and maintained a primary cell culture of *A. viridis* from gastrodermal origin, viable during the 31-day culture and capable of proliferation during the first 17 days. These results raised other questions, specifically:

Q5: Could we foresee cultivating epidermal and gastrodermal cells in an independent manner?

Q6: Is it possible to isolate and cultivate undifferentiated cells, i.e. stem cells/pluripotent cells?

3.6.1 *In vitro* primary cell culture assays from separated monolayers: epiderm *vs.* gastroderm

As described above, improvement of methodological tools to evaluate *in vivo* and *in vitro* cell proliferation in cnidarians have shown, in the adult polyp, that even if cell proliferation is equivalent between epidermal and gastrodermal cells, this proliferation pattern could be modified and regulated during the regeneration process (Passamaneck and Martindale, 2012, Fransolet et al. 2013, Amiel et al. 2015, Rabinowitz et al. 2016). We were then interested in determining if we could obtain primary cell cultures from epidermal or gastrodermal monolayers as tissue source.

Material and Methods

From regenerating tentacles of two individuals of *A. viridis,* we independently separated epiderm from gastroderm by gently scratching tentacles, and followed the protocol previously described for total cells from whole tentacle (see section 3.5). We measured cell viability and cell growth rate from 3 to 31 days in culture (epiderm: n=6 wells, gastroderm n = 12 wells).

Results

Morphological observations

After 3 days, architecture of the cell culture from both epidermal and gastrodermal monolayers was very heterogeneous with small round animal cells, *Symbiodinium* cells and cnidocytes, as previously observed for 3-day cell cultures from whole tentacle. However, cell contribution (animal viable or dead cells and symbiont cells) was different between total, epidermal and gastrodermal cells cultures.

At 3 days of culture, epidermal cells cultures were composed of 50% dead cells, a number that increased throughout the culture (Fig. 3.12). Contrasting with epidermal cultures, gastrodermal cultures were composed by less dead cells (10%) but an unsurprisingly high percentage of *Symbiodinium* cells. During the 31-day of culture, the gastrodermal culture differed from total cells culture by the continuous increase of dead cells and preservation of *Symbiodinium* cells (Fig. 3.12).

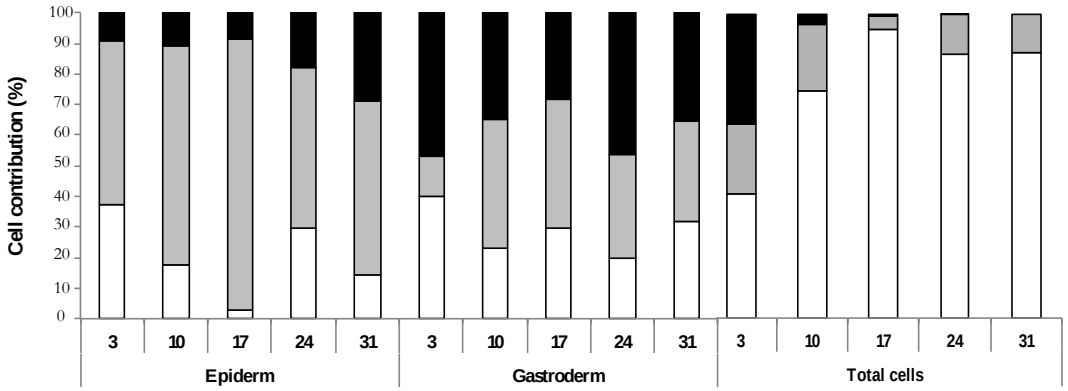

Figure 3.12 - Comparison of *A. viridis* primary cell culture contribution following the establishment of an epidermal, gastrodermal and total cells cultures.
White bars – viable animal cells, grey bars – dead animal cells, black bars – *Symbiodinium* cells.

In cell culture from gastrodermal monolayer we rarely observed cell aggregates after 10 d (2/12 wells formed aggregates) contrary to what was observed in cultures from whole tentacle, while for cell culture from epidermal monolayer formation of cell aggregates was never observed during the 31-day culture.

At 3 d, the amount of animal cells (viable and dead) *per* well was different between the type of culture, with the lowest amount of cells *per* well being observed in the epidermal culture (less than 50%; Table 4).

Table 4. Amount at 3 d of dissociated cells issued from whole tentacle, epiderm and gastroderm.
Mean data ± SE.

Type of culture	Total cells *per* well at 3 d
Whole tentacle	315.833 ± 79.652
Epiderm	170.000 ± 30.623
Gastroderm	427.500 ± 38.000

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Animal cell viability and cell growth assessment

Animal cell viability was significantly higher (72 %) for gastrodermal cells culture, contrasting with the 38% viability of epidermal cells culture ($t(12) = 4.6137$, $p = 0.000597$). In both cultures, we observed a decrease in cellular viability until the end of the 31d culture, reaching values as low as 20% (Fig. 3.13).

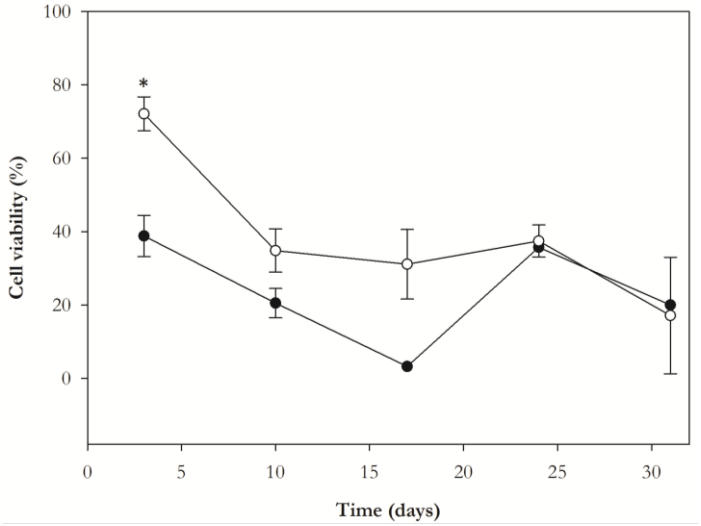

Figure 3.13 - Determination of cell viability during a 31 d cell culture issued from epidermal or gastrodermal monolayers.
Black dots – epidermal cells cultures, white dots – gastrodermal cells cultures. * $p < 0.05$. Mean data with error bars, $n \geq 6$.

In addition, no cell growth has been measured during the 31 days of culture for both epidermal and gastrodermal cells cultures (data not shown).

Discussion

Epidermal cells cultures were not viable during the 31d culture, since from the beginning we counted a low number of viable cells at 3 d and we observed a higher percentage of dead cells (60%) that continued to increase until the end of the culture (80%). No sign of cell growth was observed or quantified. At 3 d, cell dissociation efficiency and viability were higher in gastrodermal cells culture and equivalent with total cells culture from whole tentacle (Ventura et al. submitted). However, in contrast to total cells culture, the animal cell viability of gastrodermal cells culture decrease along the 31 days (at 31 day, 85% *vs.* 20%, respectively). Table 5 resumes the properties of the three different *A. viridis* cultures.

Table 5. Comparison of primary cell cultures issued from whole tentacle, epiderm and gastroderm.

Type of culture	Cell dissociation efficiency	Cell viability (3d)	Cell viability (31d)	Growth	Presence of aggregates
Whole tentacle	High	High	High	Yes	Yes
Epiderm	Low	Low	Low	No	No
Gastroderm	High	High	Low	No	Rare

In the epidermal cells cultures, the reduced efficiency in the cell dissociation is potentially linked to the dissociation method chosen. Indeed, epidermal tissue layer is relatively harder than the gastroderm, which is a soft tissue, thus easier to dissociate using enzymatic treatments. Consequently, our results suggest that enzymatic cell dissociation used in our protocol does not allow isolating a sufficient number of epidermal cells for establishment and maintenance of primary cell culture. Indeed, the increase of collagenase type I concentration (from 0.15 % to 0.5%) also tested did not result in any improvement in the cell viability or cell growth of epidermal cells culture (data not shown).

In addition, the higher mortality measured at 3d in epidermal cells culture suggests that the small number of dissociated epidermal cells and by consequence the lack of cell-cell inter-signalling is redhibitory for the *in vitro* cell culture success. Recent works of Rabinowitz et al. (2016) support that hypothesis as they succeed to maintain *in vitro* epidermal monolayers from *N. vectensis* for several months and demonstrated cell proliferation during the first 6 d after isolation. Moreover, Fransolet et al. (2013, 2014) exploring tissue regeneration after an induced stress observed epidermal tissues proliferation. Those results suggest that the monolayer integrity and the cell-cell contact are mandatory for the proliferation and the viability of epidermal cells.

While the dissociation efficiency and the viability of gastrodermal cells at 3 d were higher, the culture success was not comparable to the total cells culture. Recent *in vivo* and *in vitro* studies in the non-symbiotic sea anemone, *Nematostella vectensis*, attempted to determine the respective contribution of epiderm and gastroderm in the regeneration process. For instance, regeneration of oral tissues involves cell proliferation in both epiderm and gastroderm (Passamaneck & Martindale, 2012, Amiel et al. 2015). Nevertheless, Amiel et al. (2015) showed that regeneration of *N. vectensis* is highly dependent on the presence of the gastroderm in the amputation site and the proliferation happens first in the gastroderm. These data demonstrate the capacity of gastrodermal cells proliferation *in vivo*, and support our *in vitro* results on gastrodermal cells proliferation in cultures from whole tentacle

(Ventura et al. submitted). Nevertheless, the absence of *in vitro* proliferation of gastrodermal cell culture obtained from isolated gastroderm (this study) suggests that the lack of gastrodermal/epidermal inter-signalling is a barrier for long term gastrodermal cell culture establishment.

3.6.2 Hanging drop culture assays for isolation and cultivation of cnidarian pluripotent cells

In Ventura et al. (submitted) we determined that cells cultivated in the developed cnidarian cell culture were already in a differentiation state. However, the high regeneration and longevity capacity of cnidarians suggests a cell renewal potentially linked to the presence, in adults, of undifferentiated pluripotent cells (i.e. stem cells). Therefore, we were interested in isolation and selection of pluripotent cells from adult regenerating tentacle of *A. viridis* through a selective stem cells methodology. Hanging drop technique (inverted drop) has been used since the beginning of *in vitro* cell cultures and it was first used by Harrison (1907) to study frog neural cells. This technique has then been widely used (e.g. (Kawakami et al. 2000, Ali et al. 2004, Banerjee & Bhonde 2006) and applied to several research areas such as stem cell biology, toxicology or tumour biology. The advantage of using this technique is that cell growth and proliferation is not restricted to a two-dimensional plate. Instead, they grow in a three-dimension microenvironment where physiological conditions are more similar to the *in vivo* tissue of origin. Therefore, we chose this methodology as an attempt to select undifferentiated pluripotent cells, with regard to the high regenerative capacity of *A. viridis* tentacles.

Material and Methods

We started a hanging drop culture by selecting animal cell aggregates, issued from 10 d cultures of two independent total cells cultures obtained from whole tentacles (cf. 3.5). We seeded 20.000 cells in a 20 µl drop in the lid of 60 mm dishes (20 drops *per* dish, Nunc™ Petri Dishes, Thermo Scientific), using GMIM as a culture medium (n = 4 dishes). We assessed cell viability and cell growth rate, and we performed molecular analyses to determine epithelial tissue origin (epiderm vs. gastroderm) as well as pluripotency, from 17 to 31 d of culture (for technical details see section 3.5).

Results

Morphological observations

Drop-cultivated cells present a slightly different cell culture architecture compared to plate-cultivated cells. Indeed we observed isolated small round cells, losing its initial aggregated form (Fig. 3.14).

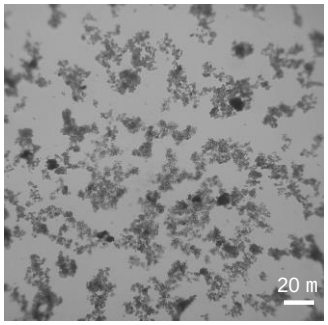

Figure 3.14 - Observation of drop-cultured cells after 7 days in drop cultures (17 d from the beginning of the primary cell culture).
Observations were done with an optic microscope with an x20 objective.

Animal cell viability assessment and cell growth rate of drop-cultures

Cell viability of drop-cultures was high (>80%) from 24 to 31 d in culture (Fig. 3.15a). Cell growth rates were however relatively low, with a maximum mean of 2-fold-increase (Fig. 3.15b).

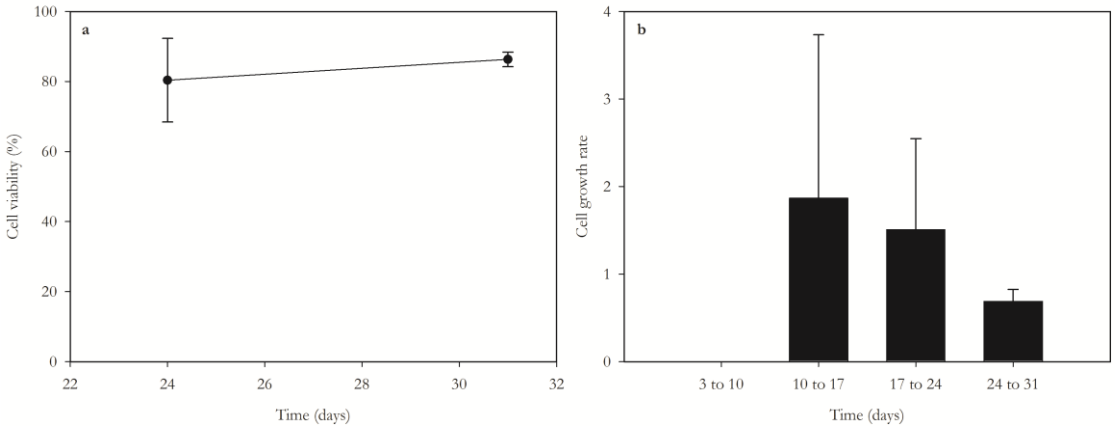

Figure 3.15 - Determination of cell viability (a) and cell growth rate (b) of drop-cultures.
Data are presented as mean ± SE, n = 4.

Determination of the differentiation state

The amplification of *A. viridis* specific gene (*AvNpc1*) on genomic DNA extracted from 17 to 31 d drop cultivated cultures confirmed the cnidarian signature of drop-cultivated cells (Fig. 3.16).

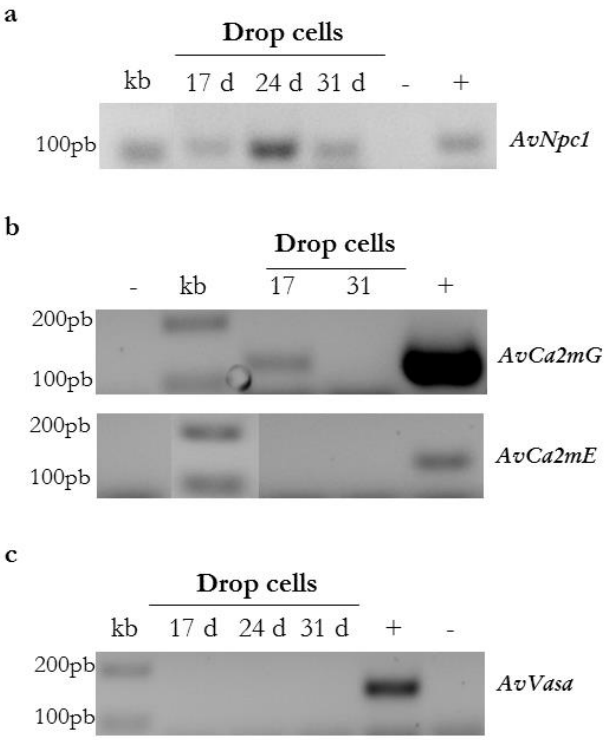

Figure 3.16 - Determination of epithelial tissue origin of *A. viridis* drop-cultured cells.
(a) Amplification of *A. viridis* specific gene. PCR was performed using specific primers of *A.viridis Npc1* gene. Genomic DNA extracted from drops at 17, 24 and 31 d of culture was used as templates. Genomic DNA from *A. viridis* whole tentacle was used as animal control (lane +); (-) corresponds to no genomic DNA template. (b) Amplification of *A.viridis* genes with tissue specific expression. RT-PCR amplifications were performed using primers of *AvCa2mG* and *AvCa2mE* genes. cDNA extracted from drops at 17 and 31 d of culture were used as templates. cDNA from *A. viridis* tentacle was used as animal control (lane +); (-) corresponds to no cDNA template. (c) Amplification of *A. viridis* gene with pluripotency specific expression. RT-PCR amplifications were performed using primers of *AvVasa.* cDNA extracted from drops at 17, 24 and 31 d of culture were used as templates. cDNA from *A. viridis* tentacle was used as animal control (lane +); (-) corresponds to no cDNA template.

Animal drop-cultivated cells showed the amplification of a specific gastrodermal gene (*AvCa2mG*) at 17 d but no amplification was observed at 31 d. No amplification was detected for the epidermal gene regardless of time points (*AvCa2mE*, Fig. 3.16)
Animal drop-cultivated cells, from 17 to 31 d, did not exhibit a molecular signature of pluripotent cells as we failed to detect expression of the pluripotency gene *AvVasa* (Fig. 3.16) and other pluripotency marker genes (*AvPiwi* and *AvPl10*, data not shown).

Discussion

The use of hanging drop technique in *A. viridis* primary cell cultures did not result in selection or enrichment in pluripotent cells, since we failed to detect expression of pluripotency markers and cells showed a gastrodermal cell signature. However, the loss of gastrodermal gene expression at 31 d might suggest that a dedifferentiation state occurs

during the drop-culture. Additionally, cells in drop-cultures were never able to reform cell aggregates that were seen in the culture plate. This observation was unexpected as one of the advantages with using hanging drop cultures is the conservation of cell-cell interaction. Up to now, stem cells, specifically intersticial-cells (i-cells), have only been identified in *Hydractinia* and *Hydra*. The recent analysis of *A. viridis* transcriptome from adult tentacle revealed the expression of pluripotency marker genes (tested in our study) reinforcing the presence of stem cell-like in adult actinians (Christen *per. comm*.). In addition, studies on tissue regeneration process in *Nematostella vectensis*, showed that the gastrodermal cells act mainly as a driving force for tissue regeneration (DuBuc et al. 2014, Amiel et al. 2015), suggesting that undifferentiated and pluripotent cells may not directly be involved. These observations could explain the absence of pluripotent cells enrichment in our cell cultures originating from adult regenerating tentacle.

Although cell viability was generally high during the drop-culture (>80%), cell growth rate was notably reduced compared with plate-cultivated cells, suggesting that cells in drops lose the capacity to proliferate. As our results are preliminary, more efforts should be carried out to improve the method and to characterize the cells maintained in drop-culture.

3.7 Conclusions

In the present chapter, we addressed several aspects of cnidarian cell culture, fundamentally characterization of cell types in culture, selection of different cell types and stemness of cells in culture. Furthermore, we were interested in evaluating the use of cnidarian primary cell cultures as a tool to assess plasticity at cellular level.

We succeed to establish a long-term cnidarian primary cell culture differentiated into gastrodermal cells, with high cell viability and growth rate, and capable of active proliferation. Both harvest protocols, selecting suspension and total cells were validated and no apparent difference was detected between them. By definition, primary cell cultures are characterized by finite life span *in vitro*. In our study, we demonstrated that 31 d is still a relevant time point (exhibiting high viability and growth) to analyse the healthy state of cultivated cells. Preliminary results have highlighted the possibility to maintain the culture over three months but a DAPI nuclear staining revealed polynucleated cells (suggesting a senescent state, data not shown). We then consider pursuing the culture kinetics over the 31 d until the first sign of senescence to decipher the mechanisms of longevity/aging in cnidarians.

Using the two different monolayers, epiderm and gastroderm as tissue source, we were unable to establish a viable and proliferative culture, suggesting that the lack of cell-cell inter-signalling and/or gastrodermal/epidermal inter-signalling is redhibitory for cell culture establishment. However, these are preliminary results and we could envisage improving the dissociation protocol for epiderm, to a mechanical protocol or to an explant culture, potentially favouring dissociation of this tissue. Additionally, we could reseed epidermal and gastrodermal cells cultures back together, in order to confirm if gastrodermal/epidermal inter-signalling is crucial to the establishment of cell culture.

Cells issued from drop-culture seem to be in a quiescent state, while plate-cultivated cells maintain high levels of growth and proliferation. Pluripotent cells were absent from both cultures, which suggests that both protocols do not favour the isolation of this cell type despite that *AvPiwi*, *AvVasa* and *AvPl10* transcripts have been shown to be expressed in *A. viridis* adult tentacle (data not shown and Fig 3.16). For the establishment of the different cultures we used tentacles in regeneration (3 days after the initial amputation). However, we cannot exclude that at the moment of the second amputation, tentacle was already completely regenerated, with cells already differentiated into different tissue layers, therefore not favouring the isolation of pluripotent cells. Further studies using earlier post-amputation time point should be carried out to potentially succeed in the isolation of cnidarian pluripotent cells. Nevertheless, two limiting factors in the isolation and *in vitro* culture of cnidarian pluripotent cells are the insufficient knowledge in this phylum on (1) pluripotent cells features, (2) potential role in regeneration and (3) the appropriate culture methods addressed for stem cells maintenance.

We determined that established cnidarian primary cell cultures could be used as a tool for *in vitro* studies from 10 d, where viability was high and cells were in active proliferation, and we validated the use of this tool to assess cellular response to environmental stress, opening up a wide range of perspectives developed in the following chapter.

4. Chapter 4 – General conclusions and perspectives

This study shows that the ability to conciliate several disciplines and approaches can greatly contribute to understand the complex processes leading to a response to environmental stress in the cnidarians. In this PhD thesis, we have analysed the potential of *A. viridis* as a model species to investigate the phenotypic plasticity of cnidarians to climate change in both whole organism and isolated cells.

In situ long-term and laboratory short-term exposures to high pCO_2 allowed us to determine *in vivo* changes in DIC absorption in part responsible for the plasticity enabling the sea anemone *A. viridis* to thrive under future predicted pCO_2 scenarios. Additionally, we observed that the response is modified when we combined increased seawater temperature with OA, environmental stressors expected to be observed concomitantly in the future predicted scenarios (IPCC, 2013).

In vitro cell culture development allowed us to obtain a viable and proliferative gastrodermal primary cell culture of *A. viridis*. We then validated the use of this powerful tool to assess the host cell sensitivity to environmental perturbations.

OA is expected to impact several marine organisms but non-calcifying cnidarians exhibit tolerance to this environment (e.g. Suggett et al. 2012, Jarrold et al. 2013, Horwitz et al. 2015). Our results support these findings by showing that for both *in situ* long-term and laboratory short-term exposures to high pCO_2 *A. viridis* changed its DIC use, from HCO_3^- user to CO_2 user, reducing energy demands of CCM which could then be reallocated to growth or reproduction. This finding is significant in the context of OA, since it suggests that *A. viridis* has mechanisms that can offset the expected negative impacts of increased pCO_2. Nevertheless, this result could be complemented by a deeper analysis of the changes in HCO_3^-/CO_2 source (i.e. tracing the preferential DIC source and/or completing the DIC transport mechanisms in high pCO_2). In our study, the comparison between *in situ* and laboratory experiments was important to determine if this observed phenotypic plasticity was due to a local selection at Vulcano or if it was rather an intrinsic property of the animal allowing colonization of fluctuating habitats. Our results show that the observed physiological plasticity is intrinsic to the animal since we observed the same decrease in CA activity of organisms that theoretically were never submitted to high pCO_2 contrary to those who were chronically exposed. The high sea anemones abundance at Vulcano could suggest an adaptation to local high pCO_2 (i.e. no energetic cost and/or high fitness; Suggett et al. 2012). However, at Plymouth, the costs to the phenotypic plasticity have not been

determined therefore we cannot conclude if we observed an adaptive (i.e. optimal phenotype) or a non-adaptive phenotypic plasticity (i.e. with a reduced fitness; Ghalambor et al. 2007). Adaptation as a strategy to overcome impacts of OA is challenging to organisms that have long generation times. Organisms with short generation times are therefore useful to understand the capacity of adaptation of those species to OA. (Lohbeck et al. 2012, 2014b) have shown that coccolithophores, a calcifying marine phytoplankton species with short generation times, can adapt to increased pCO_2 levels. Following 500 generations in high pCO_2 conditions, exposed coccolithophores show higher growth rate and calcification recovery and several genes involved in cellular pH regulation and carbon transport were up-regulating, suggesting that the observed physiological response results from adaptive evolution. The same approach cannot be used with long generation time species like *A. viridis*; however, a strategy to study phenotypic plasticity in order to identify adaptation *vs.* acclimatization mechanisms in marine organisms could be to use reciprocal incubation experiments *in situ* along natural pCO_2 gradients as those found in the Vulcano CO_2 vents (e.g. (Calosi et al. 2013, Ricevuto et al. 2015). Transplantation of individuals chronically exposed to high pCO_2 to control pCO_2 sites (H-C) and the reverse (C-H), could allow determine if *A. viridis* in Vulcano is genetically adapted to high pCO_2. If H-C individuals show the control phenotype, this means they only acclimatized and that what we observe is, like in Plymouth, a direct phenotypic plastic response. Defining the phenotypic plasticity of marine organisms is therefore fundamental to understand to what extent this phenotypic buffering is a viable strategy to environmental perturbations or whether it can favour genetic adaptation.

Ocean warming, i.e. rise of seawater temperature is an environmental factor expected to occur alongside with OA. The effect of temperature in cnidarian-dinoflagellate symbiosis has been extensively studied and it is known to induce bleaching, i.e. loss of symbionts or their photosynthetic pigments (Lesser 2011). The combined effect of both increased seawater temperature and OA has also been addressed, although poorly, but recent studies show a negative effect on metabolism (Gori et al. 2016) and calcification rates (Reynaud et al. 2003) of coral species. Our work shows that the simultaneous addition of increased temperature and high pCO_2 modified the response observed during a high pCO_2 scenario, by potentially altering the properties of the membranes but also by increasing the CCM performance (as demonstrated in free-living *Symbiodinium*; Oakley et al. 2014) and consequently countering the changes in DIC species use. However, discerning this

potential change in membrane permeability would therefore need specific studies on the effect of temperature in the diffusion of CO_2. Analysis of CCM regulation in *Symbiodinium* isolated cells and *in hospite* could also help to decorticate the mechanism of DIC absorption during temperature and pCO_2 increases. In addition, further investigations could also explore the consequences of sequential stresses as marine organisms are exposed daily to abiotic factors (e.g. temperature, pH) that can change in time and intensity (Gunderson et al. 2016). We should consider using sequential stressor experiments (i.e. high pCO_2 followed by temperature increase), a more realistic environmental approach, in order to depict patterns of phenotypic plasticity that could allow time for a compensatory response before the subsequent stressor.

To deeper understand the response to environmental stresses at cellular level it is necessary to develop new powerful tools. Although *in vivo* studies are a means to understand the plasticity of the organisms as a whole, ultimately, the plasticity of an individual is reflected in its cellular behaviour. In this PhD thesis, we developed and validated a new tool, primary single cell culture from gastrodermal tissue origin of *A. viridis*. Although a single cell type does not represent the complexity of an entire organism, single cells are advantageous in a way that they allow study a specific cell type response. In our study, we observed a rather higher rate of proliferation when compared with other cell cultures, such as the tissue balls from *Pocillopora damicornis* (23 % *vs.* 0.2%, respectively; (Lecointe et al. 2013)). To complement the analysis, it will be interesting to further analyse the cell cycle of the culture in order to differentiate the state of the non-proliferative cells: G0/G1 state, quiescent state or apoptotic state (using flow cytometry analysis).

In our study, we did not obtained pluripotent cells in culture, suggesting that our methods do not favour the isolation of this cell type. (Odintsova et al. 2005) showed that the time of regeneration of holothurian tissues influenced the different types of cell behaviour in culture. Therefore, we could envisage establishing cell cultures from tentacles at different regeneration time points (e.g. 6, 12, 24 hrs after first amputation compared to 72 hrs used in our study) to enrich cell cultures in pluripotent cells, potentially involved in the process of regeneration.

During this study we observed that isolated epidermal and gastrodermal monolayers were unable to allow viable primary cell cultures. To overcome this obstacle, we propose to test others dissociation techniques for the epiderm, favouring a combined mechanical and chemical dissociation methods and/or use an explant culture. Additionally, since we

suggested that the lack of viability could be related with the absence of epidermal/gastrodermal inter-signalling, we could propose to set up a co-culture system. Through the co-culture system, the dissociated epidermal cell culture and the established gastrodermal cell culture will be maintained in proximity potentially favouring an epidermal growing cell culture.

The perspectives of applications of cnidarian cell cultures are large and make them an attractive subject of research. Here we validated the use of primary cell culture to test *in vitro* the stress response of gastrodermal cells. We assessed a thermal stress response and observed a partial loss of cell viability after 24 hrs and 7 d of exposure. To further exploit the relevance of primary cell cultures to assess phenotypic plasticity to environmental stressors we must still determine cellular mechanisms, such as apoptosis or necrosis which are known to occur *in vivo* following a thermal stress (Richier et al. 2006). We should also compare the proliferation rate before, during and after a stressor in order to identify the existence of cellular resilience after a stress event. In complement to the studies addressed in the chapter 2 of this PhD, we could foresee study the *in vitro* cellular phenotypic plasticity to OA to be able to predict the ability to maintain cellular processes during whole culture kinetics (from 10 d to 31 d) under high pCO_2 (Fig. 4.1). In this context, a focus on the role of the carbonic anhydrases in the animal host cell phenotypic plasticity will then complete our work and could bring some new insights in the resistance of cnidarian cells to future pCO_2 scenarios. Moreover, besides environmental stressors which cnidarians are expected to suffer, symbiotic cnidarians are exposed to stressors inherent to the symbiosis itself (as oxygen or pH variations). Indeed, during a light/dark cycle there are fluctuations of O_2 concentration (from hyperoxia to hypoxia) due to photosynthesis and respiration by the symbiont (Richier et al. 2003). Therefore, to assess the mechanisms of phenotypic plasticity involved in the oxidative tolerance, we could test (1) the direct cell response to oxygen variation, (2) the processes of antioxidant regulation, (3) the impact of a pre-conditioning oxidative state (i.e. analysis of the cell sensitivity to temperature stress after a hyperoxia/hypoxia pre-exposure) (Fig. 4.1).

Moreover, as a long-term perspective we could exploite the cell culture tool to study symbiosis establishment and relationships. Cellular attraction between host cells and symbiont cells could be investigated by using a cell attraction assay (via a transwell system), to mimic *in vivo* conditions (Fig. 4.1). Thanks to this system, we can separate independent *Symbiodinium* and gastrodermal cells cultures by a microporous membrane, and we will

follow the migratory response of cells towards each other. This strategy has currently been used with mammalian cell lines and has proved useful to describe communication in individual cells in response to cell-to-cell metabolic signals (Bacchus et al. 2012). Finally, we could use the co-culture of gastrodermal primary cell cultures with *Symbiodinium* free living cells, to rebuild an *in vitro* symbiosis (Fig. 4.1), testing the symbiont "infectivity" or the host symbiotic capacity (eg. *in vitro Toxoplasma* or *Plasmodium* infectivity; Evans et al. 1999, Panichakul et al. 2007). These challenging perspectives will therefore depict the cellular mechanisms and the molecular inter-signalling involved in symbiont recognition, engulfment and maintenance with symbiont cell cylce regulation.

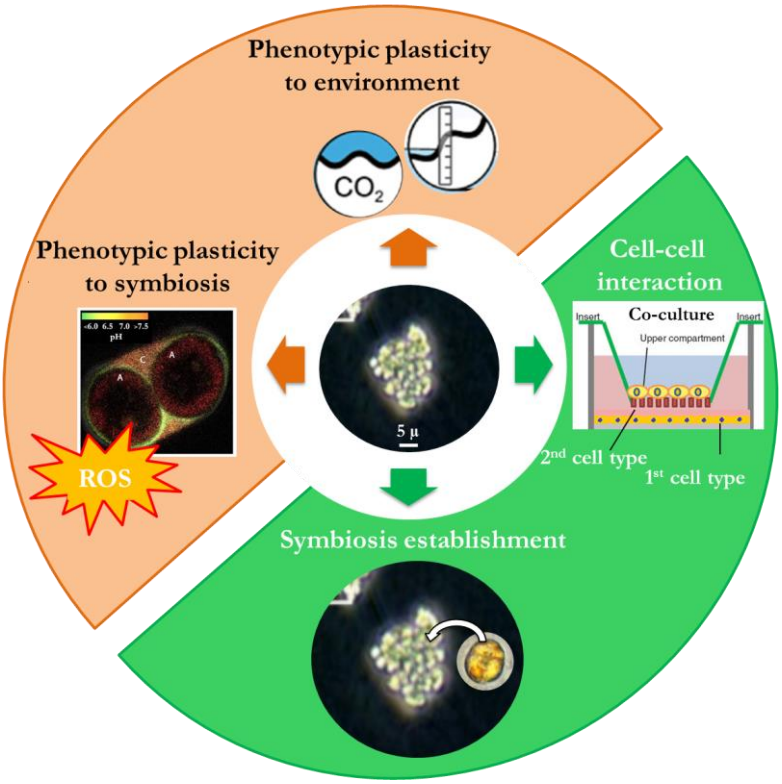

Figure 4.1 - Summary of the perspectives for the powerful new tool developed during this PhD, cnidarian primary cell culture of *A. viridis*.

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Annex I – Identification of carbonic anhydrase transcripts

Four complete transcript sequences of metazoans alpha carbonic anhydrase (α-CA) have been identified in the transcriptome of *Anemonia viridis*. The transcriptome has been obtained from the analysis of total RNAs of 12 independent tentacles tissue samples extracted from two specimens of *A. viridis*. After extraction, cleaning and quality checks of mRNA, 3 to 5 independent lanes of paired-end HiSeq Illumina sequencing were done. All R1 and R2 fastq files were pooled and Illumina adapters removed with Trimmomatic software (Bolger et al. 2014). A complete transcriptome was generated by Trinity (Grabherr et al. 2011). This generated 554 092 alternative transcripts, corresponding to 331 933 "genes".

A. viridis genes encoding carbonic anhydrase (CA) sequences were isolated from the transcriptome by Blastn using *Homo sapiens* and *Nematostella vectensis* CA family gene sequences. Data validation was then performed by tBLASTx and tBLASTn searches in the databases at NCBI (National Center for Biotechnology Information). Submission of *A. viridis* sequences to the NCBI GenBank database is in progress.

Sequence analysis was conducted firstly by aligning CA protein sequences of *A. viridis* by a multiple sequence alignment performed using MUSCLE algorithm and manual adjustment (Figure A.1).

```
          1
AvCa2mG   -------- -------- ------ME NQVLLCVL STLAAF-- -------- -HLATGAG VVYNYHLS DAT-YGPN GWAKTYPQ SCDGSNQS
AvCa2mE   -------- -------- ------MA NTFLIFLL TT-AFF-- -------- -HVSLSTK --YNYDTS DAD-YGPN GWPKTYPS -CNGSRQS
AvCa2c    -------- -------- ------MA AS------ -------- -------- -------- --WGYRPN ----NGPS HWAKNFPA -AAGTSQS
AvCa2s    NTGTEVAN NTGPVVTS NTGPVVTS NTGPVVTS KTGPVFTS KPVTGSKS AHKIKSGG PTWGYGVS ETGVYGPD DWYKMSIQ -CKGNNQS

         89
AvCa2mG   PIDIDTSK TKYDSTLV SLTLSGFD TIPAGSQF SVGNSGQS --YTVFFK PNTFFVSG GGLPGTFY TLN-VHFH WGSDDTKG SEHTVDGT
AvCa2mE   PIDIKPSE ASYDGSLG KLTFTEYD NVPAGTLV RLGNNGKS --FRVFFE ADKFLVSG GGLPGTFY TLN-FHAH WGADNDKG SEHTIDGK
AvCa2c    PIDIKTAD AQYDSTLK PLKI-QYS Q---GTDF TVSNSGHS L-VTSRKT SEGNHLSG GPL-AHNY RFEQFHFH WGKTSECG SEHLLNGK
AvCa2s    PIDIFPQA TPFKLANK PMSM-ESD NALGLVTG AFTNTGHA AQFTVNEK EGAVYLSG GPADSNNY KLSSIQFR VGCMDGSG SEHTLNSK

        177
AvCa2mG   EYPAEAHF VNVNTK-Y ANVSEALK NADGIAVL GTLLKVGD -----ENP ALTSLLKY TADVNLPS TPDIKNIT LTHTLASF LPPNITD-
AvCa2mE   KFPLEIHF VNVNQK-Y DNVTQALE QKDGIAVL GVLYEVGD -----EDV ALEPFLKY TANVTY-- VRDISNVT MTQALQSL LP-NTTD-
AvCa2c    AFPAELHL VHWNTDLF SSFGEAAS KPNGLAVL GAFVKIAG -----ENP GLKKITSL IPQVQNKG AK--QDLQ VPFNLTSM LPSNTND-
AvCa2s    PASAEMQM IFYNRK-Y KNVNEAVS QPDGIAII ATLFDYYG GFTYYANP TLERFISF LPNITEPG SSVHLDYD DRIELTRL VPELTWSY

        265
AvCa2mG   --FYRYSG SLTTPACA EVVTWTVF RNPAKISA NQLSMLRS LKESDNTT LLVNTFRP VQPLNSRV VKKSFAS-
AvCa2mE   --FYRYDG SLTTPLCQ EVVTWTVF KNYRTVSQ AQLNMLRN LRTADNVT RLVDTFRP VQPLDSRV VKKTFKT-
AvCa2c    --YWTYSG SLTTPPCY ESVTWFVF KEPIYASE EQMQQFRS LASNDG-G CIVDNYRP VMPASGRN VRASFVK-
AvCa2s    PLYYTYKG SLTTPPCY ESVTWILL KTRLSISI QQLGQFRQ LQGRNG-N QLCDNFRP IQPANSRE VTRNFTEF
```

Figure A.1 - Alignment of *A. viridis* α-CA sequences.

A second alignment was then performed, integrating human, cnidarian and bacterial complete protein sequences used for most of them in the phylogenic analysis performed by LeGoff et al. (2016). Maximum likelihood tree construction was performed using Seaview software, with bootstrap support calculated using 100 bootstrapping events (Figure A.2).

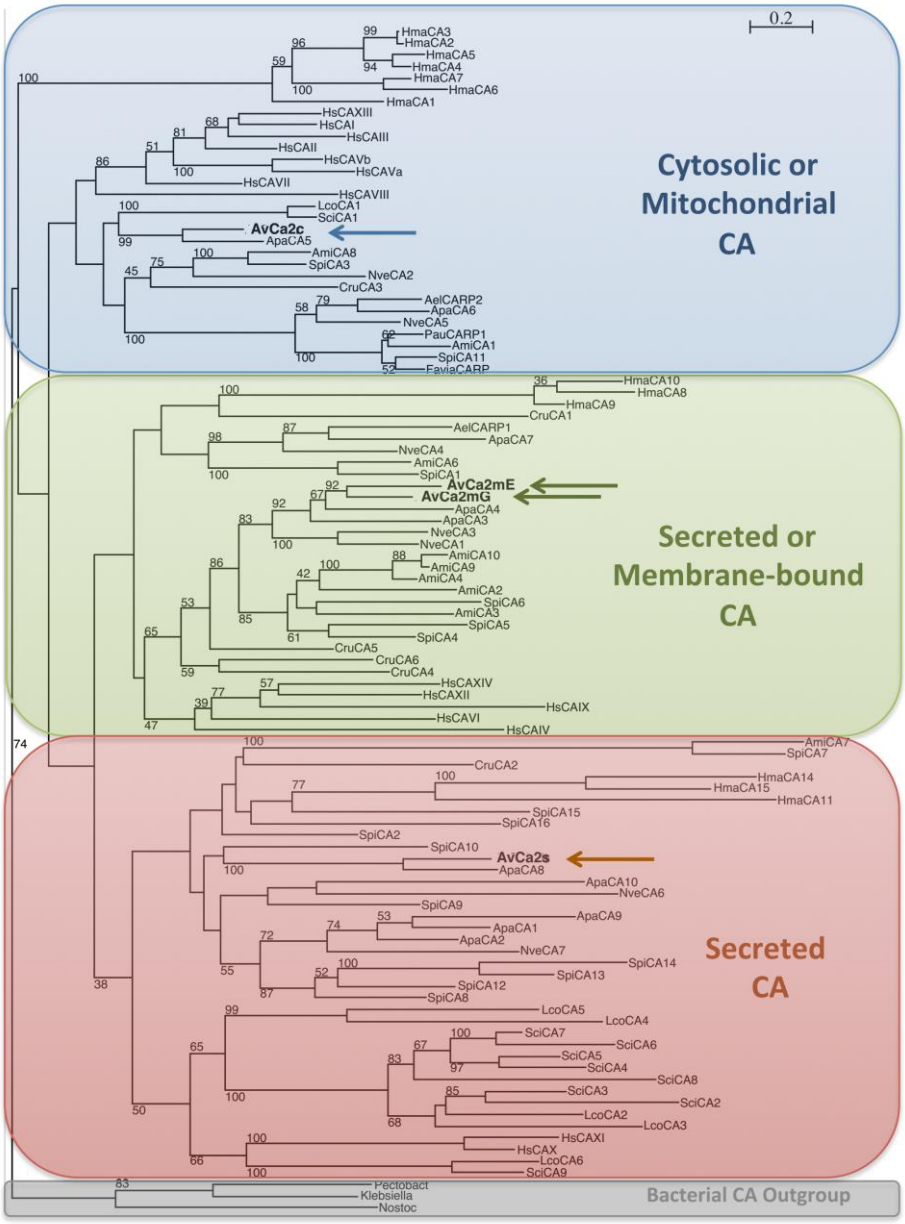

Figure A.2 - Phylogenetic tree analysis for Cnidarian α-CA proteins.
The phylogenetic tree was constructed following the same topology published by Legoff et al. (2016) with α-CAs from *Homo sapiens* (HsCA), sponges [*Sycon ciliatum* (SciCA), *Leucosolenia complicata* (LcoCA)] and cnidarians [*Anemonia viridis* (AvCa2mG, AvCa2mE, AvCac, AvCas), *Corallium rubrum* (CruCA), *Stylophora pistillata* (SpiCA), *Acropora millepora* (AmiCA), *Nematostella vectensis* (NveCA), *Aiptasia pallida* (ApaCA), and *Hydra magnipapillata* (HmaCA)], as well as the CA related proteins (CARPs) identified in different anthozoan databases, [*Anthopleura elegantissima* (AelCARP)], [*Favia* sp. (FaviaCARP)], [*Porites australiensis* (PauCARP)], [*Pocillopora damicornis* (PdaCARP)]. The bacterial CA sequences from *Pectobacterium atrosepticum*, *Klebsiella pneumoniae* and *Nostoc* sp. were used as outgroup. Bootstrap confident values are expressed as percentages with a bootstrap threshold fixed to 35%. Blue, green and red zones indicate the clusters related to the three main clusters of Cnidarian α-CAs (Legoff et al. 2016): i) the cytosolic and mitochondrial α-CAs with no disulfide bond, ii) the secreted and membrane bound α-CAs with the canonical disulfide bond, and iii) the secreted α-CAs with a canonical and a cnidarian specific disulfide bond.

ABSTRACT

During the course of their life cycle organisms are exposed to natural environment variations capable of inducing physiological, morphological and behaviour changes, thus a phenotypic plasticity. Phenotypic plasticity is the ability of a genotype to generate a new phenotype following exogeneous or endogeneous stress. Here, we investigated the phenotypic plasticity of the non-calcifying symbiotic cnidarian *Anemonia viridis* at multiple levels of structural complexity, *in vivo* and *in vitro*.

In vivo, we determined the mechanisms behind the phenotypic plasticity under expected future climate change (i.e. ocean acidification and ocean warming). Our results show physiological changes in the inorganic carbon use of the sea anemone *A. viridis* exposed to high pCO_2 during a long-term stress *in natura* or a short-term stress in controlled conditions. We then observed an equivalent decrease in carbonic anhydrase activity, a key enzyme of cnidarian carbon concentrating mechanisms. Also, we demonstrated that an increase in seawater temperature modified the response observed during a high pCO_2 scenario.

In vitro, we established a viable primary cell culture from regenerating tentacles of *A. viridis*. We determined the gastrodermal tissue origin of the cultivated cells and validated the use of this new tool to the *in vitro* study of stress response at the cellular level. The set-up of this powerful *in vitro* tool will open a multitude of perspectives for the study of cellular responses to exogeneous stress (as global change perturbations) and to endogeneous stress (as the symbiosis constraints experienced by symbiotic cnidarians).

Keywords: Symbiotic Cnidarian, Phenotypic Plasticity, Acidification, Culture Cells

RÉSUMÉ

Durant leur cycle de vie, les organismes sont exposés à des variations environnementales capables d'induire des changements physiologiques, morphologiques et comportementaux, résultant d'une plasticité phénotypique. La plasticité phénotypique est la capacité d'un génotype à générer un nouveau phénotype suite à un stress. Ici, nous avons étudié la plasticité phénotypique d'un Cnidaire symbiotique et non-calcifiant, l'anémone de mer *Anemonia viridis*, à de multiples niveaux de complexité structurale, *in vivo* et *in vitro*.

In vivo, nous avons identifié les mécanismes sous-jacents de la plasticité phénotypique potentiellement induits par les futurs changements climatiques (acidification et réchauffement des océans). Nos résultats montrent des modifications dans l'utilisation du carbone inorganique par *A. viridis* exposée à une forte pCO_2 lors d'un stress chronique *in natura* ou lors d'un stress court en conditions contrôlées. Nous avons ainsi observé une diminution des activités anhydrase carbonique, une enzyme clé des mécanismes de concentration du carbone chez les Cnidaires. Nous avons aussi démontré que l'augmentation concomittante de la température modifie la réponse observée lors d'une élévation seule de la pCO_2.

In vitro, nous avons établi une culture de cellules primaires viables issue de tentacules d'*A. viridis* en régénération. Nous avons déterminé l'origine gastrodermale des cellules cultivées et validé l'utilisation de ce nouvel outil pour l'étude de la réponse au stress au niveau cellulaire. Ce nouvel outil ouvre une multitude de perspectives pour l'étude des réponses cellulaires aux stress exogènes (changement climatique) et endogènes (contraintes dues à la symbiose).

Mots clefs: Cnidaires Symbiotiques, Plasticité Phénotypique, Acidification, Culture de Cellules