TESIS DOCTORAL

ESTADIOS TEMPRANOS DE LA OREJA DE MAR *HALIOTIS TUBERCULATA COCCÍNEA*: DESARROLLO, FIJACIÓN Y CRECIMIENTO

EARLY LIFE OF THE ABALONE *HALIOTIS TUBERCULATA COCCÍNEA:* DEVELOPMENT, SETTLEMENT AND GROWTH

GERCENDE COURTOIS DE VIÇOSE

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Index

To Eric and Maé

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ABBREVIATIONS

ANOVA: Analysis of variance

ARA: Arachidonic acid

BHT: Buthylhydroxytoluene

BZP: Biological Zero Point

CCA: Crustose coralline algae

DGR: Daily growth rates

DHA: Docosahexaenoic acid

DOM: Dissolved organic matter

DPA: Docosapentaenoic acid

DM: Dry matter

DW: Dry weight

EAT: Effective accumulative temperature

EFA: Essential fatty acids

EPA: Eicosapentaenoic acid

FA: Fatty acids

FAME: Fatty acid methyl ester

GABA: Gamma aminobutyric acid

GC: Gas chromatography

HUFA: Highly unsaturated fatty acid

PUFA: Polyunsaturated fatty acid

TRIS: Hydroxy-methylamino methane

UV: Ultra violet

The international system of units (SI) was employed in the entire document

ABSTRACT

In view of the interest in diversification of abalone species production, the identification of early life processes was necessary for the further development of spat production techniques adapted to *Haliotis tuberculata coccinea*. Therefore the main objective of this study was to "***Acquire knowledge on early life history of the abalone Haliotis tuberculata coccinea and contribute to the development of its production techniques through the improvement of larval settlement as well as growth and survival rates of juveniles***". Specifically, early development, settlement process and growth and survival of post-larvae and juveniles, as well as various factors affecting these events, were addressed in this study through the achievement of five specific objectives.

The first description of the complete embryo and larval development of *Haliotis tuberculata coccinea*, Reeve was illustrated in a micro-photographic sequence and took place in thirty-nine distinct, consecutive, stages. The occurrence rates of the different stages, observed at 23 ± 0.5ºC, differed in order of appearance to that of other species and were closer to the ones of a tropical abalone species like *Haliotis asinina* than to the ones of a temperate one as the achievement of settlement competency (emergence of the third tubule on the cephalic tentacles) appeared 62h after fertilization.

The effectiveness of different settlement induction cues as well as the effects of their age, enrichment levels and combinations, were determined based on the settlement and metamorphosis response of *H. tuberculata coccinea* larvae and post-larval survival. Larval settlement was the highest on crustose coralline algae (CCA), across experiments, confirming that CCA are among the best settlement inducing substrates. Larvae also exhibited increased settlement rates on *Ulvella lens* suggesting its use as an efficient and consistent settlement substrate for *H. tuberculata coccinea* larvae taking into account that its settlement induction efficiency was found to be reduced if colonized by diatoms. Besides, germlings of the green macroalgae, *Ulva rigida*, were discovered to greatly enhance larval settlement revealing a strong potential to be considered for future abalone spat production. Furthermore, the significant differences in larval settlement and survival rates were correlated to the biochemical composition of the algal substrates, in its turn found to be species specific as well as influenced by developmental stages, and culture conditions of the substrates. This

emphasized the ability of the larvae to distinguish between different developmental stages and proximate composition of the substrate's algae prior to settlement and metamorphosis.

On the contrary, settlement induction with 1 μM GABA and on conspecific mucus, in the absence of diatom films, led to low settlement rates and none of the diatom species tested were successful in the induction of *H. tuberculata coccinea* larval settlement, suggesting species specificity as well as the effects of age, growth phase or culture conditions of the diatoms.

High larval density had a negative effect on settlement rates and post-larval survival of *H. tuberculata coccinea* whereas post-larval growth rates were not influenced by this factor.

In addition, settlement substrate features were found to influence growth rates independently of the food source employed suggesting a competition for space between the algae from the substrate and the diatoms; specifically on CCA their high exfoliative activities could have contributed to the reduction of attached diatom cells to be ingested by the post-larvae. The present growth rates results also support the idea that settlement patterns are reflected and manifested in growth and survival of *Haliotis* larvae as the highest growth and survival rates were observed on substrate also yielding high settlement rates.

Considering benthic diatoms are the main source of food of post-larval abalone a study was performed to adapt the diatoms culture conditions to the requirements of abalone post-larvae.

The four diatom species investigated showed adequate size range, to be potentially used as feed for *H. tuberculata coccinea* post-larvae throughout the nursery stage of production and all presented fatty acid profiles characteristic of most diatoms, reflected by high proportions of 14:0, 16:0, 16:1n-7 and 20:5n-3, EPA being the predominant n-3PUFA, and low proportions of DHA, the lowest being observed in *Navicula incerta*. The results of this study revealed that low inoculum density was the best suited to sustain diatoms' GR as well as cell production and attachment and that cultures harvested in log-phase of growth would present superior nutritional values being *Amphora* sp. and *Proschkinia* sp., in their log-phase of growth, that would best fit *H. tuberculata coccinea* post-larvae nutritional requirements. *Amphora* sp. presented the highest cell attachment capacity, at all inoculums densities, a characteristic of additional interest for post-larvae nutrition.

Finally the effects of diatoms on post-larvae growth rates were assessed and *H. tuberculata coccinea* post-larvae exhibited divergence of growth rates, among diets, when reaching 0.8-1mm SL, and presented increased growth rates around 2 mm SL. These

differences in growth rates indicate first an access to diatom cell contents followed by a possible shift in nutrition towards green macroalgae germlings, as a complement of the diatom diet, when juveniles average 2mm SL. Post-larvae fed the diatom with the highest protein and lipid contents (*Amphora* sp.) presented the highest average growth rates and protein content was correlated to higher growth and final shell length. The fatty acid profiles of the post-larvae suggest that 18:2n-6 and 18:3n-3 are essencial for *H. tuberculata coccinea* and that this species is capable to absorb a selection of fatty acids from the food source or able to synthesise them from precursor dietary n-3 fatty acids.

These results provide crucial technical knowledge necessary to the adaptation of culture techniques to the requirements of *H. tuberculata coccinea.* More particularly: *Ulvaceae* species presented great potential to enhance settlement, growth and survival rates and the control of diatoms and substrates nutritional quality was shown to improve abalone post-larval growth and survival, consequently influencing the efficiency and consistency of juvenile production necessary to the development and standardization of the production methods.

EARLY LIFE OF THE ABALONE *HALIOTIS TUBERCULATA COCCINEA*: DEVELOPMENT, SETTLEMENT AND GROWTH

1. INTRODUCTION

1. INTRODUCTION

1.1 ABALONE LIFE CYCLE

Abalones are univalve (single-shelled) marine herbivorous gastropods belonging to the family Haliotidae, categorized into 56 existing species being classified in the single genus *Haliotis* (Geiger, 2000). They are present in all the oceans. Despite the fact that the study of abalone biology started long ago (Bonnot, 1930; Stephenson, 1924) it has been more intensely investigated during the last 30 years, due to the increasing interest in abalone aquaculture. Abalones are gonochoristic and broadcast spawners, with males and females synchronously liberating their gametes in the water column for reproduction (Crofts, 1930; Stephenson, 1924). Mature males and females can easily be recognized by the differences in gonad colour (Bardach et al., 1972).

Their life history can be described into five stages: embryo, larvae, post-larvae, juvenile and adult (Fig. 1).

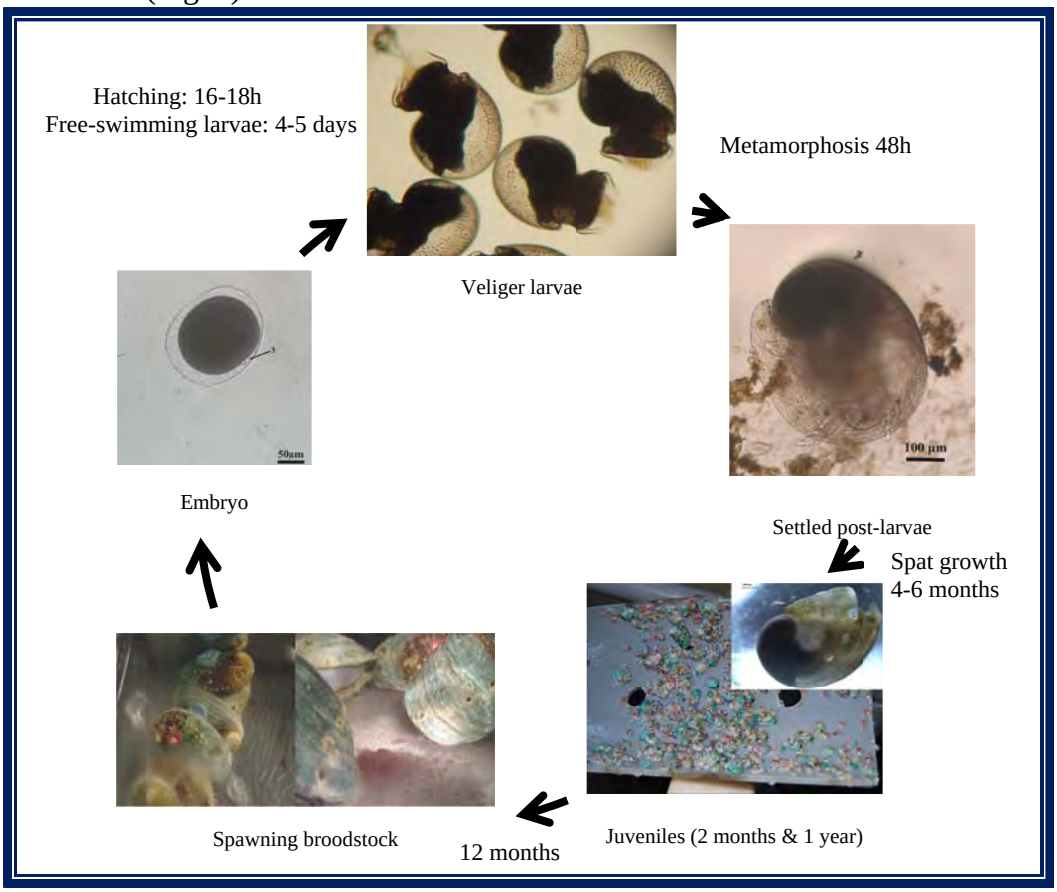


Fig. 1 Abalone life cycle.

The fertilized eggs first go through the embryonic stage, only lasting few hours, and then hatch into a ciliated trochophore larvae. The resulting larvae are pelagic and lecithotrophic, swimming in the water column for days to weeks depending on the species and water temperature before becoming competent to settle (Leighton, 1972; Sawatpeera et al., 2001). The lecithotrophic larvae rely on their energetic reserves and on the absorption of dissolved organic matter from the water until settlement (Jaeckle & Manahan, 1992; Kawamura et al., 1998b; Shilling et al., 1996). Competent larvae undergo settlement once having encountered appropriate settlement cues. Settlement is a critical phase consisting in the transition from pelagic life to benthic one and involves attachment and metamorphosis (Roberts, 2001). During attachment, the larvae stop swimming and attach to the substrate while retaining their velum, whereas metamorphosis involves irreversible changes committing the abalone to benthic life. From this point they are known as post-larvae, starting to feed on benthic microflora, mainly biofilms of diatoms and bacteria growing on the surfaces of the settlement substrates (Daume et al., 1999b; Kawamura et al., 1998b; Shepherd & Daume, 1996).

The post-larval stage will end with the appearance of the first respiratory pore after which abalone are considered juveniles until reaching sexual maturity. The size and age at which sexual maturity is attained varies between species and is dependent on temperature and food availability. From juveniles to adults, abalones shift their diet from micro to macroalgae and experience exponential growth at post-larval and juvenile stages slowing down as sexual maturity is reached. Subtropical and tropical abalone species grow faster initially, mature earlier and present smaller size than temperate species.

1.2 WORLD ABALONE PRODUCTION AND AQUACULTURE

Worldwide, total abalone production (including fisheries and aquaculture) has shown a significant increase during the last decades, passing from 20370mt in 1970 to 48405mt in 2008 (Fig. 2). The products are sold in live, fresh, frozen, canned or in dried form, with the main markets being in China, Japan, Hong Kong, South East Asia, USA, Mexico, Korea and Europe (Oakes & Ponte, 1996).

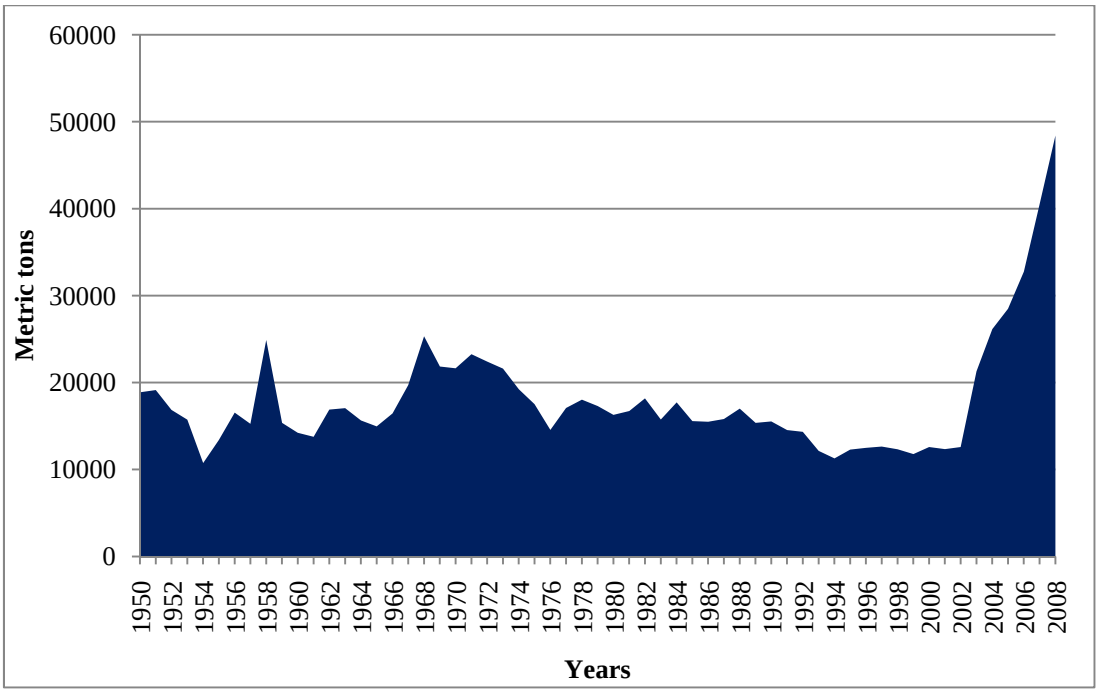


Fig. 2 World abalone production including fisheries and aquaculture (FAO, 2009).

This increase reflects the exponential contribution of aquaculture to the global abalone production since abalone fisheries have been in continuous decline over the past four decades (Fig. 3). Abalone species (*Haliotis* spp) have become the focus for aquaculture production due to their high market price and the over-exploitation of wild populations. It is estimated that in the years to come over 70% of the wild stocks will be included in the endangered species lists. As a consequence of over-exploitation, aquaculture production will be the only way to supply the growing market demand. Aquaculture products fit a different market niche to the commercial fishery products, as they can be harvested at different and smaller sizes, they also have the ability to provide fully traceable and certified products that can be identified and differentiable from wild stocks, and permit genetic improvements to optimize the production.

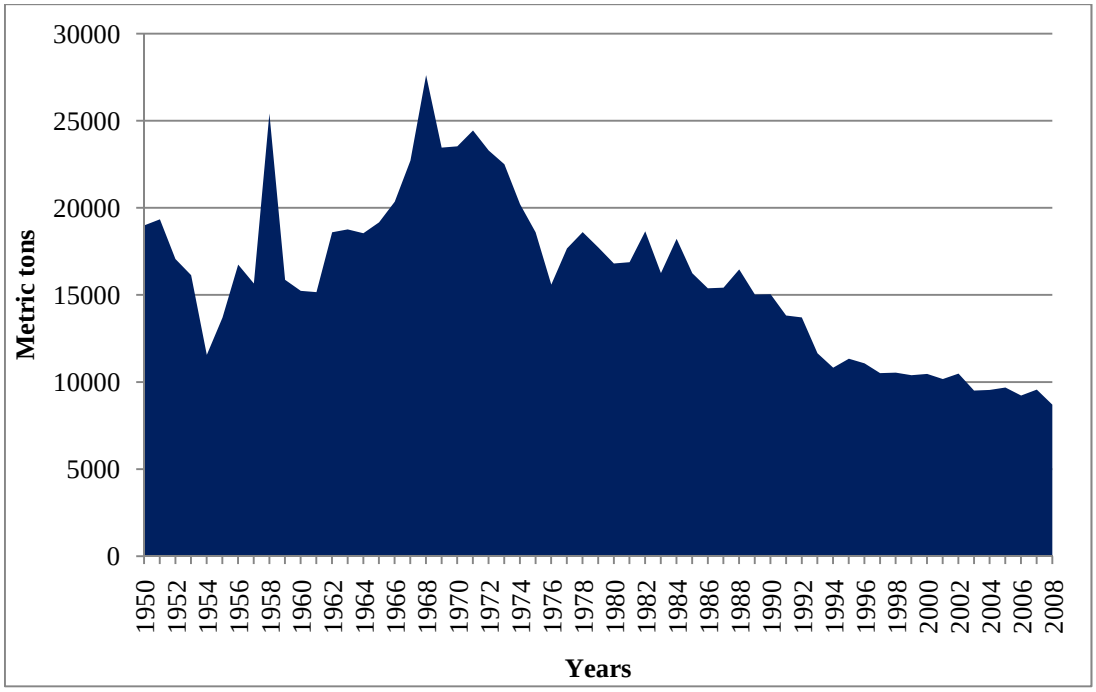


Fig. 3 World abalone wild catches (FAO, 2009).

Over fishing, disease, habitat loss, and failed governmental management of the illegal catch have all contributed to the decline of the abalone fisheries over the past four decades (Gordon & Cook, 2004). In 1970, worldwide abalone catch reached up to 23532 mt, while in 2008 this value had dropped down to 8701mt.

The situation with regard to cultured abalone is the opposite, with an increase by over 600% in the last ten years. This increase was made possible by the development of recent production practices, especially for juvenile stages, that promoted the rapid expansion of the industry (Daume et al., 2004; Roberts et al., 2004). The exponential growth of the world cultured abalone is evident when comparing the 40486 mt for the year 2008 with the just 689 mt 20 years earlier (Fig. 4).

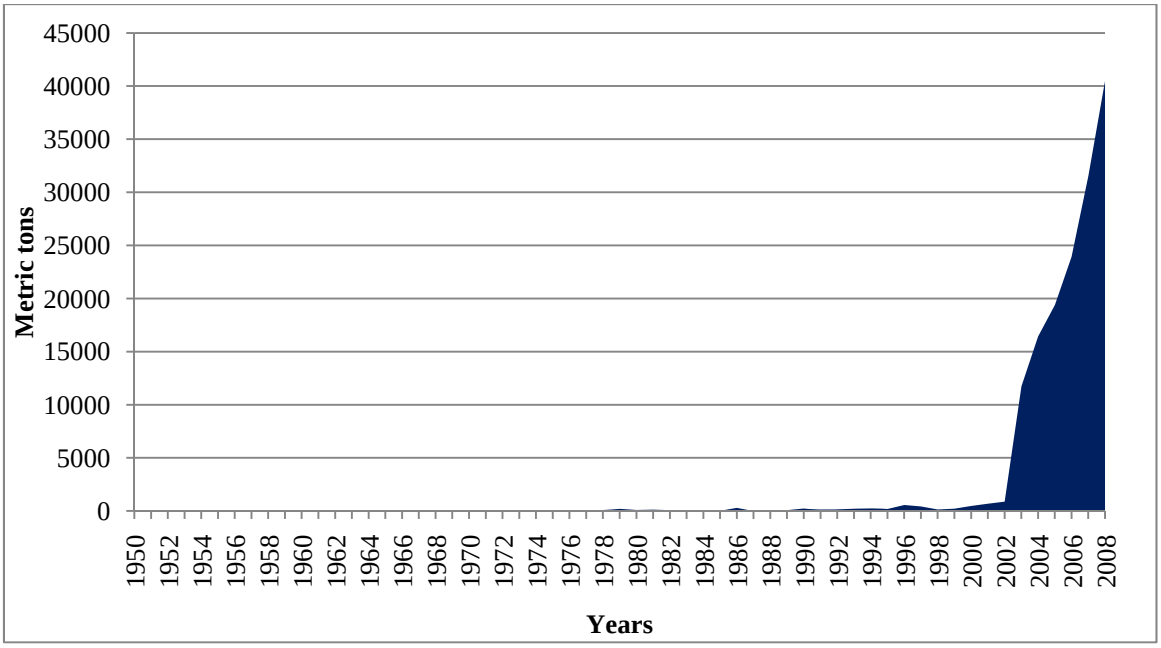


Fig. 4 World cultured abalone (FAO, 2009).

The increase in production of cultured abalone was possible thanks to the development of research on the culture of abalone that began in Japan with *Haliotis discus* (Ino, 1952). In the 1960's, hatchery systems for abalone were developed (Leighton, 2000) with the most significant contributions arousing in the early 1970's including the studies on controlled spawning by Nagahisa Uki and Shōgo Kikuchi for *Haliotis discus*, *Haliotis discus hannai* and *Haliotis gigantea* and covering topics such as the effect of temperature and nutrition on broodstock conditioning, ultraviolet (UV) induction of spawning and optimal sperm density for fertilization. These results greatly improved hatchery and nursery production (Uki & Kikuchi, 1984).

These hatchery developed techniques were soon tailored to the need of others species in other parts of the world, while it was discovered that hydrogen peroxide (H_2O_2) also induced abalone to spawn (Morse et al., 1977). Both factors have permitted the development of abalone aquaculture in several countries such as USA, South Korea, China, Taiwan, Mexico, Chile, South Africa, New Zealand, Australia and Europe.

Actually more than 15 species of abalone are commercially cultivated worldwide with production units presenting diverse order of magnitude ranging from less than one tone to 200 mt (Gordon & Cook, 2004). The main producers are China, Australia and Korea, China leading the production with 33010 mt in 2008. Australia has steadily developed its abalone

production reaching 5320mt in 2008 while Korea showed an explosive increase in its abalone production in the past ten years to a total of 5318mt in 2008 (Fig. 5).

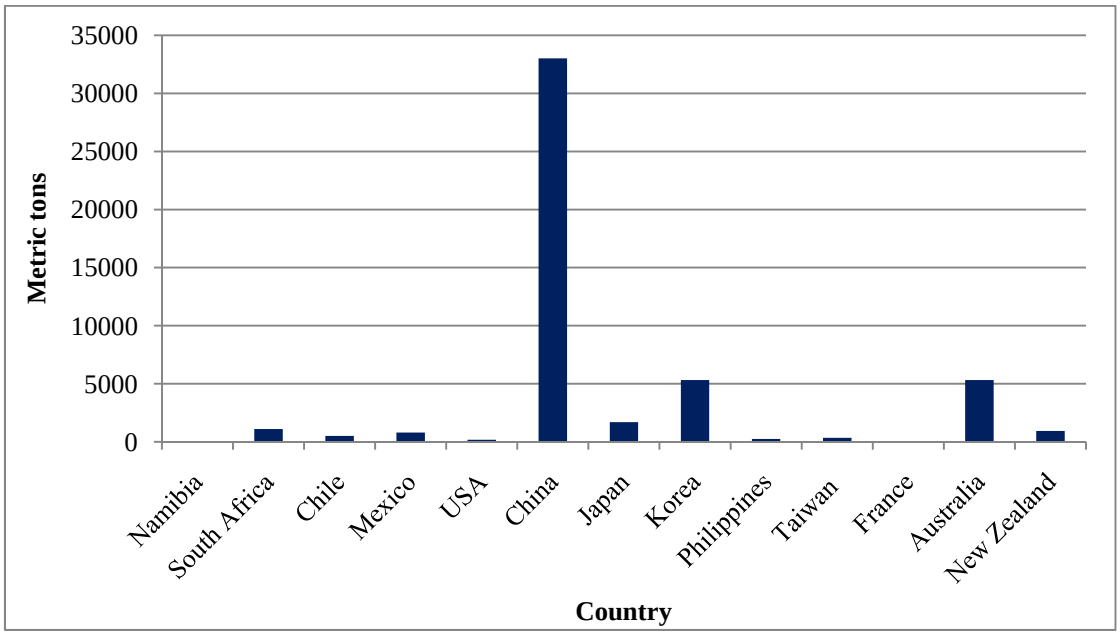


Fig. 5 World abalone producers (FAO, 2009).

1.2.1 Importance of controlling early life stages in abalone aquaculture

Despite survival rates during larval culture reaching up to 90-95%, survival during the settlement phase generally reaches only an averaged 10% (Hahn, 1989). Therefore, settlement induction and metamorphosis are considered the most critical stages on abalone seed production (Ebert & Houk, 1984). The studies on abalone larval settlement reviewed by Roberts in 2001 demonstrated that larvae ready to undergo metamorphosis need to encounter and get in contact with suitable settlement cues to shift successfully from pelagic to benthic life.

Biofilms and diatom films have traditionally been used in abalone hatcheries around the world to induce larval settlement and feed early post-larvae (Daume et al., 1999b; Daume et al., 2000; Hahn, 1989; Kawamura & Kikuchi, 1992; Seki, 1980; Seki & Kan-No, 1981b; Takami et al., 1997a). However studies, investigating growth and survival of post-larvae, revealed that the cues, necessary for larval settlement and further post-larval growth, where not efficiently provided by traditional biofilms and diatom colonized substrates (Bernal et al., 2000; Buchal et al., 1998; Daume et al., 2000; Kawamura & Takami, 1995). Moreover

biofilms are difficult to maintain on the long term as it is complicated to exert control over their species composition, their density and, thus on, biofilm quality (Hahn, 1989). The biofilm or diatom film quality and composition have been found to profoundly influence larval settlement as well as post-larval survival and growth (Daume et al., 2003; Daume & Ryan, 2004a; Gordon et al., 2006; Roberts et al., 2007; Searcy-Bernal et al., 2003; Searcy-Bernal et al., 2001; Uriarte et al., 2006; Viana et al., 2007b; Watson et al., 2004).

In parallel to the previous studies various settlement inducing cues were tested on different abalone species, including crustose coralline algae (CCA) (Daume et al., 1999b; Daume et al., 2000; Morse & Morse, 1984; Morse et al., 1980c; Roberts & Nicholson, 1997; Takami et al., 1997b), gamma amino bituric acid (GABA) (Bryan & Qian, 1998; Morse et al., 1980a; Searcy-Bernal et al., 1992; Slattery, 1992) or abalone mucus (Bryan & Qian, 1998; Seki, 1997; Seki & Kan-No, 1981a; Slattery, 1992). In Japan, *Ulvella lens* is employed in hatcheries to improve larval settlement and post-larval growth of *H. discus hannai* (Takahashi & Koganezawa, 1988) and this technique is now extended to hatcheries in Australia (Daume et al., 2004). As abalone are slow growing molluscs and since settlement rates are generally unreliable in hatcheries, any improvement in settlement success or growth rate will greatly benefit the production as it would improve the consistency of the production, reduce production time and thus, increase the cost benefit of the production. Improvements of the early life stages of the culture are essential to abalone production considering that reliable larval supply and consistent seed production are key factors for abalone aquaculture.

1.2.2 *Haliotis tuberculata coccinea*

Only one species of abalone is present in Europe, *Haliotis tuberculata* (Mgaya et al., 1995) with three sub-species being initially described based on morphological characteristics: *Haliotis tuberculata tuberculata* Linnaeus, 1758 in the Atlantic; *Haliotis tuberculata lamelosa* Lamark, 1822 in the Mediterranean Sea; and *Haliotis tuberculata coccinea* Reeve, 1846 in the Macaronesian Archipielago (Geiger, 2000; Mgaya et al., 1995). In the Canary Islands *Haliotis tuberculata coccinea* is distributed from the intertidal zone down to 15m depth in semi-exposed and exposed areas. *Haliotis tuberculata coccinea* grows to a maximum size of about 80 mm in shell length and has been commercially exploited during decades for the local market in the Canary Islands, leading to an overexploitation of its stocks, which are actually almost depleted. Preliminary studies on *H. tuberculata coccinea*

production, focusing on spawning techniques (Peña, 1986) and nutrition (Toledo et al., 2000; Viera et al., 2005), suggested the possibility of artificially reproducing this species and identified it as a species with aquaculture potential to diversify aquaculture production. However, studies have not investigated its larval settlement processes, nor the adequate settlement cues, larval densities, and their effect on post-larval growth and survival

In order to develop spat production techniques adapted to this species, it is necessary to investigate in the areas of larval production, larval settlement and post-larval nutrition. Improvement in the field of larval production require the control of reproduction and the understanding of the different induction techniques applied, as well as the study of the entire embryological and larval cycle in natural conditions. In the case of post-larvae, the effects of various settlement cues on settlement and metamorphosis and the instant at which they have to be supplied need to be studied in order to improve that stage of the culture. Finally, in the post-larval nutrition chapter; it is important to control the quality and densities of benthic diatoms, supplied as food to abalone post-larvae, and study their effect on post-larvae growth and survival. The understanding and control of these different culture phases are crucial for the improvement, development and standardization of the production of *H. tuberculata coccinea* juveniles.

1.3 ABALONE LARVAE

1.3.1 Spawning induction

Greater understandings of the processes of gonad maturation and broodstock spawning induction methods, on demand, have been developed throughout the years.

The influence of broodstock conditioning on gamete quality has been studied in various abalone species focusing particularly on the influence of external factors such as diets or temperature on gonad development and maturation (Uki & Kikuchi, 1984) as well as gamete quality and spawning success (Bautista-Teruel et al., 2001; Ebert & Houk, 1984; Grubert et al., 2004; Grubert & Ritar, 2005; Moss, 1998).

High quality gametes obtained from broodstock held in optimal conditioning systems are easily obtained through controlled spawning induction. The control of spawning induction is usually the first step in attempting to culture new species and its success is determinant for larval production, themselves essential for the development of hatchery and culture of a new species. Techniques of spawning induction are actually well developed in abalone species

and include single stimulus or combinations of several stimuli including temperature changes, seawater treated with ultraviolet rays (UV), ozone or hydrogen peroxide, handling, or air exposure. The most commonly employed and efficient methods to induce abalone to spawn are the treatment of seawater with UV or the hydrogen peroxide method (Moss et al., 1995). Morse et al. (1977) discovered that low concentrations of hydrogen peroxide (H_2O_2) induced abalone to spawn and proposed that products of the decomposition of H_2O_2, such as, hydroperoxy free radical, HOO^-, or the peroxy diradical, OO^-, acted on the enzymatic system that produces prostaglandins, which in turn initiated spawning. Uki & Kikuchi (1974) proposed the use of UV irradiated seawater to induce abalone spawning. It is thought that UV irradiation of seawater is causing the energetic decomposition of the water molecule, producing free radicals similar to the ones from the hydrogen peroxide method, the donor molecule being ozone (O_3) rather than H_2O_2.

Fertilization success relies on the quality of the gametes, which are influenced by the fertilization process employed, especially in terms of the spermatozoids-ova ratio and the fertilization time period applied. The optimal sperm density and gamete contact time for fertilization in abalone has been documented for several species (Babcock & Keesing, 1999; Baker & Tyler, 2001; Clavier, 1992; Encena et al., 1998; Kikuchi & Uki, 1974; Leighton & Lewis, 1982).

1.3.2 Embryological and larval development

Negatively buoyant fertilized eggs sink and maintain their negative buoyancy during the entire embryological development. Once hatched out, lecithotrophic larvae are mobile during two planktonic stages; the trochophore and the veliger larvae. The duration of the trochophore stage is brief (a few hours), while that of the veliger may last for days or weeks depending on the species and environmental conditions.

Being lecitotrophic, the larvae rely on their endogenous energy reserves, consisting primarily of lipids (Moran & Manahan, 2003). Nevertheless, these endogenous reserves could possibly get increased through the uptake of dissolved organic material (DOM) from seawater (Jaeckle & Manahan, 1989). Indeed DOM has been reported to energetically contribute to lecitotrophic development in various species (Anger et al., 1989; Crisp et al., 1985; Dawirs, 1987; Gallager et al., 1986; Marsh et al., 2001; Nates & McKenney, 2000).

Various studies have documented early development on a morphological basis in various abalone species, such as *H. discus hannai*, *H discus*, *H. fulgens*, *H sorenseni*, *H. tuberculata* and *H. asinina* (Ino, 1952; Leighton, 1972; Peña, 1986; Sawatpeera et al., 2001; Seki & Kan-no, 1977). These studies have recognized that larval development is a gradual process that does not occur in discrete stepwise stages but in various distinct larval stages with recognizable external features. Hence, larval stage begins with fertilization and is completed with the formation of the fourth tubule on the cephalic tentacles, although larvae are considered ready for settlement when the third tubule appears and larvae start to explore the surface (Hahn, 1989). Comparison of detail studies revealed that larval developmental sequences are similar among species, although differences in the order or moment of appearance may occur (Hahn, 1989). Thus, the occurrence of the different developmental stages is species specific and a function of water temperature and time, since abalones do not feed before settlement (Hahn, 1989).

Larval development of abalone is arrested below a certain threshold temperature known as "biological zero point" (BZP). BZP for abalone was first reported by Seki and Kan-no, (1977), for *H. discus hannai,* and is found to take place at lower temperature for temperate abalone species (Seki & Kan-no, 1977) than for tropical ones (Sawatpeera et al., 2001). At temperatures above the BZP, the appearance of each developmental stage is a function of the cumulative difference between culture temperature and the BZP, known as Effective Accumulative Temperature (EAT; expressed in ^{o}C-h). Higher temperatures are inducing more rapid development and reduce the time interval between hatch out and the achievement of metamorphic competence (the formation of the third tubule on the cephalic tentacle (Hahn, 1989), while the contrary is happening at lower temperatures. However, the rate of larval development in abalone is not twice as fast at double the water temperature (Hahn, 1989). Therefore, the knowledge on patterns of embryogenesis and larval morphology and their rates of appearance is crucial for the development of the culture of a new abalone species as it will allow to predict the onset of each developmental stage at a given temperature and identified fertilization time. The ability to predict the duration of the entire larval development is of special relevance to forecast the time of metamorphic competence achievement, as larval age and development stage affect settlement response (Barlow, 1990), and plan the transfer of the larvae to settlement tanks.

The control of the onset of these stages is of special relevance for hatchery activities since it avoids delays in metamorphosis induction in the presence of adequate settlement inducing cues, which as to be differentiated from the reduce larval survival and growth,

observed in the absence of suitable settlement inducing cues, caused by exhaustion of endogenous reserves provoked by prolonged failure to contact with an appropriate environmental cue (Roberts & Lapworth, 2001; Takami et al., 2002).

The early life history of *H. tuberculata* has received little attention (Crofts, 1938; Koike, 1978) and, the study of embryonic and larval development of *H. tuberculata coccinea* is restricted to the study of Peña (1986), who briefly described the developmental stages at a temperature range (14-15ºC) not occurring in its natural habitat. Although several studies have been conducted to better understand the physiology of this species and its behaviour under captive conditions (Peña, 1986; Toledo et al., 2000; Viera et al., 2003; Viera et al., 2005) none of them have focused on the early development of *H. tuberculata coccinea* in its natural thermal range (18-24ºC).

Hence, the aim of the study of *H. tuberculata coccinea* early development is to determine its stages of development, order of appearance and timing at a given temperature. Such knowledge is essential to develop and improve larval rearing techniques, prevent delays in metamorphosis induction and improve seed production, a phase remaining a major bottleneck for the production of this species.

1.4 ABALONE SETTLEMENT AND METAMORPHOSIS

Once competent to undergo settlement, after the apparition of the fourth tubule on the cephalic tentacles, the larvae will transform into benthic organisms. Settlement of abalone involves first larval attachment followed by the metamorphosis. During attachment larvae are attached to the substrate by their foot but still retain their velum to be able to resume swimming if exploration denotes an unsuitable substrate. On the contrary, irreversible physical changes are taking place during metamorphosis. These changes include the loss of the velum, the development of enlarged gills and foot, the opening of the mouth and the beginning of feeding as well as the deposition of peristomal shell. The metamorphosis stage of abalone larvae has received special attention, as it is a crucial developmental stage involving the reorganization of existing tissue and the construction of new one, increasing the risk of suffering high mortalities rates. As a consequence it is considered as the most critical stage of abalone culture.

The processes involved in metamorphosis are taking place after the competent larva settle onto the benthos in response to environmental cues (Hadfield, 1986). These cues are

providing chemical or nutritional information relative to the habitat the post-larvae and juvenile will subsequently inhabit. Knowledge of these cues and their settlement inducing effect is critical in abalone culture where complete, rapid and predictable settlement is desired but hard to achieve (Roberts, 2001). The absence of such cues leads to low settlement and survival rates in early post larvae (Daume et al., 1999b; Roberts et al., 1999b; Searcy-Bernal et al., 1992; Slattery, 1992; Takami et al., 2002).

A broad range of cues and their effects on abalone larval settlement have been studied, for various abalone species, in the last decades. These include crustose coralline algae (CCA) and their chemical extract **(**γ aminobutyric acid, GABA), biofilms, including diatoms, bacterial films, pure chemicals or abalone mucus and macroalgae.

Published literature on settlement and metamorphosis induction of these substrates has documented contradictory results and wide variation in abalone settlement rates, depending on the abalone species or the experimental methodology employed in different studies. These results, reported for each set of cues, are detailed below.

1.4.1 Crustose Coralline algae and γ aminobutyric acid (GABA)

Crustose coralline algae (CCA) are calcareous red algae that occur abundantly on hard substrate in subtidal communities from tropical to polar latitudes. Initial research on the abalone settlement induction properties of CCA was based on field observations and reported that juvenile abalones are closely associated with them in the wild (Day & Branch, 2000; McShane et al., 1988; Shepherd & Daume, 1996; Shepherd & Turner, 1985). The strong and rapid settlement inducing activity of CCA was confirmed by laboratory experiments that proved that they are among the best abalone settlement inducers, regardless the abalone species tested (Daume et al., 1999a, b; Morse & Morse, 1984; Morse et al., 1980c; Moss, 1999; Moss & Tong, 1992a; Roberts et al., 2010 ; Roberts et al., 2004; Roberts & Nicholson, 1997; Takami et al., 1997b). However, the use of CCA in hatcheries in not practical as coralline algae are generally slow growing and are not easily propagated on a large scale (Daume, 2006).

Morse et al. (1979) found that γ aminobutyric acid (GABA), an amino acid and neurotransmitter in higher animals, could be extracted from crustose red algae and induced settlement of abalone larvae. This discovery enabled to better understand the settling mechanisms of abalone larvae, the biochemical pathways involved and the receptors

implicated in settlement induction (Barlow, 1990; Morse, 1985; Trapido-Rosenthal & Morse, 1986). Besides it was found that attachment and metamorphosis could be artificially triggered through the use of various chemical substances thought to induce larval attachment or metamorphosis by affecting signal transduction pathways such as trans-membrane ion transport (Jensen et al., 1990; Morse, 1992). GABA's efficiency for settlement induction has been a case of controversy, in the published literature, that could be related to the susceptibility of GABA to marine microbes degradation and probably explain the different results obtained among studies (Kaspar & Mountfort, 1995; Morse, 1992; Slattery, 1992). Another question arousing about the use of GABA is the concentration at which it has to be employed as well as the time of exposure for the larvae. Morse (1979) reported effective settlement induction of GABA at a concentration of 10^{-6} M for *H. rufescens* larvae while higher concentrations have been found to be toxic and inhibit shell growth or metamorphosis(Morse et al., 1980b; Searcy-Bernal & Anguiano-Beltran, 1998). On the contrary, Searcy-Bernal & Anguiano-Beltrán (1998) showed that GABA concentrations could be doubled or triple without compromising metamorphosis or post-larval survival and growth in *H. rufescens*. Settlement results obtained through GABA induction are also variable among the different abalone species tested (Roberts & Nicholson, 1997), suggesting that chemical signalling for settlement induction could be species specific.

1.4.2 Other pure chemicals

Abalone settlement induction investigations with GABA enabled a better understanding, at the molecular level, of the way that inducers provoke a response in abalone and revealed that this was happening through the binding of a substance to a specific larval receptor. As a consequence, other pure chemicals thought to have a possible effect on signal transduction pathway were subsequently tested as settlement inducing substances for abalone. The other pure chemicals tested and reported to induce a settlement activity in abalone are dibromomethane and the manipulation of potassium chloride (KCl) concentration in seawater affecting the potassium ion (K^+) concentration. The effect of KCl addition to natural seawater was found to induce attachment but no metamorphosis in *Haliotis iris* and *Haliotis virginea* (Roberts & Nicholson, 1997) and its effects on metamorphosis induction for *Haliotis rufescens*, *Haliotis diversicolor* and *Haliotis discus hannai* were describe as low and function of the concentration and time exposure employed (Baloun & Morse, 1984; Bryan & Qian,

1998; Kang et al., 2004). Dibromomethane, a volatile chemical naturally released by coralline and other algae, was reported to induce metamorphosis in sea urchin larvae (Taniguchi et al., 1994). Dibromomethane has been implicated in metamorphosis induction of *H. discus hannai* (Kang et al., 2004) whereas its induction effects were negligible for *H. iris* (Roberts & Nicholson, 1997). However, combining it with abalone mucus provoked rapid and complete settlement of *H. discus hannai* and *H. iris* larvae, suggesting synergetic effects between these cues (Roberts & Nicholson, 1997; Seki, 1997).

1.4.3 Benthic diatoms biofilms

Diatom biofilms have long been used to induce larval settlement in abalone hatcheries worldwide. Diatoms species composition of the biofilms is broad and diatoms are associated to bacteria, this combination makes difficult the precise description of biofilm features and the identification of the specific characteristics responsible for larval settlement induction. In the first settlement induction studies, where there was no control on diatoms biofilm composition, and filamentous diatoms were found to be unsuitable for abalone settlement induction (Seki & Kan-No, 1981b). This observation has led to various studies on the effect of biofilm age, diatoms species composition, diatoms growth form or substrate colonizing density on abalone larvae settlement in order to better understand the mechanisms involved in settlement induction by diatoms biofilms. The response of abalone larvae to different diatoms strains and densities have revealed marked differences in the percentage of abalone undergoing metamorphosis among diatoms strains or density (Kawamura & Kikuchi, 1992). Moreover these diatoms characteristics also influence the time required to complete settlement (Kawamura, 1996; Kawamura & Kikuchi, 1992). Eight types of diatoms growth form have been identified (Fig.6) and the strains forming flat communities have been found to induce high settlement, while the ones with three-dimensional growth forms entangled larvae and prevented them from metamorphosing (Kawamura, 1996). Besides the effect of diatoms growth forms on settlement induction, the abundance and age of the diatom biofilm are also considered as influencing factors. Frequently, older and denser biofilms induce higher settlement of abalone larvae (Daume et al., 1999b; Kawamura & Kikuchi, 1992; Moss & Tong, 1992b; Roberts et al., 2007). As a consequence, the selection of attached prostrate forms of diatoms to grow on settlement plates in culture is performed by the pregrazing of plates by juvenile abalone (Bryan & Qian, 1998; Searcy-Bernal et al., 1992; Seki & Kan-No,

1981a) or, in the case of ungrazed biofilms, by filtering the incoming seawater or culturing specific diatoms species (Gallardo & Buen, 2003; Gordon et al., 2004; Hahn, 1989). Despite the widespread use of diatoms in abalone nurseries, metamorphosis rates on diatom films are often low (Daume, 2006; Roberts, 2001; Roberts & Watts, 2010) and research towards the identification of potentially ideal diatoms inducing reliable and efficient settlement, revealed significant differences among strains and abalone species. For instance, *Cocconeis* spp. has been reported as suitable diatoms, being often dominant on the pre-grazed plates that induce settlement in Japanese abalone hatcheries (Kawamura et al., 1998b; Roberts, 2001; Seki, 1997). However, some strains of *Cocconeis* have given poor settlement induction in other species, implying differences in settlement requirements among abalone species (Daume et al., 1999b; Daume et al., 2000). Other diatoms of relevance in abalone hatcheries are *Cylindrotheca* spp., *Nitzschia* spp. and *Navicula* spp., all leading to variable settlement induction performance in function of diatom strains or abalone species tested (Daume et al., 1999b; Kawamura & Kikuchi, 1992; Kawamura & Takami, 1995; Ohgai et al., 1991; Roberts & Nicholson, 1997). Thus, it appears that diatom films offer low consistency in settlement induction what could be related to physical or chemical variations. As a matter of fact, other factors that often covary with diatom abundance in the biofilm include the growth phase of the film, mobility and adhesive strength of the diatoms and the density of bacteria in the film. The settlement induction effectiveness of diatoms strains has also been tested based on their physico-chemical properties in an attempt to better understand the chemical basis of the chemosensory settlement response of abalone larvae (Gordon et al., 2004).

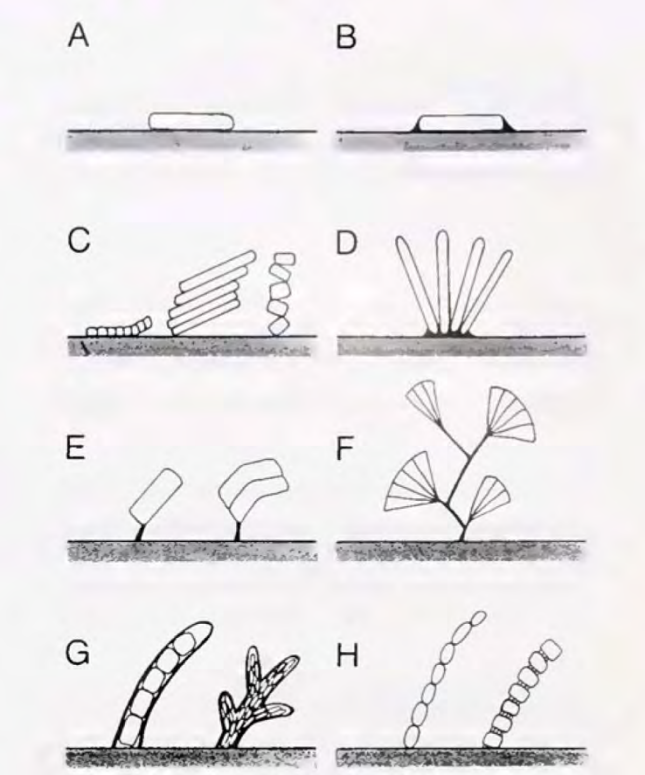


Fig. 6: Schematic diagram of eight types of diatom growth forms (Kawamura, 1994).

1.4.4 Bacteria

Bacterial films are described as settlement inducers for other mollusc species (Fitt et al., 1990). Bacteria are also alleged to play a role in abalone settlement induction together with diatoms, since diatoms films are colonised by a wide range of bacteria in culture tanks. Attachment of *Haliotis diversicolor* larvae was induce in low proportions by three species of bacteria (Bryan & Qian, 1998) and some bacterial strains induced 50% metamorphosis in *H. iris* larvae (Roberts et al., 2010). Bacteria were also reported to induce *Haliotis virginea* larval settlement but the response was slow, taking 1 week to reach 50% metamorphosis (Roberts, 2001) and bacterial activity was demonstrated to greatly influence the diatom film effect by triggering settlement (Roberts et al., 2007). However, only limited studies were performed on abalone settlement induction by bacteria and it remains unclear if the settlement inductive substances are synthesised by the bacteria independently or if these last ones synthesize them from compounds derived from the diatoms.

1.4.5 Abalone's mucus

Diatom biofilms grazed by juvenile or adult abalone are widely use as effective inducers of abalone larval settlement (Roberts, 2001). It seems that the action incited by the mucus is contact dependant and genus-specific as larval attachment of *H. discus hannai* is induced by mucus from four Japanese abalone species but not by mucus from other gastropods (Seki & Taniguchi, 1996). For *H. australis* the grazing effect appeared to be species specific, as pre-grazing by *Haliotis iris* has no effect on settlement induction of *H. australis* larvae (Roberts & Watts, 2010). However, variable mucus induction efficiencies are reported between abalone species (Bryan & Qian, 1998; Seki, 1997; Slattery, 1992). This variability could be explained by the fact that larvae are able to distinguish between different types of mucus secreted by the gastropod's foot (Hahn, 1989). Four different types of mucus ("grazed", "crawled", "adhered" and "rubbed") are described and investigated, settlement of *H discus hannai* being only observed on the "crawled" and, particularly, the "grazed" types of mucus, (Seki & Taniguchi, 1996). These differences may result from variations in the physical or chemical properties of the several types of mucus (Seki & Kan-No, 1981a) or by bacteria associated with abalone's foot (Bryan & Qian, 1998).

1.4.6 Macroalgae

Few studies are reporting the use of macroalgae as settlement inducer of abalone larvae and have investigated the potential of various macroalgae for settlement induction and post-larval nutrition. *H. rubra* larvae show high settlement response to the green algae *Ulva australis* and *Ulva compressa* (Huggett et al., 2005). Seki (1997) also reported that brown, red and green macroalgae induce metamorphosis of *Haliotis discus hannai* and (Strain et al., 2006) suggested that spores of *Ulva* sp. could provide a suitable food source for abalone juveniles. Besides, various studies have shown that abalone can settle successfully on the crustose green algae, *Ulvella lens*, growing as prostrate rosettes (Daume et al., 2004; Daume et al., 2000; Daume & Ryan, 2004a; Takahashi & Koganezawa, 1988). *U. lens* is suitable to improve the settlement of *Haliotis laevigata* larvae on commercial scale and is actually grown to be used as larval settlement induction substrate by a majority of the commercial scale abalone farms in Australia (Daume, 2006). Daume (2006) also points out the influence

of age and biochemical characteristics of *U. lens* on abalone larval settlement, based on the clearer preference of *Haliotis rubra* and *Haliotis laevigata* larvae for older rather than for younger *U. lens*. However, no studies have investigated the use of macroalgae's spores as abalone settlement inducers besides the investigation performed with *U. lens* (Daume et al., 2004; Daume et al., 2000; Daume & Ryan, 2004a; Takahashi & Koganezawa, 1988). Such research would be useful to determine new possible settlement cues that could be easily propagated and handled at commercial scale and that could also be a potential food source for juvenile abalone.

1.4.7 Artificial hatchery culture techniques

As previously discussed, the quality of algal biofilm is highly variable in function of its density, age, and diatom strains. In the quest for reliable and stable settlement induction substrate, alternative systems, replacing live algae, have been recently proposed in Japan for *H. discus discus* and *H. diversicolor*, as a means of settlement and growing post-larvae. The proposed systems consist in spraying an agar solution mixed with dried algae powder onto settlement plates conditioned with mucus from juvenile abalone (Stott et al., 2004b). No significant difference in settlement rates of *H. discus discus* between the microalgae powder treatments and the living natural biofilm are observed and settlement rates are significantly higher than the ones of the negative control (Stott et al., 2004a). This is an interesting system that can serve the double purpose of settlement induction and feed provision for post-larvae, although mechanized and cost-efficient ways of spraying the plates need to be developed before it becomes commercially viable.

1.5 Abalone post-larval nutrition and growth

1.5.1 Factors influencing post-larval growth and survival

Benthic diatoms are the main source of food for abalone post-larvae once metamorphosis has taken place (Daume, 2006; Hahn, 1989; Kawamura, 1996; Seki & Kan-No, 1981b). Adequate nutrition is crucial for growth and survival of abalone post-larvae and juveniles (Hahn, 1989; Kawamura et al., 1998b). Growth and survival rates during the early post-larval stages reported in the literature are variable and generally low (Daume et al.,

1999b; Daume et al., 2000; Kawamura, 1996; Kawamura et al., 1998a; Kawamura et al., 1998b; Kawamura & Takami, 1995; Roberts et al., 1999b; Searcy-Bernal et al., 2001). These variations have been attributed to differences in the biofilm on which post-larvae feed, such as, diatoms species and strains, their cell density, culture conditions, developmental stage of the culture and abalone species (Daume et al., 2000; Kawamura et al., 1998b; Kawamura & Takami, 1995; Roberts et al., 1999b; Searcy-Bernal et al., 2003; Searcy-Bernal et al., 2001). Therefore, maintaining a suitable diatom film is a critical factor in the success of abalone hatcheries worldwide (Hahn, 1989).

The quantity of food available for post-larvae is an important factor determining their growth. Grazing and growth rates of *H. fulgens* post-larvae increase as the density of the diatom *Navicula incerta* increases from 500 to 4,000 cells/mm^2 (Searcy-Bernal et al. 2001). However, high diatom densities may have a negative impact on post-larval growth and the various potential reasons proposed to explain this include: smothering (Tong & Moss, 1992), reduced mobility, harbouring of predators (Ebert & Houk, 1984) and poor water quality (Searcy-Bernal, 1996).

However, ingestion of diatom cells does not imply that they are utilized as food (Kawamura et al., 1995). The proportion of diatom cells that are broken during grazing appears to be a critical factor controlling the nutritional value of diatoms for post-larval abalone, as it is suggested that the post-larvae are unable to utilise diatom cell contents for nutrition unless the diatom is broken during grazing (Kawamura et al., 1998a). The difference in digestion efficiency among diatoms is reported as an important factor determining their nutritional value for post-larval abalone. Diatom's digestion efficiency induced variations in growth rates, with digestible strains producing a significantly faster growth than inefficiently digested diatoms, and is linked to the physical characteristics of the diatoms cells such as size and attachment strength (Kawamura et al., 1998b; Kawamura et al., 1995; Matthews & Cook, 1995; Seki & Kan-No, 1981b). Post-larvae of 0.8-2 mm in shell length grow ca 40-60 μm day-1 on "digestible" diatoms, but only ca 15-30 μm day-1 on "indigestible" diatoms (Kawamura et al., 1998a)

Various other factors may also influence the nutritional value of diatoms. These include the nature and quantity of their extracellular polysaccharide, the associated microbial flora (Daume, 2006; Kawamura, 1996; Roberts et al., 1999b; Searcy-Bernal et al., 2001) and biochemical and fatty acid composition of the diatom cell contents (Dunstan et al., 1994), all varying between diatoms species. Besides, it is hypothesized that the influence of

biochemical difference will only have an effect on growth from the moment the post-larvae acquire the capability to digest and benefit from diatom cell content (Kawamura et al., 1998b)

Another important feature influencing post-larval growth and survival is the stage of post-larval development as it affects the selective consumption of diatoms species, and the abalone ability to digest them (Kawamura et al., 1998a; Roberts et al., 1999b). Three major transitions have been reported in post-larval and juvenile feeding: the first one is the transition from lecitotrophy to particle feeding, the second one correspond to the beginning of effective diatoms cells ingestion and digestion and appears around 600-800μm shell length, finally the third one is the nutritional shift from diatoms to macroalgae (Kawamura et al., 1998b). These transitions in feeding, together with the post-larvae ability to consume diatoms are linked to the development of the digestive system as well as the state of morphological development of the radula, a circular mouth with a tongue-like organ with rows of tiny teeth that allow abalone to rasp algal particles (Chitramvong et al., 1998; Johnston et al., 2005; Onitsuka et al., 2004; Roberts et al., 1999a; Takami et al., 2000; Takami et al., 1998).

Differences in growth rates in response to different algae have been observed only from the second feeding stage and onwards as there appears to be a residual benefit of the yolk supply during the first transition to early ingestion. (Daume et al., 2000; Kawamura et al., 1998b; Roberts et al., 1999b).

Within 2 days of the initiation of abalone metamorphosis the mouth opens and feeding begins (Norman-Boudreau et al., 1986; Roberts et al., 1999b; Seki & Kan-No, 1981b) on bacteria and extracellular secretions (Garland et al., 1985; Kawamura, 1996) and suitable diatoms (Martinez-Ponce & Searcy-Bernal, 1998; Roberts et al., 1999b). Adequate nutrition at this stage is a key element to survival, since the rearrangement of tissues at metamorphosis is potentially energetically costly and may result in depletion of endogenous reserves. If suitable diatoms are not available or food consumption increase drastically, resulting in food shortage considering hatcheries struggle to meet the increasingly higher post-larvae food demand (Ebert & Houk, 1984; Hahn, 1989), starvation periods were observed (Onitsuka et al., 2010; Roberts, 2001) and may lead to high mortalities (Roberts et al., 1999b). Food shortage at this stage is diminished by using *U. lens* to feed juveniles as early as 2-3 mm shell length (Daume & Ryan, 2004a). The low growth rates observed on *U. lens* before post-larvae reached 2-3 mm size (Daume et al., 2000; Kawamura et al., 1998b) suggest their inability to utilize this food source probably due to the developmental stage of the digestive system and the radula before reaching this size (Roberts et al., 1999a).

1.5.2 Algae's quality

The biochemical composition of algae varies among species and is known to be influenced by the culture conditions and developmental stage of the culture (Brown et al., 1996; Liang et al., 2002; Liang et al., 2001; Mercado et al., 2004; Simental-Trinidad et al., 2001; Thompson et al., 1993). The biochemical compositions of the algae are directly related to their nutritional values (Brown et al., 1997; Renaud et al., 1999), and are therefore having a direct effect on animals' growth and survival. The effect of the biochemical composition of algae has been studied on juvenile and adult abalone (Boarder & Shpigel, 2001; Dunstan et al., 1996; Mai et al., 1996, 1995b, a, 1994; Shpigel et al., 1999). However, only few studies have investigated the effects of varied dietary proximate and fatty acid composition on post-larvae and early juveniles' growth and survival (Daume et al., 2003; Daume & Ryan, 2004a; Gordon et al., 2006; Uriarte et al., 2006; Viana et al., 2007a). Post-larvae rely solely on diatom films and its associated microbial flora as a source of food, until they reach 2-3mm when they can be weaned onto macroalgae spores.

Culture conditions were found to affect diatom's nutritional value and consequently may strongly affect post-larval and juvenile abalone growth and survival (Daume, 2006). An increase in nitrate amount of the culture medium raised the protein content of *Navicula* sp., leading to an increase in growth rates of 1-3 mm shell *H. rubra* juveniles (Daume et al., 2003). When cultured under different light intensities and nitrate concentrations *Navicula jeffreyi* biochemical composition was found to vary, presenting lower protein contents under high light conditions, and when fed *N. jeffreyi* of different biochemical composition juveniles of *H. Laevigata* were reported to graze a larger number of diatoms when the protein content of the diatoms was low, possibly compensating for the lower protein levels (Watson et al., 2005). Besides, optimal levels of dietary protein for juveniles and adults abalones were reported to vary from 20% to 35% depending on the species and age (Britz & Hecht, 1997; Mai et al., 1995a).

Abalones have a carbohydrate-based metabolism and their digestive enzymes are capable of hydrolyzing complex carbohydrates making carbohydrate a suitable dietary energy source for abalones. In fact their natural diet consists of 40-50% carbohydrate (Fleming et al., 1996).

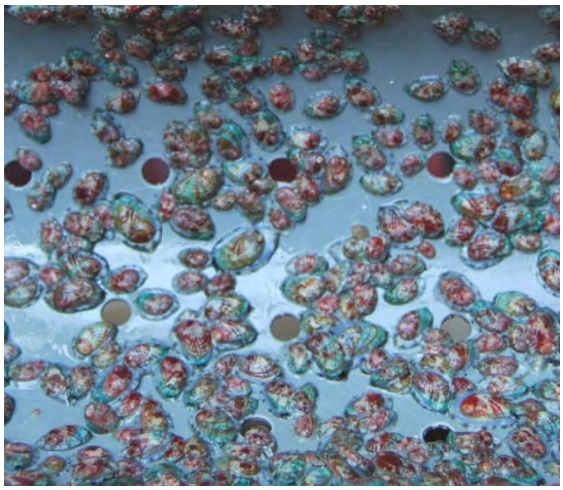
Lipids are important dietary constituents not only for their high energy value and source of essential fatty acids, but also because they are vehicles of fat-soluble vitamins (Fleming et al., 1996). It has been found that lipid requirement of abalones is very low as corresponding to herbivorous animals (Mai et al., 1995b) and that an increased lipid level in the feed can decrease their growth rates as they are believed to reduce the digestibility of other nutrients (Britz & Hecht, 1997). Lipid levels of 4% to 5% have been shown to be optimal to maximize growth rates and health of abalone and, like proteins, their requirements are species specific (Britz & Hecht, 1997).

Fatty acids (FA) are perhaps the most important lipids as they are sources of energy and cell membranes components. As all animals, haliotids cannot synthesize all the FA required for normal cellular function and growth (Uki et al., 1986), and rely on dietary sources of these essential fatty acids (EFA) to fulfil their requirements. Restricting the intake, either through reduced feed rations or provision of feeds low in EFA, results in suboptimal growth of abalone (Floreto et al., 1996; Mai et al., 1996; Uki et al., 1986). The type of macroalgae consumed was found to significantly affect abalone growth rates (Uki et al., 1986) and offer different proportions of the nutritionally important polyunsaturated fatty acids (PUFA). One feature that distinguishes abalone from the rest of other marine animals is their dietary requirements in docosapentaenoic acid (22:5n-3, DPA) rather than docosahexaenoic acid (22:6n-3, DHA) (Dunstan et al., 1996). This was observed for abalone fed different diets by Dunstan et al. (1996) who concluded that this C_{22} PUFA distribution could be the results of an adaptation to a macroalgal diet, implying a low lipid diet, as well as a low rate of conversion. PUFA of both n-3 and n-6 families are reported essential for the growth of juvenile *H.discus hannai* (Mai et al., 1996). Among the highly unsaturated fatty acids (HUFA), eicosapentaenoic acid (20:5n-3, EPA) is reported to promote fast growth in *H. discus hannai* juveniles (Dunstan et al., 1996; Mai et al., 1996). EPA has also been suggested to be of special relevance during post-larval feeding (Viana et al., 2007a) as it seems that this fatty acid could readily be absorbed from the diatoms (Gordon et al., 2006) known to offer high levels of lipids and PUFAs, especially EPA (Brown et al., 1997; Dunstan et al., 1994). Arachidonic acid (20:4n-6, ARA) was also suggested to be of importance considering it was found in high proportion in abalone tissues (Fleming et al., 1996) and reported to increase in post-larvae tissues once they start to graze on diatoms (Viana et al., 2007a). On the contrary, DHA was found in low proportion in abalone's tissues and therefore presumed of a lesser quantitative importance in abalone's nutrition (Dunstan et al., 1996; Mai et al., 1996). Moreover diatoms are known to be low in DHA (Dunstan et al., 1994). Hence, the

development of optimum culture techniques, providing and maintaining suitable density of algae of good nutritional quality, for the first stages of the life cycle is a key factor to abalone hatcheries success.

EARLY LIFE OF THE ABALONE *HALIOTIS TUBERCULATA COCCINEA*: DEVELOPMENT, SETTLEMENT AND GROWTH

2. OBJECTIVES



2. OBJECTIVES

In view of the interest in diversification of abalone species production and the identification of early life processes as main constraints for the further development of this production, the main objective of this study was to "***Acquire knowledge on early life history of the abalone Haliotis tuberculata coccinea and contribute to the development of its production techniques through the improvement of larval settlement as well as growth and survival rates of juveniles***". Specifically, early development, settlement process and growth and survival of post-larvae and juveniles, as well as various factors affecting these events, were addressed in this study. Therefore, to achieve the general objective five specific objectives were selected:

Objective I: To study the embryonic and larval development of *Haliotis tuberculata coccinea,* in comparison with other species, and adapt culture techniques to developmental rates, a critical factor for production success.

Objective II: To study larval settlement of *Haliotis tuberculata coccinea* in response to different inducing cues.

Objective III: To improve *Haliotis tuberculata coccinea* settlement using novel and modified induction cues jointly promoting early growth and survival

Objective IV: To improve benthic diatoms culture techniques and determine the potential nutritional value of diatoms species for *Haliotis tuberculata coccinea* post-larvae.

Objective V: To study early nutrition of *Haliotis tuberculata coccinea* focusing on the effect of selected benthic diatoms on post-larvae and juveniles' growth and survival.

These objectives were addressed by the following studies:

Study I: Embryonic and larval development of *Haliotis tuberculata coccinea* Reeve: an indexed micro-photographic sequence

In order to achieve objective I this study described the complete embryonic and larval development of *Haliotis tuberculata coccinea* under natural temperature conditions, illustrating the morphological features characteristic of each stage and the time required for their apparition. It also addressed aspects of early morphological development with the

objective to evaluate the time period necessary for the larvae to acquire settlement competence and enable the transfer of competent larvae to settlement substrate at an adequate time to ensure higher possibilities of settlement success.

Study II: Larval settlement of *Haliotis tuberculata coccinea* in response to different inductive cues and the effect of larval density on settlement, early growth, and survival

To study the larval settlement of *Haliotis tuberculata coccinea* proposed in objective II four experiments were conducted. On one hand, the settlement induction efficiency of different substrates, including different diatom species, reported to induce settlement in other abalone species, was studied. On the other hand, the effect of larval density on post-larval performances was also investigated. This study allowed to identify suitable settlement substrates for this abalone species and to determine the influence of larval density on settlement rates and post-larval performance in terms of growth and survival rates.

Study III: Larval settlement, early growth and survival of *Haliotis tuberculata coccinea* using several algal cues.

To address objective III improving *Haliotis tuberculata coccinea* settlement by using novel and modified induction cues, study III tested for the first time the use of spores of macroalgae other than *U. lens*, as abalone settlement inducers. The study also investigated the effects of age and proximate and fatty acid composition of *U. lens* and *Ulva rigida* germlings, in addition to crustose coralline algae, on larval settlement, survival and growth of *H. tuberculata coccinea* post-larvae. This study allowed a better understanding of *H. tuberculata coccinea* settlement processes induced by macroalgae to further improve recruitment and post-larval growth for spat production.

Study IV: Potential value of *Navicula incerta, Proschkinia* sp.*, Nitzschia* sp., and *Amphora* sp. as feed for *Haliotis tuberculata coccinea* post-larvae: Effect of density on algal growth rates.

This study examined the characteristics of four benthic diatoms species and the effect of density on their growth response and nutritional quality for abalone, in terms of their proximate and fatty acid composition. This study was addressed with the objective to

improve benthic diatoms culture techniques to provide the abalone species studied with a reliable, both in quality and quantity, and nutritious source of feed during the first stages of the life cycle to sustain better growth and survival rates.

Study V: Improving nursery performances of *Haliotis tuberculata coccinea:* Nutritional value of four species of benthic diatoms

The last study aimed to determine the effect of different benthic diatoms, and their proximate and fatty acid composition, on post-larval growth and survival, when fed to abalone *H. tuberculata coccinea* throughout the post-larval period up to juvenile stage. The objective of this study was to gain a better understanding of the nutrition and its relation to early growth of *H. tuberculata coccinea* post-larvae from the moment they pass onto an exogenous feeding mode, in order to improve growth and survival for the development of standardized post-larval rearing protocol for this abalone species.

EARLY LIFE OF THE ABALONE *HALIOTIS TUBERCULATA COCCINEA*: DEVELOPMENT, SETTLEMENT AND GROWTH

3. MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 LOCATION AND GENERAL FACILITIES

The studies were carried out at the culture facilities of the Aquaculture Research Group (GIA) in the Canarian Institute of Marine Sciences (ICCM). This institute is located in Melenara, Telde, Las Palmas de Gran Canaria (Canary islands, Spain) and its geographic coordinates are 27º59'31''N and 15º22'31''. The abalone culture facilities consist in: a broodstock conditioning, spawning induction and larval rearing area (Fig. 7), nursery area, for post-larvae production (Fig. 8), grow out and feeding trial area (Fig. 9), microalgae production area (Fig. 10) and biofiltering units recycling fish tanks effluents for macroalgae production (Fig. 11).

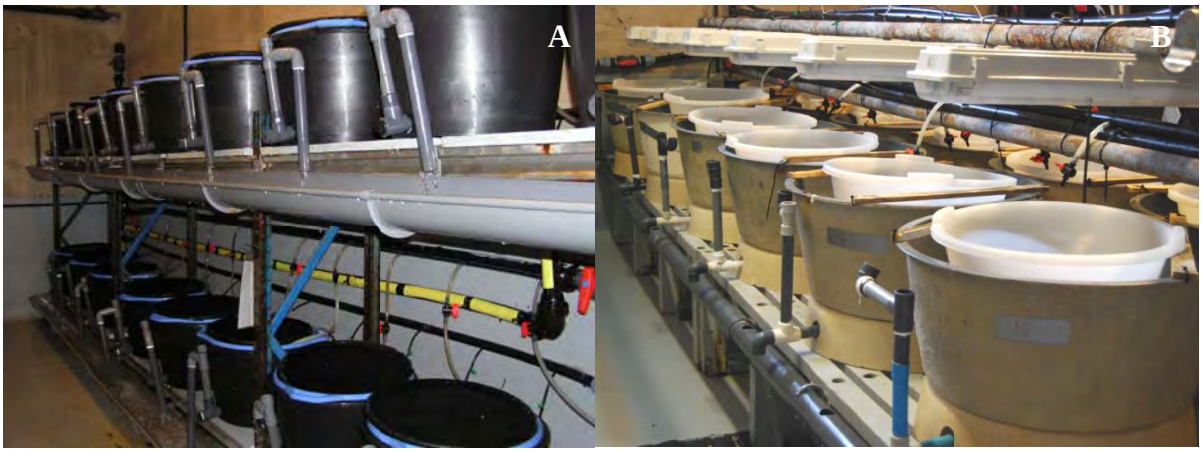


Fig. 7: Brood-stock conditioning (A) and larval rearing facilities (B).

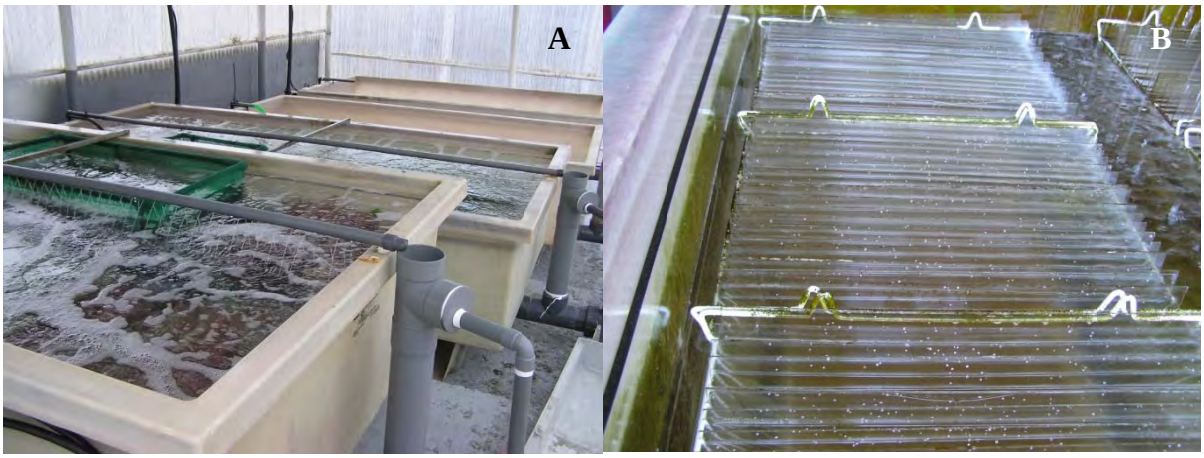


Fig. 8: General view of the experimental Abalone nursery area at ICCM (A). Detail of the settlement plates (B).

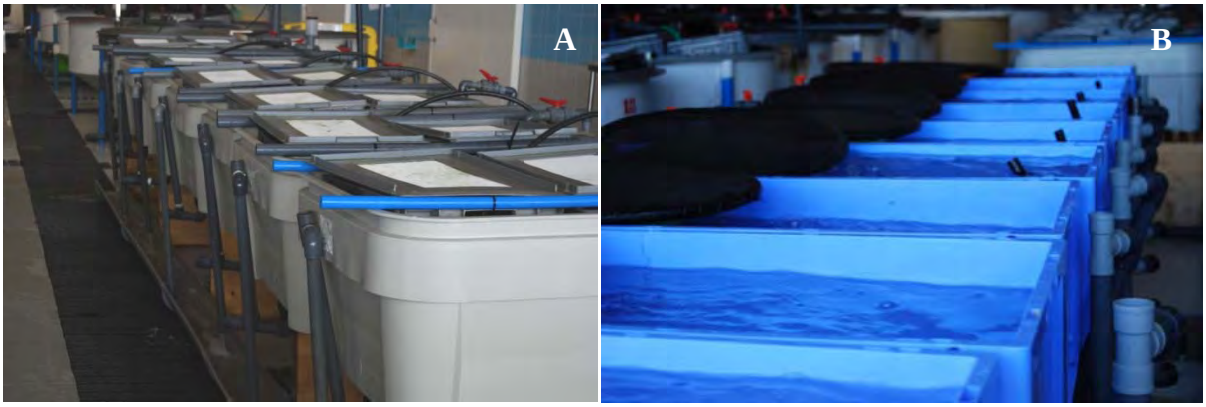


Fig. 9: Abalone grow out (A) and feeding trial area (B).

Fig. 10: Benthic diatoms production.

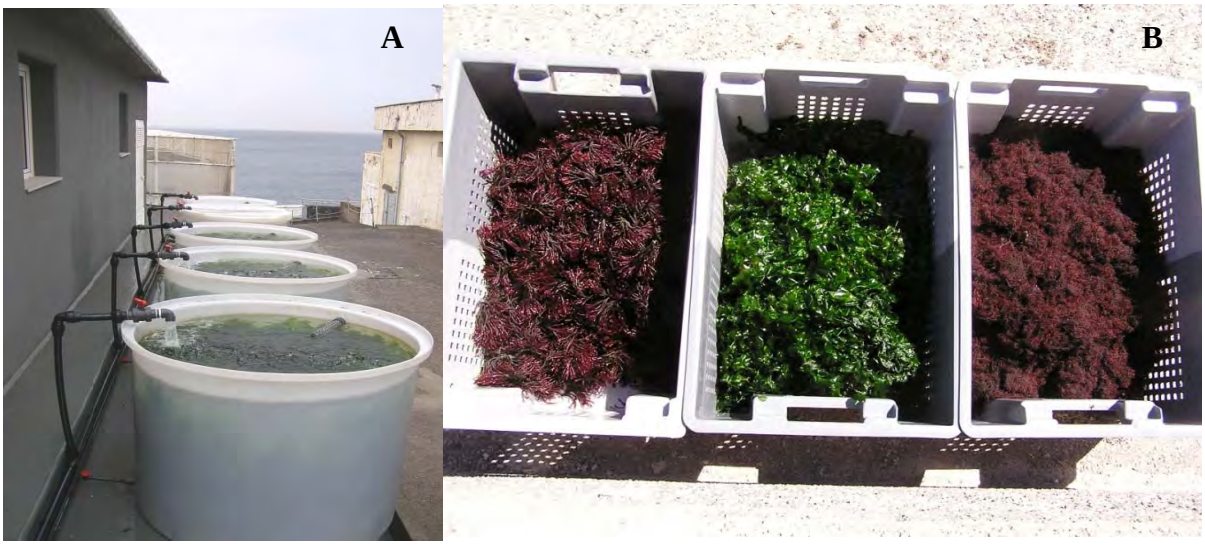


Fig. 11: Biofiltering units (A) and the resulting macroalgal production (B).

3.2 BROOD-STOCK CONDITIONING AND SELECTION

Captive *Haliotis tuberculata coccinea* brood-stock were kept under natural photoperiod conditions and ambient seawater temperature, in shaded 60-L tanks, in the flow through broodstock conditioning system (Fig. 7, Fig. 12). Inside each of the 24 broodstock holding tanks, 10-15 animals (depending on the size) were kept under PVC tiles that provide them shelter, and were separated from debris, on the tank's bottom, through a perforated divider. Males and females, differentiated by the colour of their gonads (creamy white for the males and dark grey to violet in the females) (Fig. 13), were maintained in separate holding tanks. Broodstock was fed twice a week with a mix diet of *Ulva rigida, Gracilaria cornea* and *Hypnea spinella* produced in the biofiltering units. Abalone selected to be induced to spawn were the ones showing mature gonads in stage 3 or between stage 2 and 3 (Fig. 13).

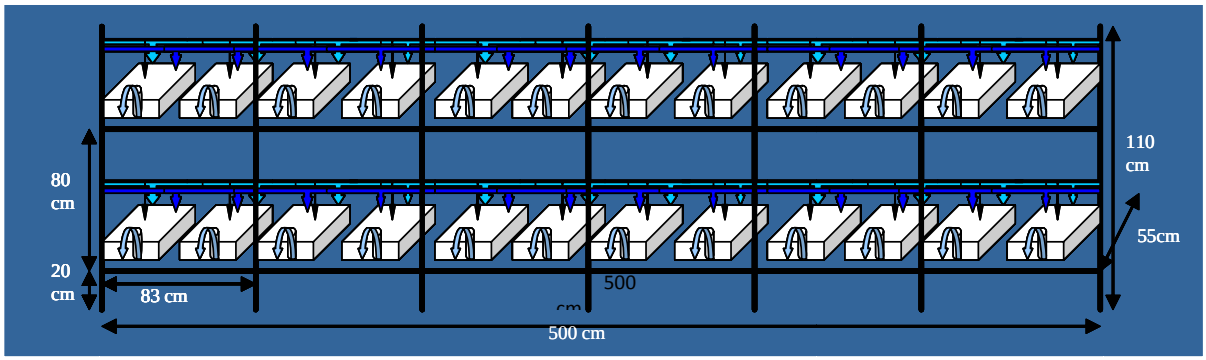


Fig. 12: Brood-stock conditioning system diagram.

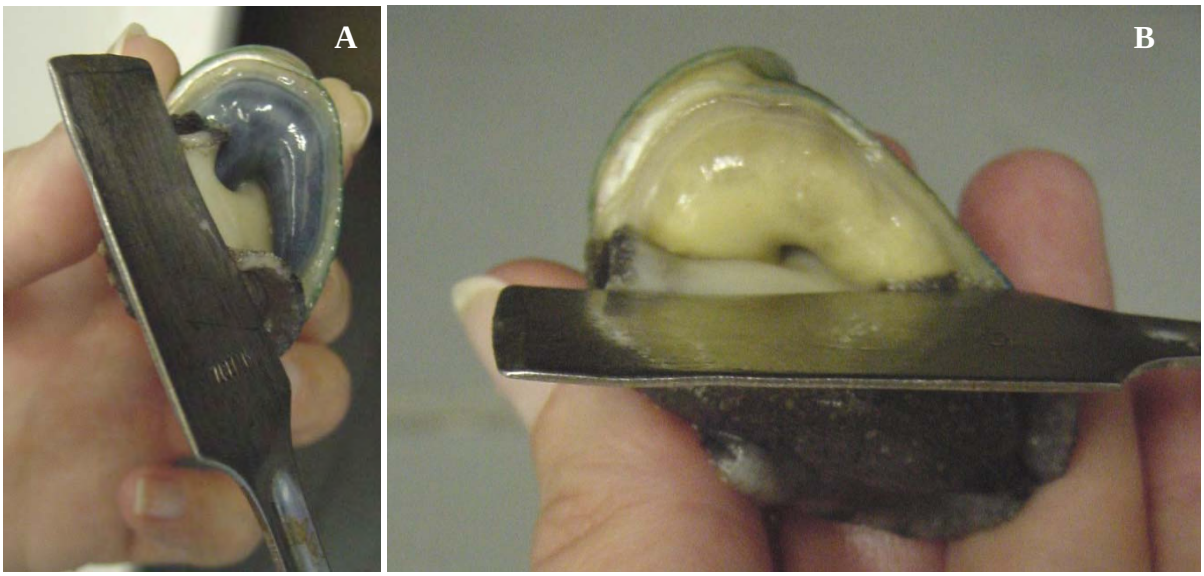


Fig. 13: Female (A) and male (B) *H. tuberculata coccinea* in stage 3 of the gonad index.

The stages of abalone gonad development have been identified by Ebert & Houk (1984), who differentiated the gonad developmental stages according to the following gonad index:

- 0 – Immature, sex indeterminate, the digestive gland is easily viewed as a greyish-brown mass.
- 1 – Gamete development initiated; gametes appear in a patchy pattern, colour differentiated, on the surface of the digestive gland. Sex determination is easy for males at this stage because of the creamish colour; however, female sex determination is difficult.
- 2 – Gametes fully envelop the conical appendage, sex easily determined but gonad not bulky.
- 3 – Same condition as for index stage 2 except that the gonad is quite bulky. This bulk extends to the gonad tip.

3.3 SPAWNING INDUCTION

Mature males and females, with a male to female ratio of 1:2, were induced to spawn, separately by sex, into spawning containers filled with 1-µm cartridge filtered and UV sterilized seawater. The UV sterilization of the water was realized at 254nm wavelength with industrial equipment (Mod REX 1x36HO15-PE; Wedeco, AG, Herford, Germany). Gametes from different sex were obtained separately in order to control the ratio of gametes employed during fertilization and have better control over the fertilization process. During spawning induction the containers were left in the dark. Two spawning induction methods were employed to carry out these studies, the hydrogen peroxide spawning induction method (Morse et al., 1977) and the ultraviolet spawning induction method (Kikuchi & Uki, 1974).

3.3.1 Hydrogen peroxide spawning induction method

This spawning induction method is described to be incited by the hydroperoxy free radical $HOO^{\cdot}$ or the peroxy diradical $^{\cdot}OO^{\cdot}$ that can be produced when hydrogen peroxide (H_2O_2) is added to water. Moreover high pH enhances the decomposition of the added hydrogen peroxide into these highly reactive and short-lived free radical oxidants.

Males and females of *H. tuberculata coccinea,* to be spawned, were placed in 10-L spawning containers with 1-mm cartridge filtered and UV sterilized seawater and aeration. The pH of the water was first increased to 9.1 by adding 6.6 ml of 2 M Tris - (hydroxy-methylamino) methane per litre of seawater in the spawning containers. The 2M solution of Tris was made by adding 24.2g of Tris to 75ml of distilled water and topping this up to 100ml when fully dissolved. Fifteen minutes after adding the Tris, 3 ml of freshly prepared 6% hydrogen peroxide solution was added for each litre of water in the container. 6% hydrogen peroxide solution was prepared by diluting 30% reagent grade hydrogen peroxide, by adding 20 ml of 30% hydrogen peroxide to 80 ml of distilled water, giving a final volume of 100 ml of 6% hydrogen peroxide. Water in the container was then thoroughly stirred to mix the solution. Abalone were left undisturbed in this solution mix during 1.5 to 2 hours after which the solution was decanted from the containers and the abalone were thoroughly washed in isothermal seawater to remove any traces of the chemicals. This is essential as the chemicals will destroy the released gametes. The containers were then refilled with isothermal seawater and after 30 minutes to an hour and a half later the abalones were observed shedding their gametes (Fig. 14).

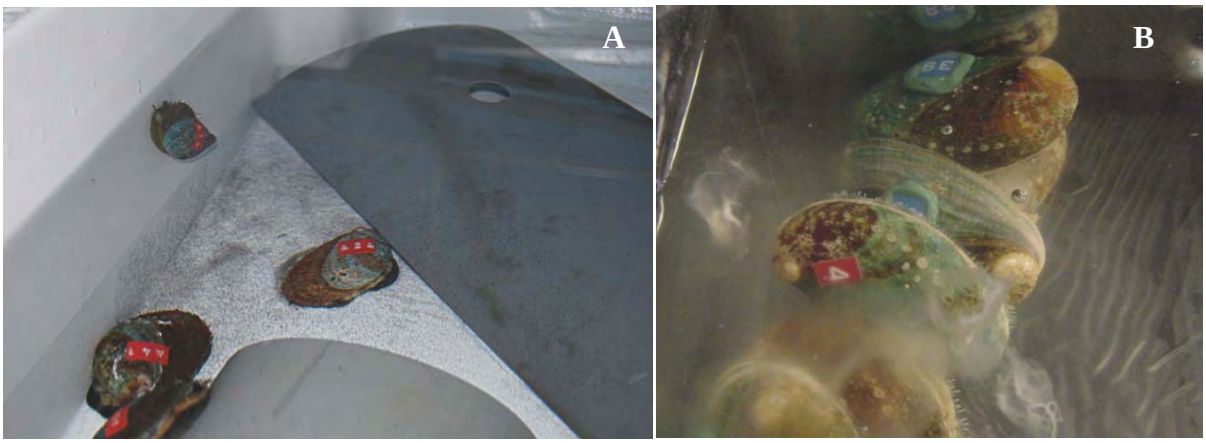


Fig. 14: Gametes expulsion from females (A) and males (B).

3.3.2 Ultraviolet spawning induction method

This spawning induction method was first described by Kikuchi & Uki (1974) who stated that the UV irradiation causes the water molecule decomposition into hydroperoxy free radical $HOO^{\cdot}$ or peroxy diradical $^{\cdot}OO^{\cdot}$, responsible of spawning induction (Hahn, 1989). This

method is generally considered the best and most reliable method of spawning induction in the majority of abalone culture facilities worldwide.

Males and females, *H. tuberculata coccinea*, to be spawned were placed in spawning containers, filled with flowing 1-mm cartridge filtered and UV sterilized seawater and aeration, and left undisturbed in the dark until the beginning of spawning. The UV irradiation of the water was conducted at 254nm wavelength with industrial equipment (Mod REX 1PE10; Wedeco, AG, Herford, Germany). The time between initiation of UV stimulation and spawning is reported to be proportional to the amount of UV irradiation; therefore the ideal UV strength recommended for spawning induction is 800 milliwatt hours per litre. Using that method, broodstock of *H. tuberculata coccinea* was observed to begin spawning between 2 to 3 hours after the beginning of the UV stimulation (Fig. 14).

An advantage of using UV for induction of spawning is that it is completely harmless to the gametes. As a result water changes during the spawning process are not necessary.

3.4 FERTILIZATION METHOD AND ESTIMATION

Once the gametes were obtained, the released oocytes, which are negatively buoyant, were siphoned from the spawning containers and passed through a 300 μm mesh screen to retain faeces or other debris (Fig. 15). The oocytes were collected in 10-L containers and fertilized with a final sperm concentration of 10^5/ml during 30 minutes. Sperm concentration was controlled in order to ensure maximal fertilization rate without risk of polyspermy, observed in the case of excess sperm density (Hahn, 1989). In order to determine the sperm concentration, a drop of Lugol solution was added to samples of sperm suspension and the number of spermatozoids was contabilized with a hemacytometer (Mod. Neubauer, Germany) (Fig. 15). Another parameter to consider during fertilization is the gametes viability; therefore in order to maximize the success, fertilization was performed with freshly spawned gametes not older than one hour post spawning. After the 30 min of fertilization period, eggs were rinsed with fresh seawater to remove excess sperm and fertilization rates were determined by recording the proportion of eggs showing dividing cells 1 h after fertilization. Fertilization rate estimate was performed by taking three 3-ml samples from fertilization containers and recording the number of fertilized eggs observed in the counting slide under the dissecting microscope (Mod. SL 260004, Optech, Germany). Fertilized eggs, from the same batch and obtained from fertilized gametes from various males and females, were placed in trays (hatching trays) with a wide flat bottom to form a monolayer of eggs and

remained inside until reaching the larval trochophore stage. Hatching trays were 60-L rectangular trays fitted with a water intake at one end and a water exit at the opposite end directing the out flow into the larval rearing container over which it was fixed (Fig. 16). Gentle water flow was turned on in the hatching trays once all the eggs were observed to be spread evenly over the bottom of the trays.

Once hatched, trochophore larvae observed swimming to the surface were separated by overflow from the unhatched eggs and discarded egg cases, and were then, transferred with the out flow from the hatching trays into the larval rearing tanks.

Fig. 15: Egg collection (A) and their screening to free them from debris (B). (C) Sperm concentration estimation.

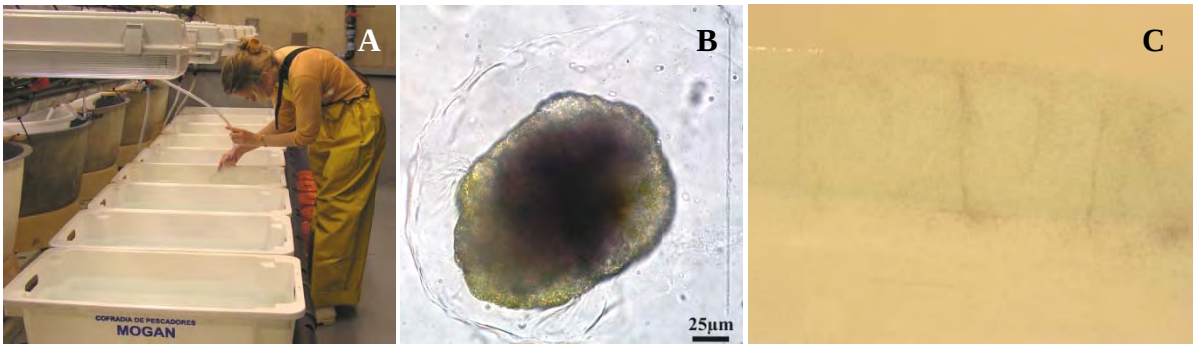


Fig. 16: Hatching trays (A) and hatching out trochophore larvae equipped with cilia (B) enabling swimming behaviour (C).

3.5 LARVAL CULTURE

The abalone larvae enter the larval culture phase from the trochophore stage up until they are characterized as competent for settlement and metamorphosis.

Two different methods can be used during larval rearing: static and flow-through culture. In the static method, the larvae are reared in large tanks (5-20 m^3) without a continuous inflow of seawater and the water in each container is changed periodically, every one to two days. In the present studies *H. tuberculata coccinea* larvae were reared, under natural photoperiod and ambient water temperature, in a flow through larval culture system consisting of twelve 100-L larval tanks further described (Fig. 17).

During that phase of the culture special care should be given to water quality and, therefore, the larval culture facility was fitted with filtration and sterilization equipment. Water supplied to the larval tank was filtered through 1μm cartridge filters and sterilized by UV irradiation at 254nm wavelength with an industrial equipment (Mod REX 1x36HO15-PE; Wedeco, AG, Herford, Germany) (Fig. 17).

Each 100-L larval rearing tank was fitted with wide 63μm mesh screens to prevent the loss of larvae as culture is realized in flow through. The mesh screens were submerged in the culture water to ensure the maximum surface area for larvae and mesh contact to prevent clogging. Gentle aeration was provided through air stones and water renewal was regulated to provide an approximate 20% exchange rate per hour (Fig. 18).

The surface swimming larvae from the hatching trays were directly transferred by overflow into the larval rearing tanks at a density of 10-20 larvae ml^{-1} (Fig. 18). The density in the larval rearing tanks was controlled by recording the number of fertilized eggs introduced into each hatching tray and by an evenly distribution among the hatching trays.

The oligotrophic larvae remained unfed in the same tanks during the entire larval rearing period, being transferred for settlement after the appearance of the third tubule of the cephalic tentacle, an indication of larval competency for settlement. Settlement competency was observed between 62 to 72 hours post-fertilization depending on the larval rearing temperature.

Larvae used in each experiment always belonged to the same batch and were obtained from fertilized gametes from various males and females.

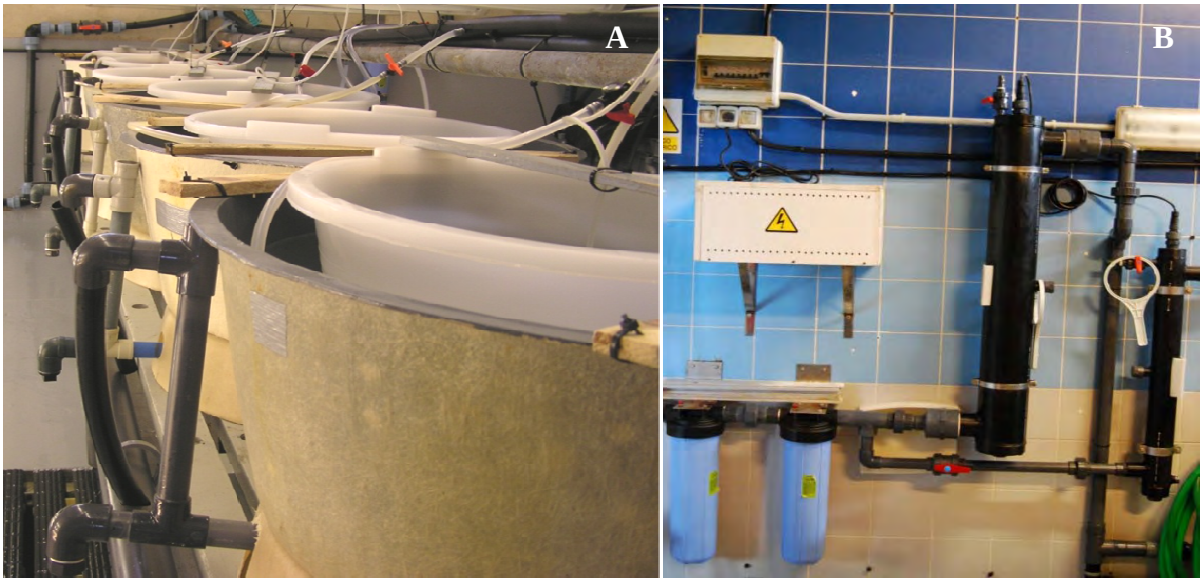


Fig. 17: Larval rearing facility (A) and filtration and sterilization equipment (B).

Fig. 18: Larval rearing tanks (A) and hatching tray linked to larval rearing tank in flow through system (B).

3.5.1 Experimental set up

The study on the embryonic and larval development of *H. tuberculata coccinea* was started immediately after fertilization and was carried out continuously until the appearance of the third tubule on the cephalic tentacles could be observed. All observations were performed with pooled larvae in 5-L containers. The larvae sampled originated from the same fertilization period and the same batch and were regularly sampled from the hatching trays and the larval rearing tanks. All observations were performed continuously throughout the entire surveyed development and were realized under transmitted light with a Leitz DMRBE microscope (Leica, Wetzler, Germany) at a magnification of x400 (Fig. 19). The time required by the eggs and larvae to reach each stage was recorded. Pictures of each stage were taken by a digital camera (Evolt, E-300, Olympus) attached to the microscope. Egg's diameter and larvae's length and width were measured from photographs (Fig. 19).

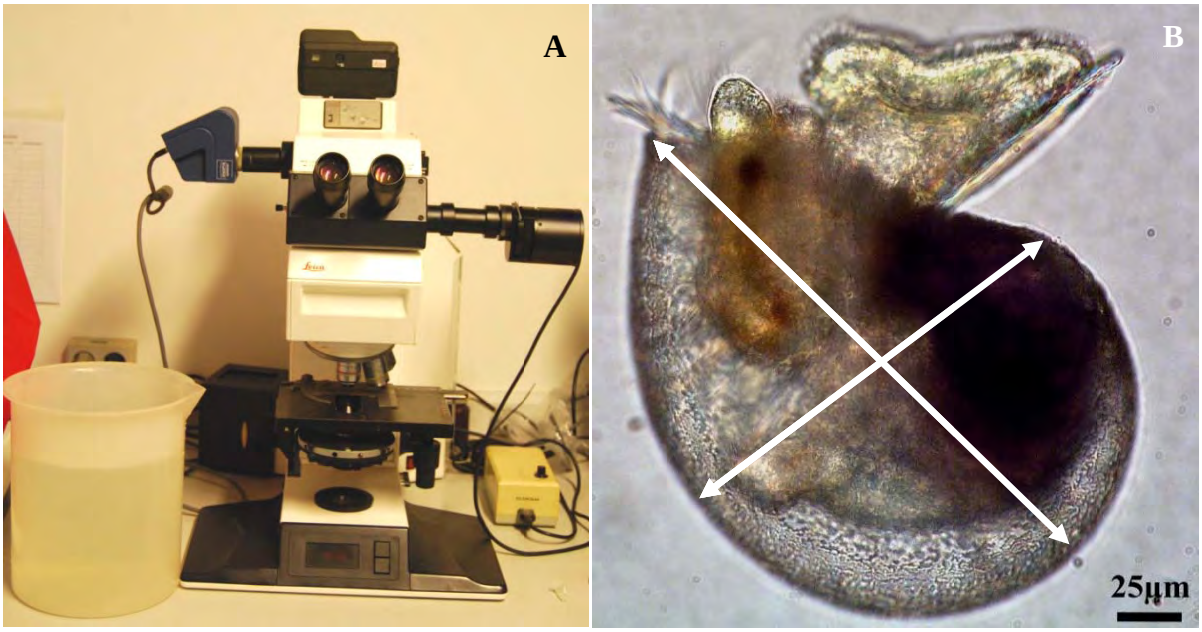


Fig. 19: Leitz microscope (A) and measurements of larvae's total length and width (B).

3.6 LARVAL SETTLEMENT AND POST-LARVAL GROWTH

Settlement induction of *H. tuberculata coccinea*, in the nursery, was performed using vertical settlement plates located within baskets inside the settlement tanks. Each settlement tank had a 2500-L capacity and contains 12 baskets with 20 settlement plates (60x30 cm) each. Aeration was provided through four airlines located on the bottom of the tank, along the sides of the baskets, and perpendicularly to the plates (Fig. 20). Each tank was run in flow through with filtered seawater. Seawater was mechanically filtered through a sand filter (Mod. 00689; Astra pool, Barcelona, Spain) followed by cartridge filtration located at the intake of each settlement tank (Fig. 21). Once settled, the post-larvae relied on mix benthic diatoms, cultured and provided to the post-larvae on a weekly basis. The diatoms species and culture conditions will be further described.

In order to perform small scale settlement, growth and survival experiments in culture conditions being representative of the nursery ones, all the settlement experiments performed in these studies were conducted according to the description of the following sections.

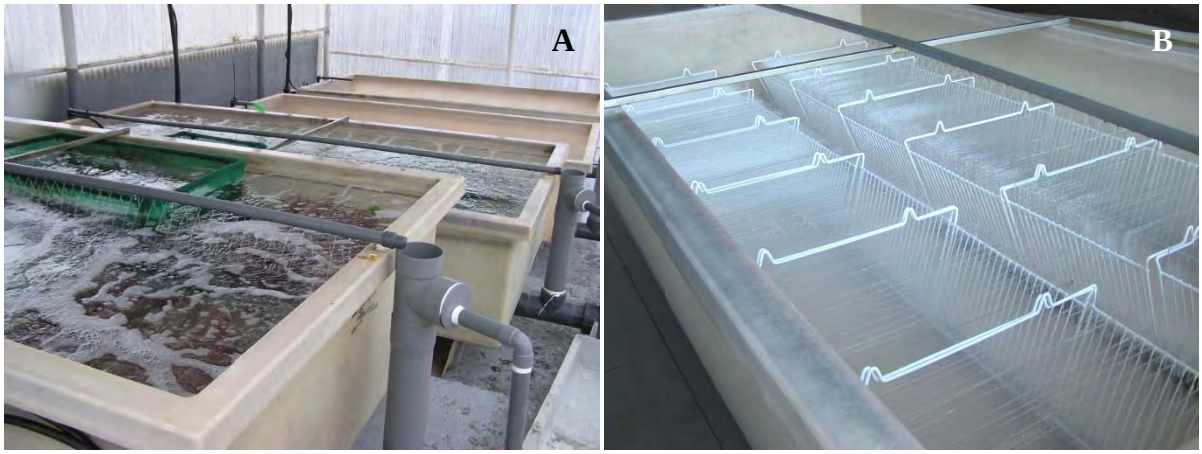


Fig. 20: *H. tuberculata coccinea* nursery area (A) and detail of settlement tank (B).

Fig. 21: Seawater filtration units in the nursery area.

3.6.1 Experimental set up

In the first set of settlement studies, the experimental settlement plates consisted of 50 cm^2 plastic squares colonized by the settlement cues to be tested, as opposed to negative control plates that were not colonized. For each treatment tested, each replicate consisted of four experimental settlement plates placed vertically in 12 L containers filled with 1µm cartridge filtered seawater and supplied with low aeration (Fig. 22). The density of larvae to be introduced into the containers was estimated by counting them in three 3-ml subsamples drawn from the entire larval batch. Larvae in all experiments came from the same batch and the densities tested were 2000 and 200 larvae per 12-L container, corresponding to 10 and 1 larvae cm^{-2} of substrate respectively. Twenty four h after the introduction of the larvae, the flow was initiated in the experimental containers at a rate of 1% exchange rate per hour, being increased up to 20% exchange rate per hour after 72h. The seawater outlet was fitted with 125-µm mesh screens to prevent the loss of larvae cultured in flow through conditions (Fig. 22). Settlement experiments were run at ambient seawater temperature (19-21ºC) and under an artificial photoperiod 12:12 hours L: D provided at a light intensity of 2000 Lux and measured using a Digital Light Meter, (HT170N, HT ITALIA, Italy).

Once settled and metamorphosed, the post-larvae were weekly fed with 200ml of mix diatoms inoculums (10^6 cells ml^{-1}). The diatoms species, culture conditions and cell count

estimations are further described. Post-larval growth and survival was followed during 4 weeks after settlement and the methodology employed is further described.

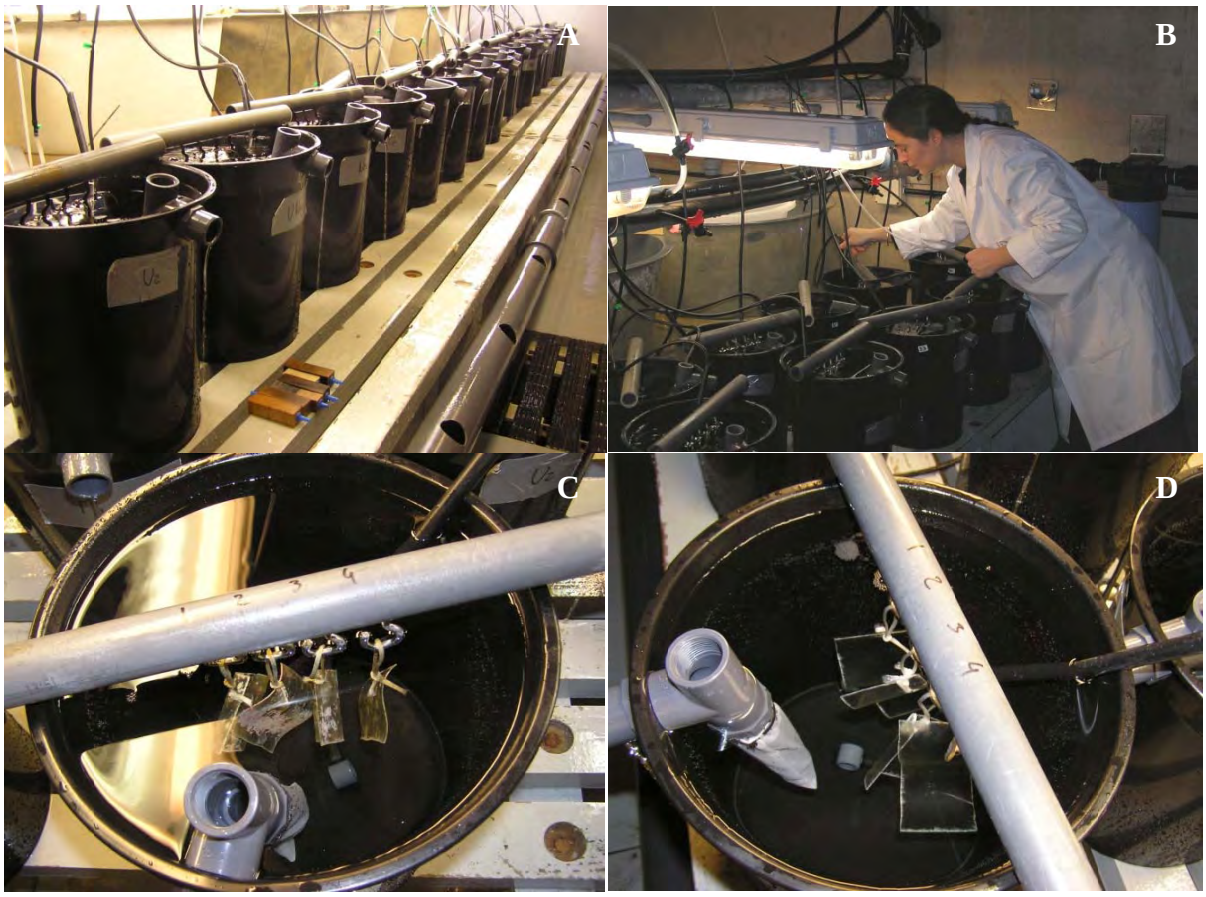


Fig. 22: General view of the settlement induction experimental set up (A & B). Detail of the settlement plates in treatments replicates, aeration and container outlet (C & D).

In the other set of settlement studies, competent larvae were induced to settle on settlement plates that had been colonized with 45-days-old mix of *U. lens* and *U. rigida* germlings. For each treatment tested, each replicate consisted of 100-L tank containing four colonized settlement plates (60 x30 cm) plus four 50 cm^2 platelets. Settlement plates and platelets were placed vertically in the settlement tanks filled with 1µm cartridge filtered seawater and supplied with low aeration. Competent larvae, from the same batch, were introduced at a density of 7200 larvae 100 L^{-1} tank, representing 0.5 larvae cm^{-2} of settlement substrate. Twenty four hour after the introduction of the larvae, flow was initiated in the experimental containers at a rate of 1% exchange rate per hour and was increased up to 20% exchange rate per hour after 72h. The seawater outlet was being fitted with 125-µm mesh screens to prevent the loss of larvae cultured in flow through conditions. Three days after

settlement induction diatom cultures were added as food supply to the 100l tanks. Four different diatom treatments, *Amphora* sp., *Navicula incerta*, *Nitzschia* sp. and *Proschkinia* sp., were tested, in triplicate, as food for *H. tuberculata coccinea* post-larvae. In each replicate, post-larvae were weekly fed with 2L of respective diatom inoculums (10^5-10^6 cells ml^{-1}) which culture conditions and cell count estimations are further described. Seawater temperature in the rearing containers was 21±1 ºC and the experiment was performed under an artificial photoperiod 12:12 hours L: D provided at a light intensity of 2000 Lux and measured using a Digital Light Meter, (HT170N, HT ITALIA, Italy). Post-larval growth and survival was followed during 10 weeks after settlement and the methodology employed will be further described. The proximate composition and fatty acid analysis of feed sources and juveniles samples was performed once completed the experimental period.

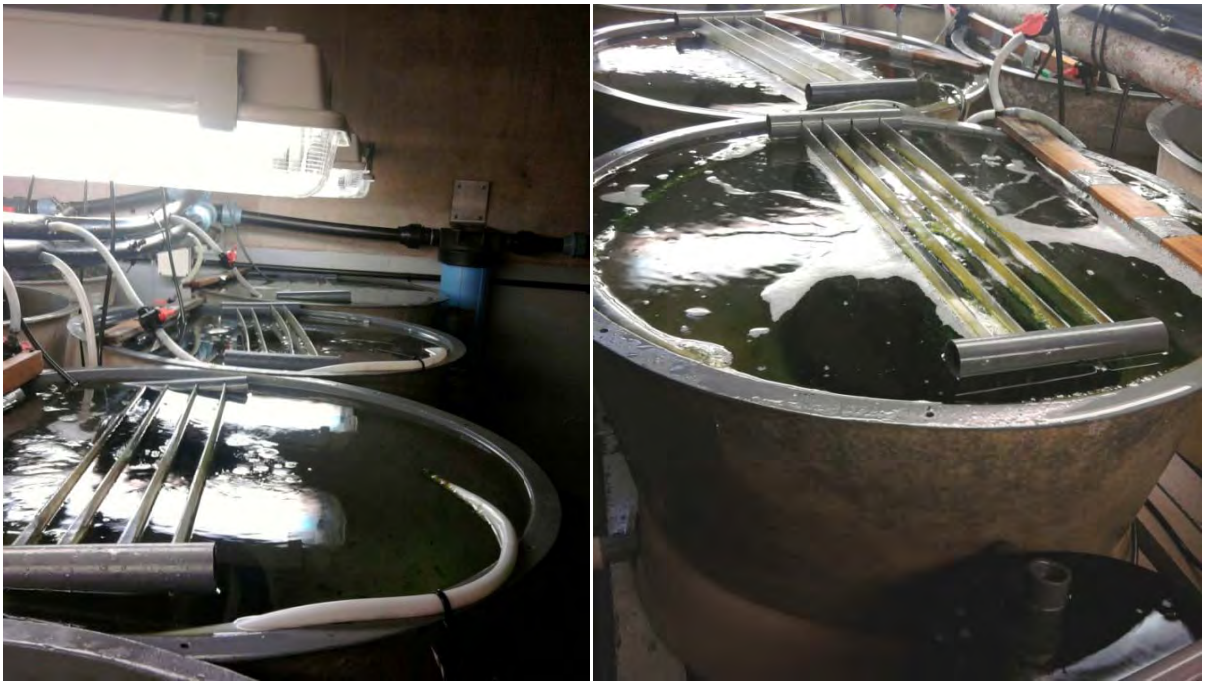


Fig. 23: 100-L experimental settlement tanks.

3.6.2 Measurements

The number of settled post-larvae and their survival were estimated by recording the number of live post-larvae on settlement plates of each replicate under a dissecting microscope (Mod. SL 260004, Optech, Germany) (Fig. 24). Settlement rate was estimated 48 hours post introduction of the larvae to the settlement containers while survival was

monitored on a weekly basis. Larvae were considered as settled once permanently attached to the substrate after shedding the velum to complete metamorphosis.

Post-larval growth was monitored by weekly or fortnightly sampling the shell length of randomly selected post-larvae per treatment. Measurements were performed with a profile and measuring projector (Mod. PJ-H3000; MITUTOYO; Japan) (Fig 24 & 25). Plates were kept immersed at all times during observation were and replaced immediately after observation and measurement (Fig. 25). Based on the obtained data, daily growth rate (DGR) was calculated according to the formula:

$$\frac{Lf - Li}{t}$$

Where *Lf* is final shell length in micrometers, *Li* is initial shell length in micrometers, and *t* is time in days.

Fig. 24: Dissecting microscope (A) and profile and measuring projector (B).

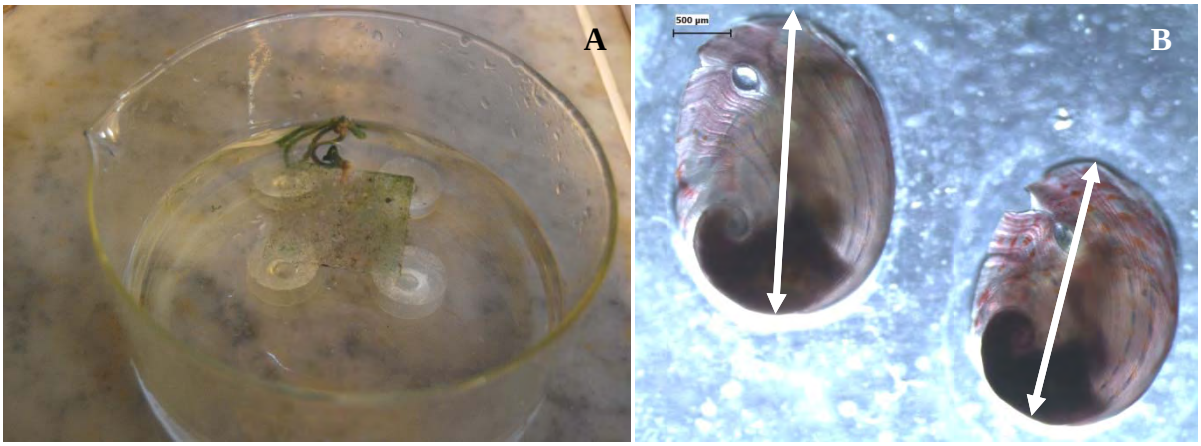


Fig. 25: Immersed settlement plate (A) and shell length measurements (B).

3.6.3 Estimation of cell density and percentage cover of algae

For the purpose of inoculation and in order to monitor growth rate, diatoms cell number was recorded. Cells were counted in a hemacytometer (Mod. Neubauer, Germany). To avoid possible cell aggregations, ultrasound (Mod. 3510 E-MT, Branson, USA) (1 or 3 min) was applied to the samples prior to evaluation of cell concentration. Average growth rates were estimated using the following formulae:

$$\mu = Ln\ (N_1/N_0)/\ t_1\text{-}t_0$$

Where N_1 = cell density at time t_1 and N_0 = cell density at time t_0, (Guillard, 1973).

Percentage cover of settlement substrates tested as well as the number of attached diatoms cells cm^{-2} were estimated by randomly choosing ten fields of view of the settlement plates, from each treatment, and photographing them at a magnification of 400 x at the time of settlement. Cell number and percent cover were then calculated by processing the images with Image J Software (National Institutes of Health, USA, Image J 1.42q) (Fig. 26).

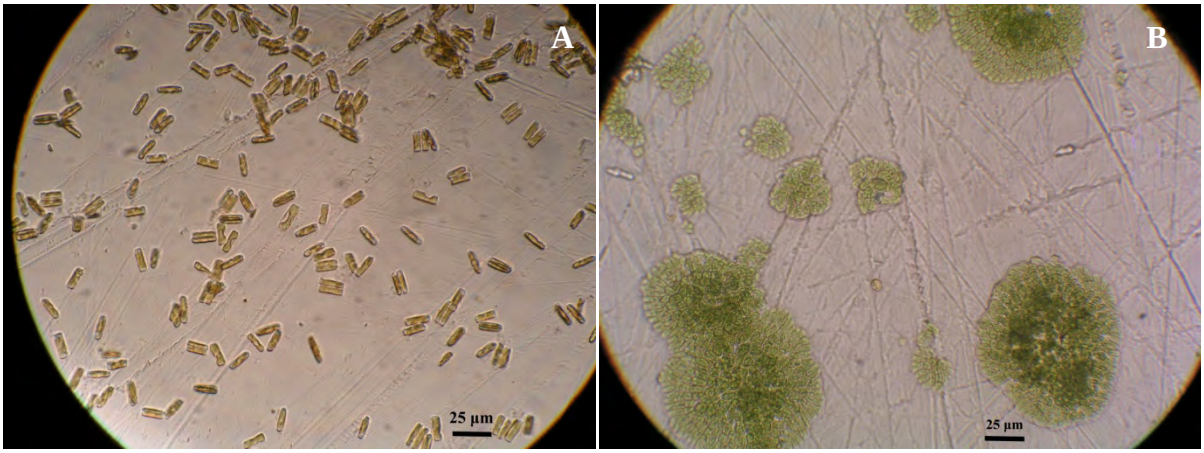


Fig. 26: Fields of view, at magnitude x400, of diatoms (A) and *U. Lens* (B) colonised settlement plates.

3.7 ALGAL CULTURE

3.7.1 Crustose coralline algae (CCA)

In order to colonize the experimental settlement plates with crustose coralline algae, settlement plates were left to be colonized in nursery tanks until reaching an approximate 50% percent cover (Fig. 27).

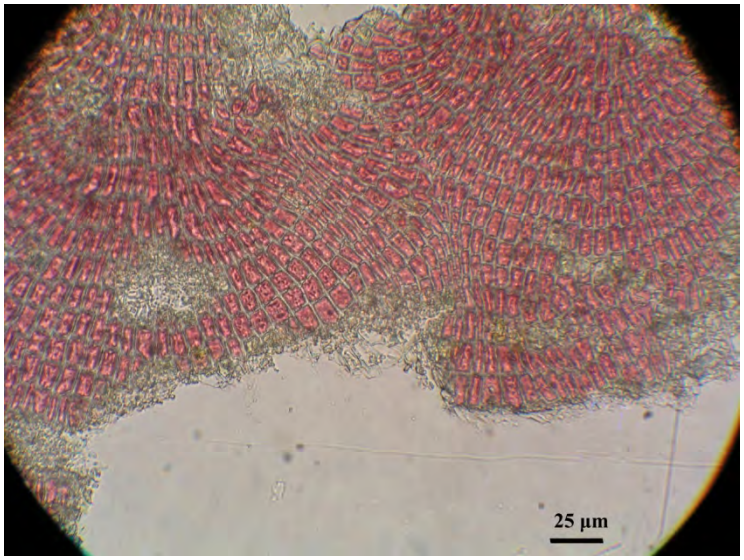


Fig. 27: Field of view of CCA colonizing experimental settlement plate.

3.7.2 Green macroalgae

The spores of green macroalgae tested to be used as settlement induction substrates were *U. lens* and *U. rigida*. Germlings from both species were obtained from mature (spore producing) *U. lens* and *U. rigida* thalli. *U. lens* was maintained in culture under natural photoperiod and with fortnightly addition of f/2 mix (Guillard, 1975) (Fig. 28). *U. rigida* was cultured under natural photoperiod in biofiltering units located outside and receiving water from fish tanks effluents (Fig. 28).

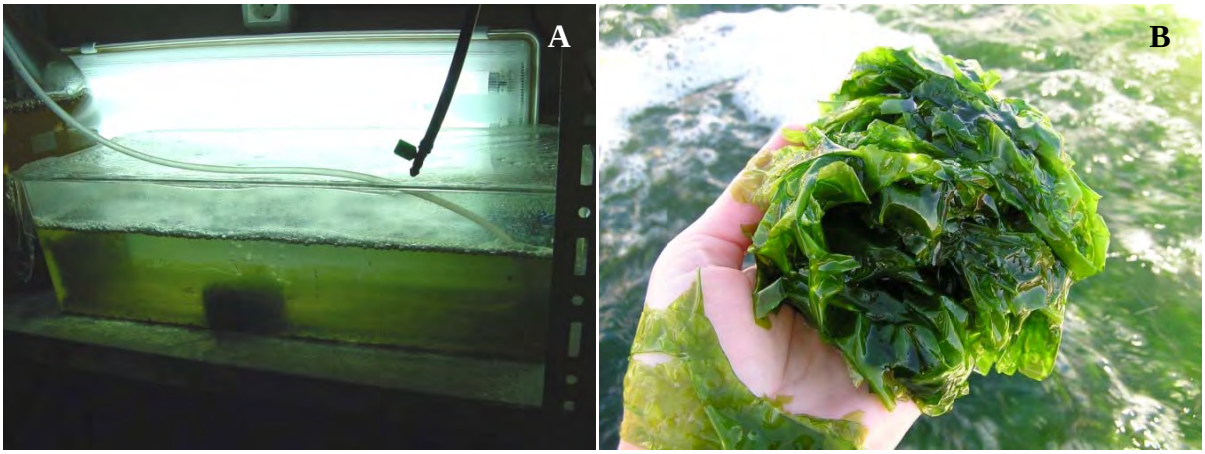


Fig. 28: Stock cultures of (A) *U. lens* and (B) *U. rigida.*

Plates with large, dark green, mature patches of *U. lens* were freed from diatom film and left in the dark during 2 weeks. *U.rigida* thalli were subjected to 7 days of cold treatment (4ºC) prior to starting the conditioning of the experimental plates. In order to induce them for spore release each species were placed in tanks, holdings the experimental plates, maintained with no water flow, low aeration and to which a complete f/2 mix, was applied. The largest spore released was observed 4-5 days after the introduction of the mature macroalgae into the tanks holdings the experimental plates. The methods for germlings production and their culture were adapted from Daume et al. (2004) and Strain et al. (2006). The germlings on the experimental settlement plates were maintained in culture during different periods to evaluate the effect of substrate age and quality on settlement induction. During that period the germlings were maintained aerated, under natural photoperiod and with a weekly renovation of the f/2 culture medium (Fig. 29).

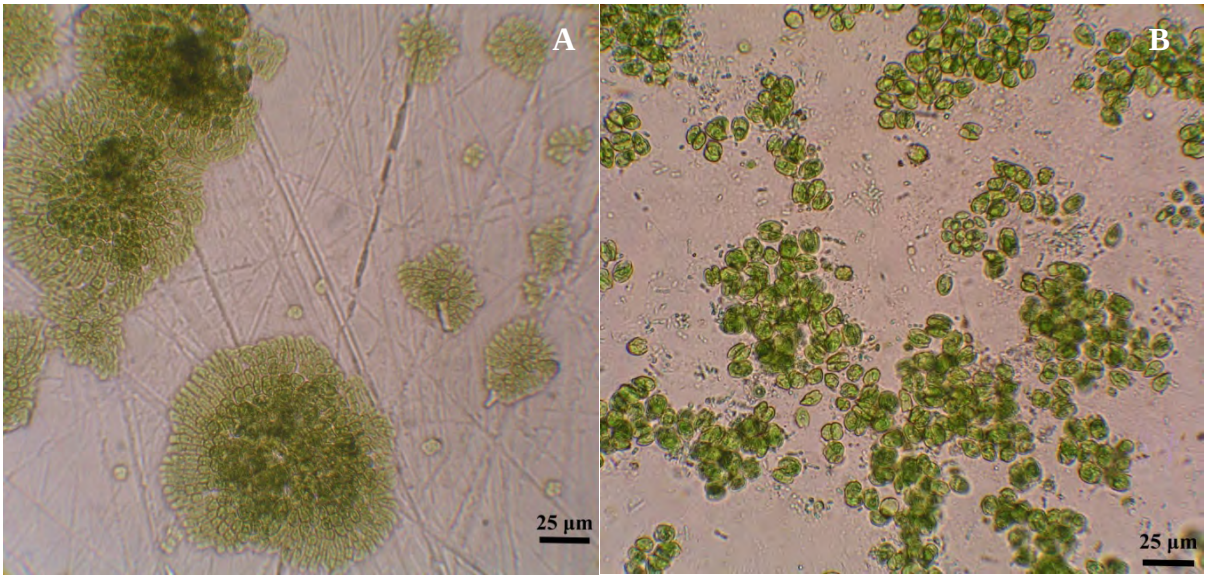


Fig. 29: Germlings from old *U. lens* (A) and young *U. rigida* (B).

3.7.3 Benthic diatoms

Large scale culture of benthic diatoms took place in horizontally laid 40-L polyethylene algal bags. Four species of diatoms *N. incerta*, *Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp. were cultured individually in a scaling up process until reaching large scale culture. They were maintained and their growth was estimated in 10-ml test tubes, they were further grown in 4-L Erlenmeyer flasks and finally cultured in 40-L algal bags (Fig. 30). The diatoms were grown in f/2 medium plus silicate ($1 mgL^{-1}$) (Guillard 1975), at ambient temperature and under continuous light of 62 ± 8 µmol photon m^{-2} s^{-1}. In the "Batch culture" protocol, all diatoms were cultured with initial inoculums of 10^5 cells ml^{-1} and harvested after 5 days of culture corresponding to the exponential phase of growth. The cultures were unialgal but not axenic, they were maintained neither aerated nor agitated. Diatom cell density was evaluated with a hemacytometer (Mod. Neubauer, Germany). Prior to the counts ultrasound (Mod. 3510 E-MT, Branson, USA) (1 or 3 min) was applied to the diatoms samples to avoid possible cell aggregations. Photon flux density (irradiance) was measured using a Digital light meter (HT170N, HT ITALIA, Italy). Water employed for the benthic diatoms culture was mechanically filtered through a cartridge filter and sterilized by UV irradiation as previously described.

The diatoms cultured were employed as a nutritional source for post-larvae, and in the case of being used as settlement inducing substrate; diatoms were harvested after 5 days of

culture and transferred to containers holding experimental settlement plates to be colonized by attaching diatoms until reaching an approximate density of 10^5 cells cm^{-2}.

Fig. 30: Batch culture of benthic diatoms.

3.8 BIOCHEMICAL AND FATTY ACID ANALYSIS

In the course of the different studies, triplicate samples of diatom cells, algal cues, larvae and post-larvae were collected to be analyzed for proximate composition including dry matter, ash, protein and lipid content, as well as fatty acids. Collection methods of the samples differed according to their nature. Samples diatoms cells were collected by filtration from the algal bags and rinse with fresh and distilled water to eliminate salt particles before being stored. Algal cues were collected by scraping the surface of settlement plates at the start and end of the experiments. Larvae were siphoned from the larval tanks, collected in a 63µm mesh and rinsed with fresh and distilled water. Finally, post-larvae were collected from the settlement plates. All samples were stored at -80ºC prior to be analyzed. Before analysis all samples where homogenized with mortar and pestle before being weighted for further analysis. All the analyses were performed at the laboratory of the Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA, ULPGC).

3.8.1 Dry matter content

Dry matter content was determined by drying a known fresh sample quantity (0.5-10g) (*Pi*) in an oven at 105ºC until obtaining a constant weight (*Pf*). Before being weighted the samples were submitted to desiccation during 30 minutes until reaching ambient temperature. The dry matter content was calculated according to the following formula:

$$\%DM = \frac{(Pi - Pf) \times 100}{Pi}$$

3.8.2 Ash content

Ash content was determined gravimetrically after incinerating a known amount of sample (1-2g) (*Pi)* in an oven at 600ºC during 24h. The remaining amount of ashes was recorded and weighted until reaching constant weight (*Pf*) according to the established recommendations (2005). Final ash content of the samples was calculated according to the following formula:

$$\%\ Ash = \frac{Pi \times 100}{pf}$$

3.8.3 Protein content

Total protein was calculated from total nitrogen content of the samples by the Kjeldahl method in agreement with AOAC (2005). The samples (0.2-0.4g) are digested by boiling, at 400ºC during one hour, a homogeneous sample in concentrated sulphuric acid (H_2SO_4) together with a copper catalyst. The end result is an ammonium sulphate solution ($(NH_4)_2SO_4$). Excess base (NaOH) is added to the digestion product to convert NH_4 to NH_3 which is recovered by distilling the reaction product. In the distillation unit (Mod. Foss Tecator, 1002, Höganäs, Sweden) direct titration is performed, using boric acid (H_3BO_3) as the receiving solution, to quantify the amount of ammonia in the receiving solution. Titration is performed with HCl at 0.1M. Analysis of a blank was run in parallel to the samples analysis. The protein content of the samples was calculated according to the following formulae:

$$\%\ Protein = \frac{(Vs - Vb) \times N \times 14.007}{W} \times F$$

Where:

- *Vb* = mL titrant for the blank
- *Vs* = mL titrant for the samples
- N = Normality of the acid titrant (HCl)
- 14.007 = Nitrogen molecular weight
- W = Weight of sample in milligrams
- F = the factor for converting the percent nitrogen in a sample to percent protein, its value was 6.25.

3.8.4 Lipid content

Lipids were extracted according to the methodology described by (Folch et al., 1957). 50 to 200mg of sample mixed with 5 ml of a solution of chloroform/methanol (2/1) plus 0.01% of the antioxidant Butylhydroxytoluene (BHT) were homogenized in an Ultra Turrax (IKA-Werke, T25 BASIC, Staufen, Germany) during 5 min. The obtained homogenate was then washed with 5 ml of chloroform/methanol filtered and KCl at 0.88% was added to increase the water phase polarity. The mixture was centrifuged at low speed (2000 rpm) during 5 min to separate the watery phase from the organic one. The watery phase on the upper part was discarded while the lower one, containing the lipids, was filtrated and evaporated with Nitrogen steam until complete dryness was obtained. The dry lipid content was then gravimetrically determined.

3.8.5 Fatty acids content

Fatty acids from the lipid extracts were trans-esterified to methyl esters (FAMEs) with 1% sulphuric acid: methanol complex (Christie, 1982) and preserved in N_2 atmosphere. The mixture was left in shaking incubation during 16 hours at 50ºC then cooled. Distilled water together with hexane diethyl ether 1:1 and BHT at 0.01% were then added. The purified FAMEs samples were evaporated until complete dryness with N_2 and hence weighted. Finally the FAMEs were extracted into hexane and stored at -80ºC. FAMEs were analyzed in a Thermo Finnigan- GC Focus gas chromatograph (Mod. Shimadzu GC-14A; Analytical instrument division Kyoto, Japan) equipped with a flame ionization detector (260ºC). FAMEs were separated with capillary column (Supercowax 28m x 0.32mm x 0.25 i.d.) using helium as the carrier gas under the following gas pressures: He 1kg cm^{-2}, H_2 0.5 kg cm^{-2}, N_2 1kg cm^{-2}, air 0.5 kg cm^{-2}. The conditions were the following: injector temperature 260ªC, column temperature 180ºC during 10 minutes, increasing to 215ºC at a rate of 2.5ºC min^{-1} and maintained at 215ºC during 15 minutes. Fatty acids were identified by comparison to the reference EPA 28.

3.9 STATISTICAL ANALYSIS

Statistical analysis was performed using the Statgraphics Plus 5.1. Software. Analyses of variance (one-way ANOVAs) were performed to compare proximate biochemical composition, as well as cell number, settlement, survival and daily growth rate (DGR) between treatments. Data showing significant differences ($P<0.05$) were analyzed by paired comparisons using Tukey's HSD test. Assumption of normality and homogeneity of variance were assessed with standardized skewness and kurtosis and Bartlett's test. When variances were heterogeneous and/or in the case of non normal distribution data was transformed to logarithms or with arc sine function.

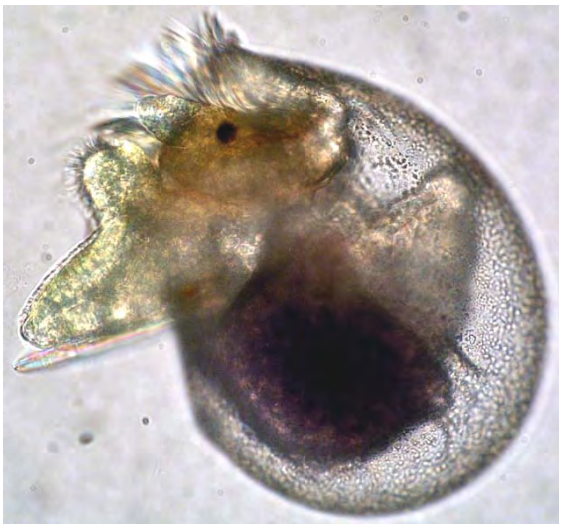
Multiple regression analyses were carried out to explain the variation in settlement, growth and survival between treatments. The proximate biochemical composition of the algal cues and diatoms; proteins, lipids, ash and carbohydrates contents, were the factors selected for the analyses.

Principal component analysis was performed on the data of fatty acids composition of the algal cues and diatoms and principal components were identified.

Kendall rank correlation between the main principal components and settlement, growth and survival data was then performed in order to identify the effects of fatty acids composition determinant among treatments.

Cluster analysis was carried out on the larvae, post-larvae, diatoms and substrates fatty acid composition data in order to establish groups by proportion of similarity.

4. STUDY I: EMBRYONIC AND LARVAL DEVELOPMENT OF *HALIOTIS TUBERCULATA COCCINEA* REEVE: AN INDEXED MICRO-PHOTOGRAPHIC SEQUENCE



Embryonic and larval development of *Haliotis tuberculata coccinea* Reeve: an indexed micro-photographic sequence.

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Abstract

First description of the complete embryo and larval development of the Canarian abalone (*Haliotis tuberculata coccinea* Reeve.) was conducted along 39 stages from fertilisation to the appearance of the third tubule on the cephalic tentacles and illustrated in a micro-photographic sequence. Eggs obtained by induced spawning with hydrogen peroxide from the GIA captive broodstock were stocked at a density of 10 eggs/ml and kept at 23±0.5 ºC for 62 h until the formation of the third tubule. Live eggs and larvae were continuously observed on a 24 h basis at a 400x magnification under transmitted light. At each stages, specific morphological features, illustrated by microscopic photographs, were described, as well as the time required for their apparition. Fertilised eggs diameter was 205±8µm (mean ± SD), whereas length and width of larvae ready to undergo metamorphosis were 216.6±5.3µm and 172±8.8µm, respectively. Knowledge on the larval morphological development acquired through this study will contribute to the improvement of larval rearing techniques for this abalone species.

Keywords: Abalone, embryonic and larval development, *Haliotis tuberculata coccinea*.

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Introduction

Abalone's life cycle includes larval, post-larval, juvenile and adult stages. Larval development is a gradual process that does not occur in discrete stepwise stages. However, various stages can be recognized during larval development and larval development rate is determined by the time larvae require to show certain distinctive features (Hahn, 1989). In *Haliotis discus hannai*, Ino, (1952) identified distinct larval stages with recognizable external features. These morphological features and the order of their appearance in larval developmental stages provided the guidelines for studies of larval development in other abalone species (Oba, 1964; Leighton, 1974; Seki and Kan-no, 1977; Koike, 1978; Owen, et al., 1984; Peña, 1984, 1986). Hence, larval stage begins with fertilisation and is completed with the formation of the fourth tubule on the cephalic tentacles, although larvae are considered ready for settlement when the third tubule appears and larvae starts to explore the surface (Hahn, 1989).

Haliotis tuberculata coccinea Reeve; an abalone subspecies present in the Macaronesian Archipelago grows to a maximum size of about 80 mm in shell length and is considered gourmet seafood in the Canary Islands. During decades this subspecies has been commercially exploited for the local market in the Canary Islands leading to an overexploitation of its stocks which are presently almost depleted. Therefore, there is a great interest to develop the culture techniques of this species due to its high market value and the interest in recovering its wild populations by re-stocking. Several studies have been conducted to better understand the physiology of this species and its behaviour under captive conditions (Peña, 1986; Toledo, et al., 2000; Viera, et al., 2003, 2005). However, production of seeds remains a major bottleneck for the commercial production of this species, studies related with its embryonic and larval development being scarce and insufficient to allow the development of larval rearing techniques. Certain stages of the embryonic development of *Haliotis tuberculata coccinea* were partially described by (Peña, 1986) with their respective developmental rates being determined at 15ºC, a temperature out of the natural range, 17-24ºC, for this subspecies. Thus, the present research was conducted to study the complete embryonic and larval development of *Haliotis tuberculata coccinea* under natural temperature conditions, describing and illustrating for the first time; with photographic support; the morphological features characteristic of each stage and the time required for their apparition.

Materials and methods

Captive *Haliotis tuberculata coccinea* broodstock were kept under dark conditions in 60-L tanks with a flow through system at the Instituto Canario de Ciencias Marinas (Canary Islands, Spain). Water temperature was kept at 23±0.5 ºC along the experiment which was carried out during November 2005. Broodstock were fed a mix diet of *Ulva rigida, Gracilaria cornea* and *Hypnea spinella*, twice a week. Abalone showing mature gonads in stage 3 (Ebert and Houk, 1984) were transferred to be induced to spawn.

8 males and 14 females (58.2±4.54 mm and 32.9±8.36 g) were placed separately by sex into two 10-L spawning aquaria filled with 1µm filtered and UV sterilized seawater. They were induced to spawn using the hydrogen peroxide method (Morse, et al., 1977). Aquaria were kept in the dark during spawning. Released oocytes were collected in 10-l containers and fertilised, with a final sperm concentration of 10^5/ml, during 30 minutes. After that period eggs were rinsed with fresh seawater refills to remove excess sperm and fertilisation rates were determined by the proportion of eggs showing dividing cells 1 h after fertilisation. Fertilisation rate estimate was performed by taking three 3ml sample from fertilisation containers.

Fertilised eggs were allowed to develop until the trochophore stage in the hatching containers. Trochophore larvae were reared at a density of 10 larvae/ml in 70-L flow-through larval rearing tanks filled with 1µm filtered and UV sterilized seawater.

Observation of embryo and larval development was carried out under transmitted light with a Leitz DMRBE microscope (Leica, Wetzler, Germany) at a magnification of 400X. The study started immediately after fertilisation and was continuous for the next 62 h. All observations were performed with pooled larvae in 5-L containers until the appearance of the third tubule on cephalic tentacles. Time required by the larvae to reach each stage was recorded. Pictures were taken with a digital camera (Evolt, E-300, Olympus) attached to the microscope. Egg diameter and larvae's length and width were measured from photographs.

Results

Spawning was achieved in 2 h for females whereas it took 1 h 30 for the males. General fertilisation rate, recorded one hour after fertilisation, during the experiment was 77±3.6%.

H. tuberculata coccinea eggs were dark violet, whereas the larvae showed an orange-yellowish foot, velum, and cephalic tentacles and a violet-coloured visceral mass. The average size of unfertilised eggs was 196±8 µm. After fertilisation, egg size increased up to 205±8 µm and it did not change for the rest of the pre-hatching period. Length and width of the free swimming trochophore larvae were 166.6±7.6 and 124.7±4.75, respectively. From the larval shell completion stage until the apparition of the third tubule on the cephalic tentacles larval size was constant with a length of 216.6±5.3µm and a 102 width of 172±8.8µm.

Thirty nine distinct, consecutive, larval stages, with recognizable external features, were observed from fertilisation until the apparition of the third tubule on the cephalic tentacles. Table 1 shows the time required by eggs and larvae to reach each larval stage at a water temperature of 23±0.5 ºC. Twenty five min after fertilisation (stage 1), the first polar body was discharged (stage 2) followed by the discharge of the second polar body (stage 3) (Fig. 1 A&B). Cleavage began after discharge of the polar bodies, the first and second one being along the vertical axis of the egg (stages 4&5) and the third in the horizontal plane just above the axis (Fig. 1 C&D). At the third division, micromeres and macromeres could be differentiated and egg development progressed to the gastrula with cell cleavage being total, unequal and spiral (stages 6 to 11) (Fig.2 A to D and Fig.3 A). At stage 12, cilia grew along the top of the embryo and began beating, causing the rotation of the embryo within the egg membrane (Fig. 3 B). The stomodeum appeared at stage 13 and the prototrochal girdle with its cilia were completely formed during stage 14 (Fig. 3 C&D). At this stage, the embryo could be identified as a trochophore like larva inside the egg. Then, the egg membrane became thinner and larvae started to move more intensively inside the egg, finally bursting the egg membrane with the help of the apical cilia, causing the hatch out and starting to swim (stage 15) (Fig. 4 A).

Table 1. Larval development rate of *H. tuberculata coccinea* at 23±0.5ºC.

Sequence	Larval Development Stage	Time (h)
1	Fertilisation.	0.00
2	Discharge of first polar body.	0.25
3	Discharge of second polar body.	0.39
4	First cleavage (2 cells).	0.49
5	Second cleavage (4 cells).	1.26
6	Third cleavage (8 cells).	1.32
7	Fourth cleavage (12 cells)	1.55
8	Fifth cleavage (16 cells).	2.00
9	Morula.	2.30
10	Blastula.	3.13
11	Gastrula.	4.30
12	Appearance of cilia forming the prototrochal girdle.	6.21
13	Stomodeum.	7.22
14	Complete formation of prototochal girdle and cilia.	8.20
15	Trochophore larvae ready to hatch out.	9.11
16	Larval shell formation.	9.35
17	Veliger larvae exhibiting flat apical region and completely developed velum with cilia.	11.45
18	Appearance of larval retractor muscle.	15.20
19	Appearance of integumental attachment to larval shell.	15.20
20	Development of foot mass.	15.20
21	Appearance of eye spot.	17.20
22	Completion of larval shell.	18.17
23	90 degree torsion of the cephalo-pedal mass.	18.38
24	180 degree torsion of cephalo-pedal mass.	21.29
25	Spines at the end of metapodium and formation of operculum.	22.38
26	Operculum.	25.00
27	Appearance of cilia on foot sole.	26.29
28	Vertical groove formation in velum.	30.20
29	Appearance of propodium.	36.10
30	Appearance of cilia on propodium.	39.00
31	Appearance of cephalic tentacles.	40.20
32	Appearance of cilia in mantle cavity up to the anterior edge of the velum.	43.00
33	Appearance of apophysis on propodium.	47.50
34	Formation of epipodal tentacles.	52.32
35	Appearance of otolith.	53.20
36	Appearance of spines on cephalic tentacles.	54.20
37	Protrusion of snout underneath the velum.	54.40
38	Appearance of two tubules on cephalic tentacles.	58.05
39	Third tubule appearance on cephalic tentacles.	61.42

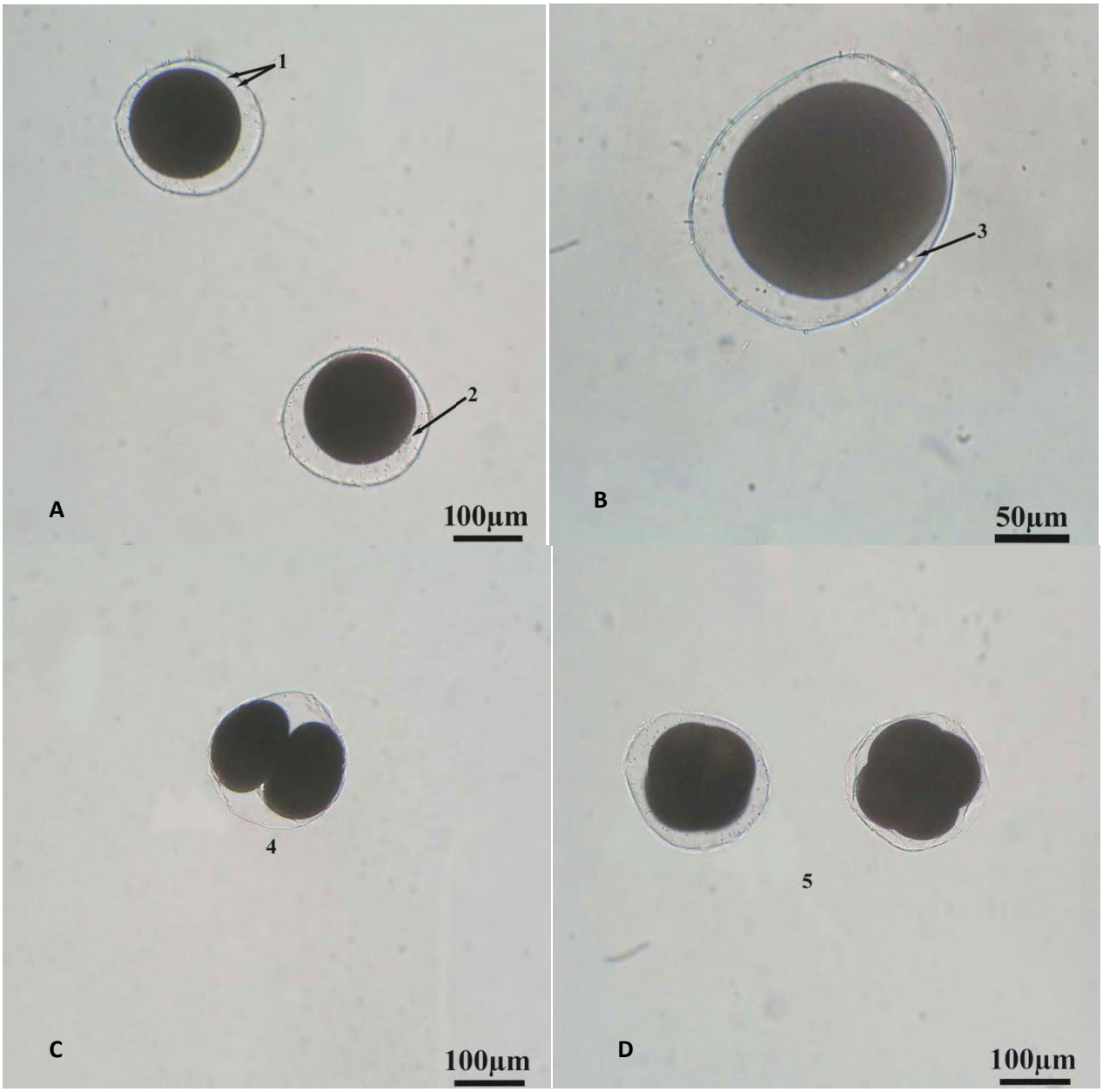

Figure 1. (A) Stage1 Spermatozoids. Stage 2 Discharge of first polar body. (B) Stage 3 Discharge of second polar body. (C) Stage 4 First cleavage (2 cells). (D) Stage 5 Second cleavage (4 cells).

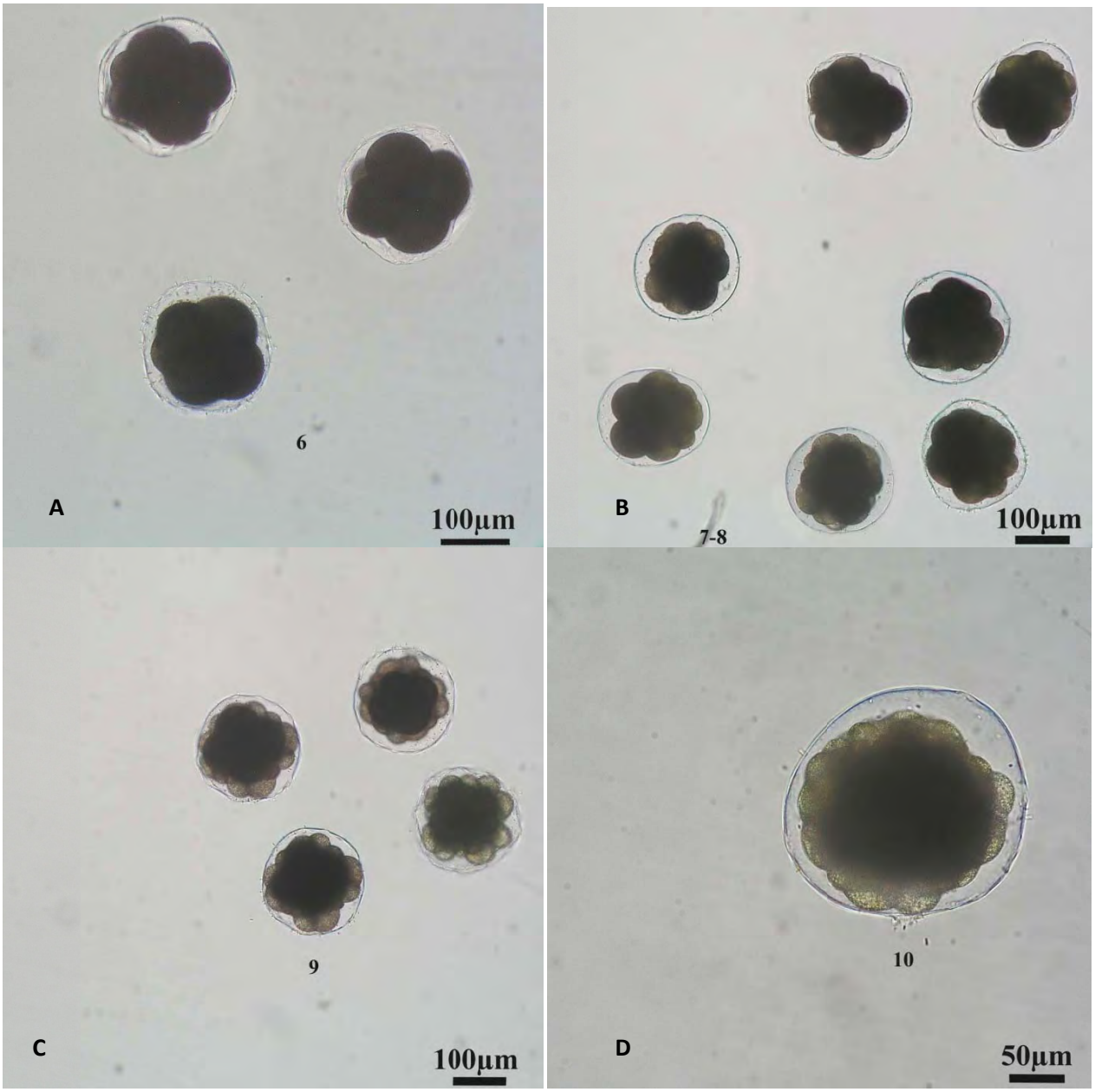

Figure 2. (A) Stage 6 Third cleavage (8 cells). (B) Stages 7&8 Fourth and fifth cleavages (12-16 cells). (C) Stage 9 Morula. (D) Stage10 Blastula.

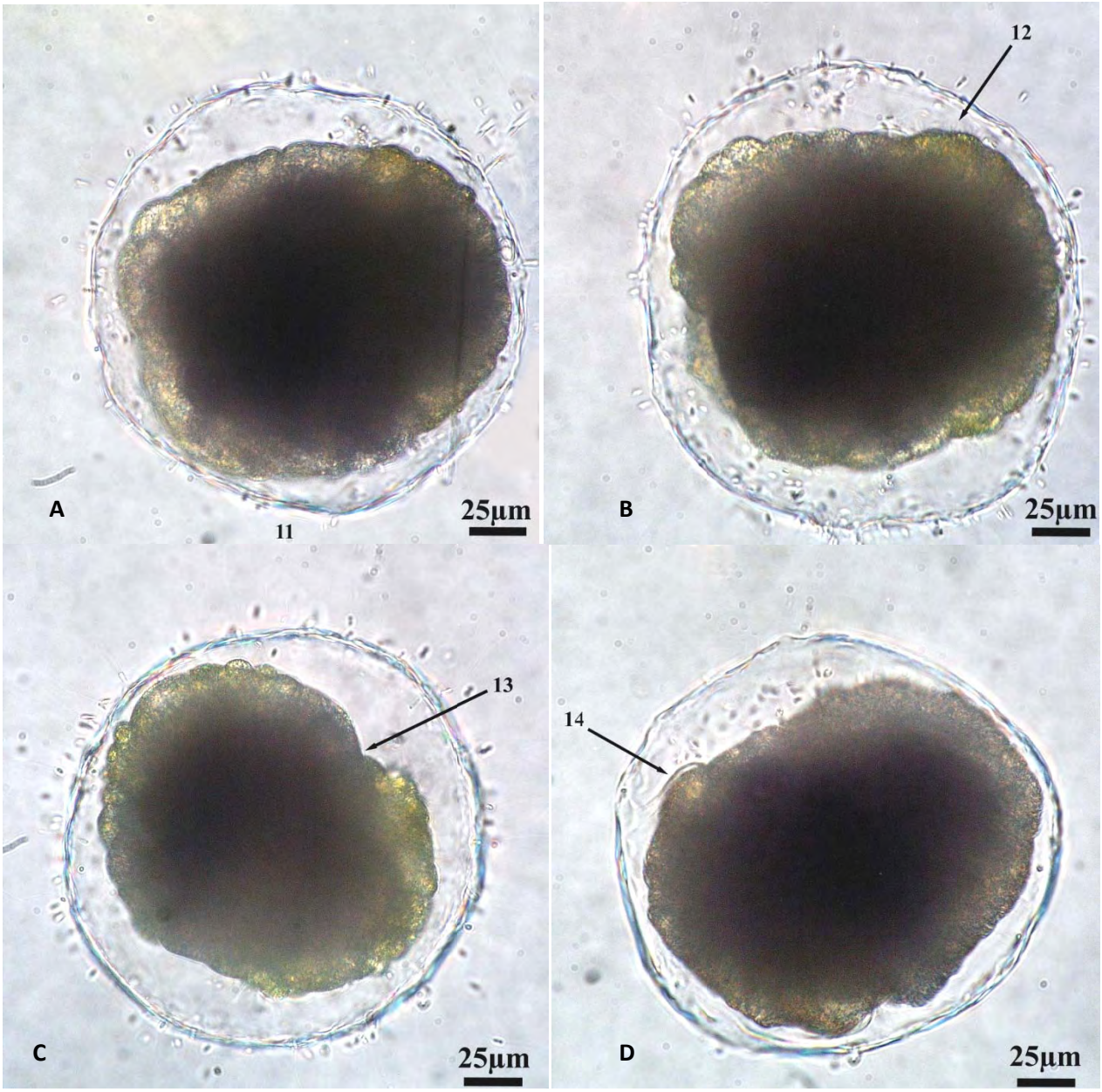

Figure 3. (A) Stage 11 Gastrula. (B) Stages 12 Appearance of cilia forming the prototrochal girdle. (C) Stage 13 Stomodeum. (D) Stage14. Complete formation of prototochal girdle and cilia.

Swimming trocophora larvae swam in groups forming spirals from the bottom of the container up to the water surface. Soon after hatch-out, the larval shell began to be secreted at the back of the larvae (stage 16) (Fig. 4 B). Larval development continued with the flattening of the apical region and the completion of the velum presenting cilia (stage 17) (Fig. 4 C), larva being then identified as a veliger. The following steps in larval development consisted in the formation of the retractor muscle (stage 18) and the integumental attachment to the larval shell (stage 19) (Fig. 4 D).

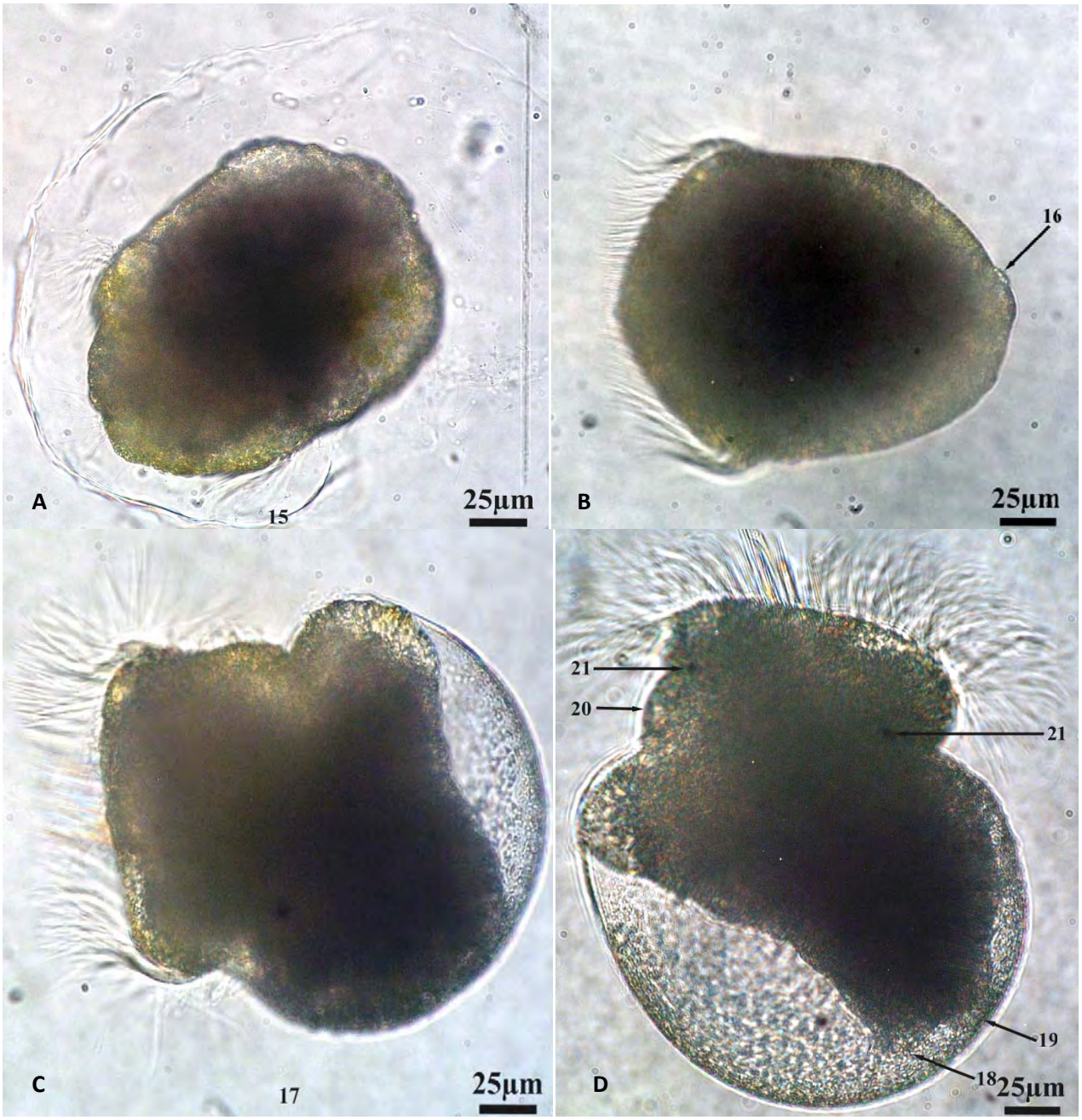

Figure 4. (A) Stage 15 Trochophore larvae ready to hatch out. (B) Stages 16 Larval shell formation. (C) Stage 17 Veliger larvae exhibiting flat apical region and completely developed velum with cilia. (D) Stage18 Appearance of larval retractor muscle. Stage 19 Appearance of integumental attachment to larval shell. Stage 20 Development of foot mass. Stage 21 Appearance of eye spot.

During stage 20, the foot mass started to protrude to the top of the shell and the eye spot appeared (stage 21) (Fig. 4 D) followed by the completion of the larval shell which grew from dorsal to ventral, until covering the body just below the velum (stage 22) (Fig. 5 A).

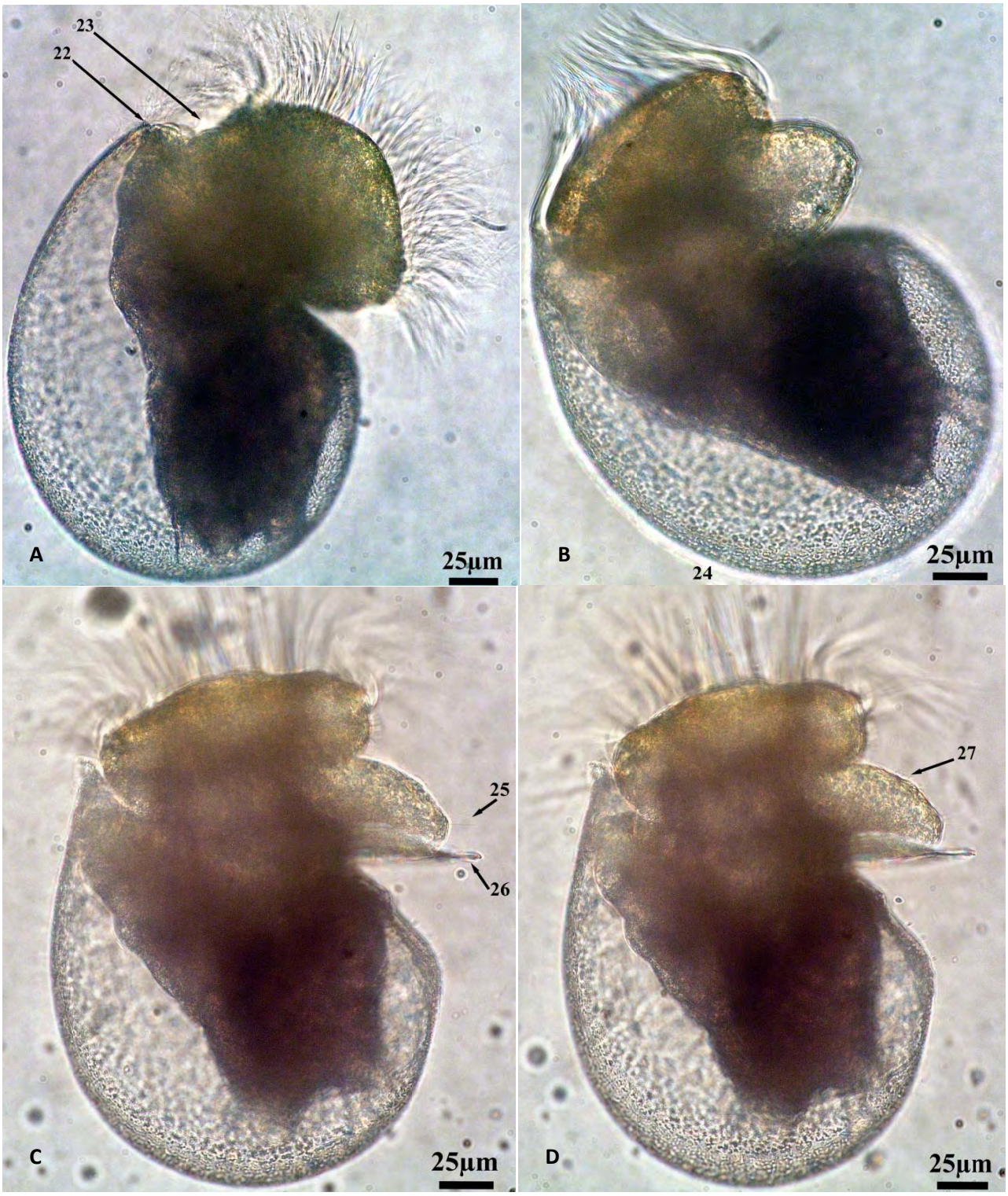

Figure 5. (A) Stage 22 Completion of larval shell. Stage 23 90 degree torsion of the cephalo-pedal mass. (B) Stage 24 180 degree torsion of cephalo-pedal mass. (C) Stage 25 Spines at the end of metapodium and formation of operculum. Stage 26 Operculum. (D) Stage 27 Appearance of cilia on foot sole.

During torsion, the cephalo-pedal mass first rotated 90º (stage 23) (Fig. 5 A), followed by the rotation of the region to become mouth and foot, until finally reaching a 180º rotation from its original position (stage 24) (Fig. 5 B). After torsion, spines could be observed at the end of the

metapodium (stage 25) as well as a developed operculum (stage 26) (Fig. 5 C). At that moment, the cephalo-pedal mass could be retracted into the shell that would be closed by the operculum.

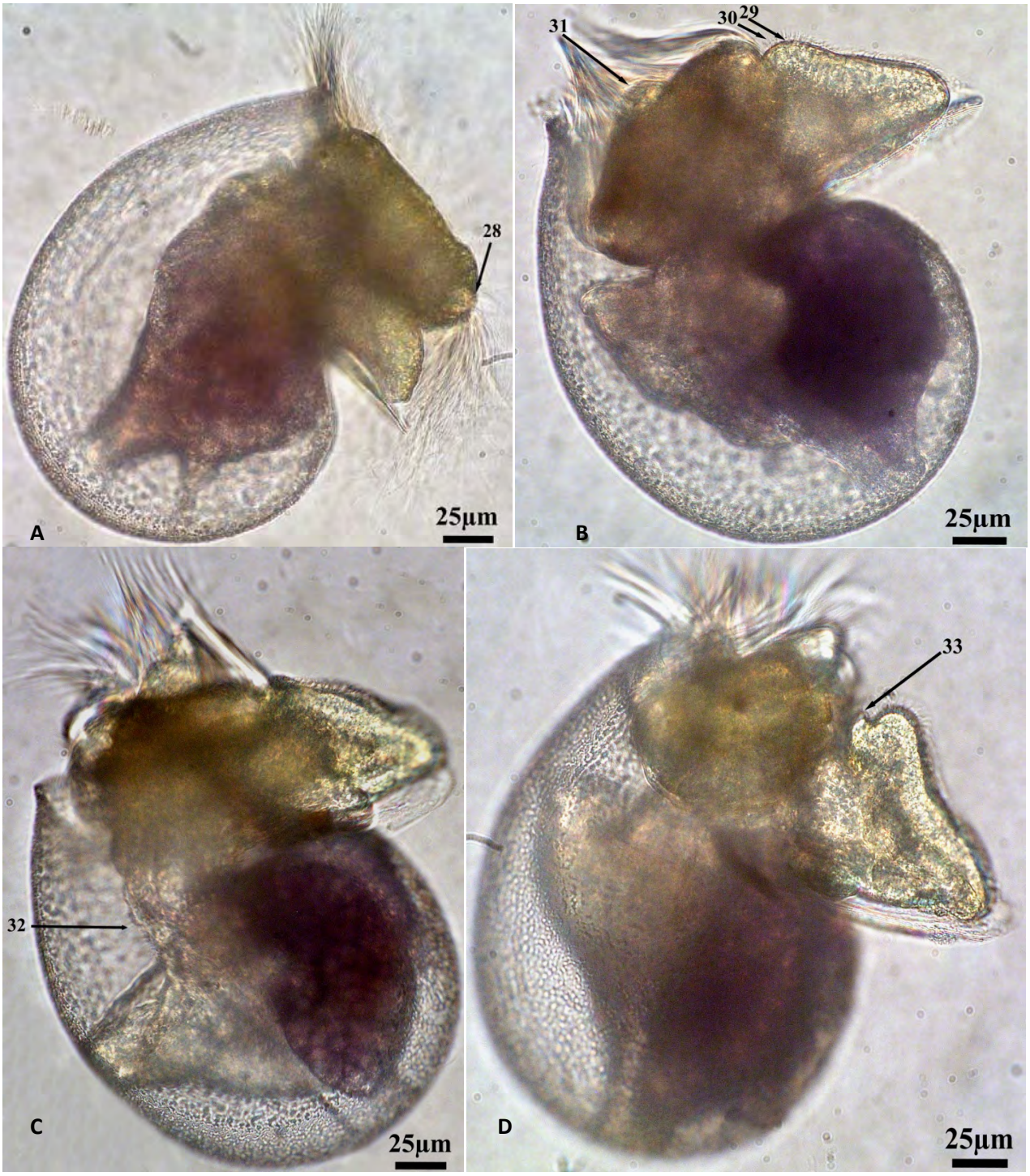

Figure 6. (A) Stage 28 Vertical groove formation in velum. (B) Stage 29 Appearance of propodium. Stage 30 Appearance of cilia on propodium. Stage 31 Appearance of cephalic tentacles. (C) Stage 32 Appearance of cilia in mantle cavity up to the anterior edge of the velum. (D) Stage 33 Appearance of apophysis on propodium.

In succession, fine cilia developed on the foot sole and began beating (stage 27) (Fig. 5 D), a groove appeared in the velum (stage 28), the propodium got formed (stage 29) with

cilia growing on it (stage 30) and a cephalic tentacle developed on the velum (stage 31) (Fig. 6 A&B). Cilia appeared in the mantle cavity and began beating (stage 32) (Fig. 6 C). Apophysis could be observed on the propodium (stage 33) (Fig. 6 D) and a pair of epipodal tentacles formed on both side of the foot (stage 34) (Fig. 7 A).

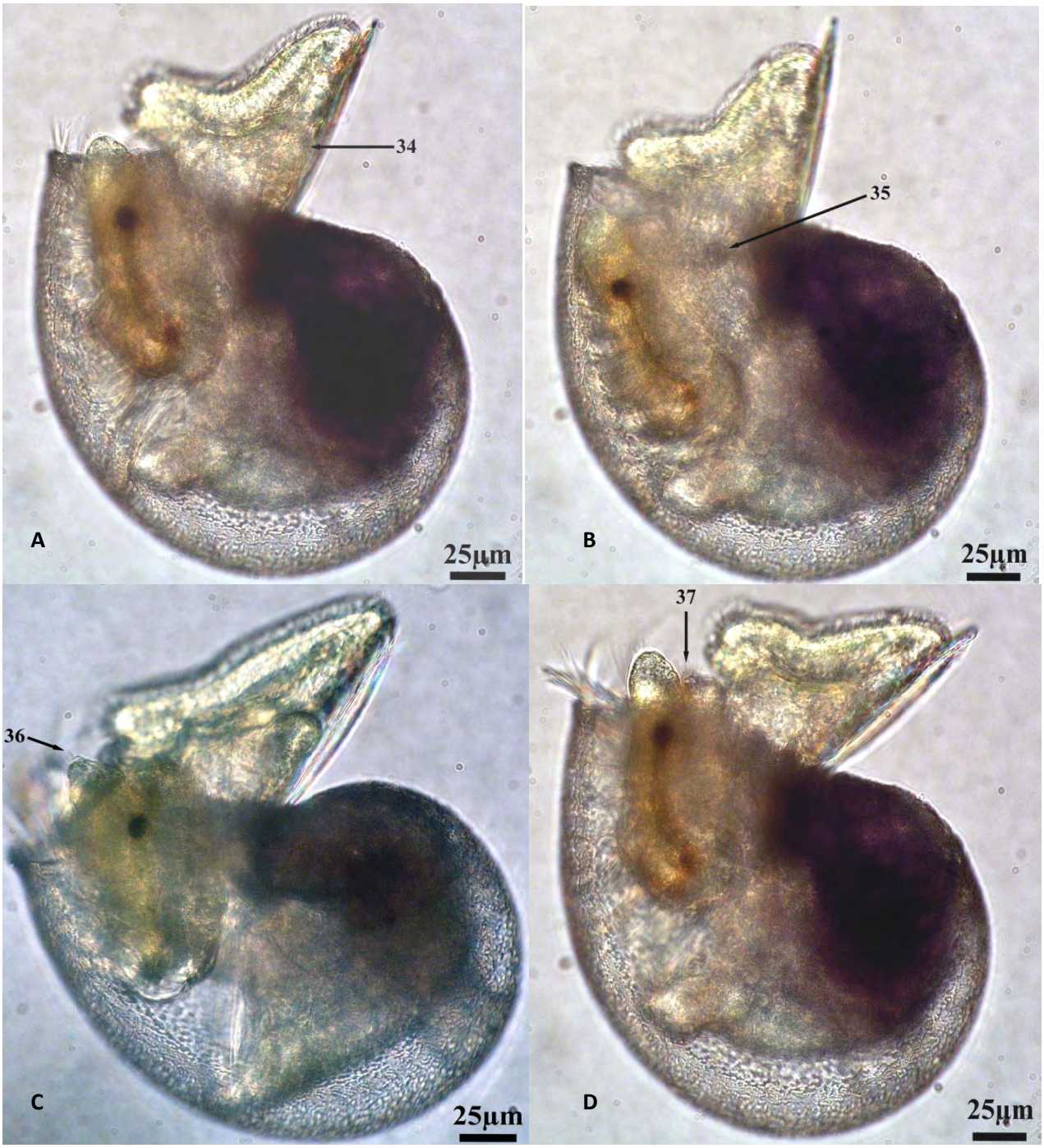

Figure 7. (A) Stage 34 Formation of epipodal tentacles. (B) Stage 35 Appearance of otolith. (C) Stage 36 Appearance of spines on cephalic tentacles. (D) Stage 37 Protrusion of snout underneath the velum.

From this point larvae started exploring the surface with their foot but did not stop their swimming behaviour considering they had not shed their velum, no suitable settlement substrate being present. The otolith appeared (stage 35), then short spines appeared on the cephalic tentacles (stage 36), followed by the snout protrusion from underneath the velum (stage 37) (Fig. 7 B to D).

Two tubules appeared on the cephalic tentacles (stage 38) followed by the apparition of a third tubule on the cephalic tentacles (stage 39) (Fig. 8 A&B).

Figure 8. (A) Stage 38 Appearance of two tubules on cephalic tentacles. (B) Stage 39 Third tubule appearance on cephalic tentacles.

Discussion

The time required by *H. tuberculata coccinea* to start spawning in this study is in agreement with those observed by Peña, (1986). Eggs and larvae colours reported among abalone species vary among species of *Haliotis* genus. For instance *H. rufescens*, *H. cracherodii*, *H walallensis*, *H. assimilis* and H. *kamtschatkana* have dark green eggs and green larvae, whereas *H. fulgens*, *H. sorenseni* and *H. corrugate* produce brown, beige and olive eggs, respectively, and larvae colour reflect those egg pigments (Hahn, 1989). Pigmentation of *H. tuberculata coccinea* eggs was dark violet as stated by Peña, (1986). However, the colours described by Peña, (1986) for trochophore and veliger larvae, violet-coloured foot, velum and cephalic tentacles and an orange-yellowish-coloured visceral mass, are opposite to the ones observed in the present study.

Size of fertilised abalone eggs also varies from species to species. Diameter of *H.*

tuberculata coccinea eggs observed in this study (205±8µm) was nearly the double of the one found by Peña, (1986) (102.8±0.82) for the same abalone species but similar to the one of *H. rubra* (200µm) (Harrison and Grant, 1971), *H. sorenseni* (200µm) (Leighton, 1972) and *H. tuberculata* (210µm) (Koike, 1978). The size of trochophore and veliger also varies depending on the abalone species. The length and width of *H.tuberculata coccinea* larvae, found varied from the ones reported in the literature for other species, and were also different from the ones reported by Peña, (1986) who observed 201x160µm trochophore larvae and 264x206µm larvae having completed their larval shell. These differences could be attributed to distinct broodstock feeding and conditioning temperatures as well as dissimilarities in broodstock size and age.

Previous studies on larval development of *H. discus hannai* (Seki and Kan-no, 1977), *H. sieboldii*, *H. discus* (Ino, 1952) and *H. diversicolor supertexta* (Oba, 1964) showed that there were no differences in the development from fertilisation to trochophore larvae, between these species. In this study we could confirm this statement, no differences in the development to the trochophore larvae having been observed in *H. tuberculata coccinea* comparing to the rest of the species. However from the trochophore larvae onward, differences were noticed.

Larval development of *H. tuberculata coccinea*, from trochophore stage to torsion differs to that of other species since the formation of the larval retractor muscle, the integumental attachment and the development of the foot mass occurred simultaneously in the present species. Moreover the apparition of the eye spot was observed before torsion, earlier in the development in comparison to all the other abalone species. However like in *H. discus hannai*, *H. sieboldii*, *H. discus* and *H. diversicolor supertexta*, the development of the operculum occurred in *H. tuberculata coccinea* after these stages.

Many differences were noted from torsion to metamorphosis. As in *H. discus hannai* and *H. asinina* (Sawatpeera, *et al.*, 2001), larval shell of *H. tuberculata coccinea* was completed before torsion occurred. Conversely in *H. sieboldii* Ino, (1952) found that torsion occurred before larval shell completion. In *H. tuberculata coccinea* similarly to *H. asinina*, cilia in the mantle cavity were seen after the formation of the first epipodal tentacle whereas the contrary was observed in *H. discus hannai* and the first epipodal tentacle could only be observed after metamorphosis in *H. gigantea*.

Otolith could be observed after the formation of the first epipodal tentacle contrary to the reverse order reported in *H. tuberculata*. Seki and Kan-no, (1977) found that snout protrusion and ciliary process on the roof of the mantle cavity happened before the formation

of the third tubule on the cephalic tentacles. In agreement with their finding this study confirm a similar order of appearance in *H. tuberculata coccinea* as opposed to Sawatpeera, et al., (2001) who found that they form after the apparition of the third tubule on the cephalic tentacles in *H. asinina*. In *H. tuberculata* ciliary process on the roof of the mantle cavity could only be observed after metamorphosis.

Finally the fact that development rate is only partially described for several abalone species summed to the fact that larval development were studied at different water temperature for different species did not allow the comparison of all reported morphological features between species. Nevertheless the comparison between specific developmental stages such as hatch out and torsion could provide a general tendency about time required for *H. tuberculata coccinea* development in comparison with other species. In that case the timing of embryonic and larval development stages of *H. tuberculata coccinea* observed at the reported temperature was more similar to the one of a tropical abalone species like *H. asinina* than to the one of a temperate one. Hahn, (1989) stating that temperature is an important factor in many stages of development such as gonad maturation, spawning and larval development could help explain the significant differences observed in eggs and larvae characteristics of *H. tuberculata coccinea* between this study and the one of Peña, (1986). Studies of *H. tuberculata coccinea* embryology and larval development at different water temperatures would allow to clarify the importance of this parameter and its consequences on larval production and rearing potential of this sub-tropical species in comparison with the ones of other abalones species from other regions.

Acknowledgments

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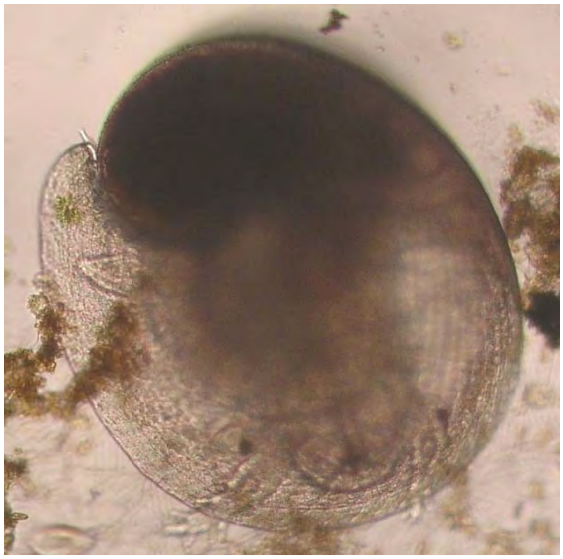
5. STUDY II: LARVAL SETTLEMENT OF *HALIOTIS TUBERCULATA COCCINEA* IN RESPONSE TO DIFFERENT INDUCTIVE CUES AND THE EFFECT OF LARVAL DENSITY ON SETTLEMENT, EARLY GROWTH AND SURVIVAL



Larval settlement of *Haliotis tuberculata coccinea* in response to different inductive cues and the effect of larval density on settlement, early growth and survival

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Abstract

Settlement and metamorphosis of *Haliotis tuberculata coccinea* larvae were examined in the presence of different settlement cues reported effective in larval settlement of other abalone species (crustose coralline algae (CCA), *Ulvella lens*, conspecific mucus, γ-aminobutyric acid (GABA), and four benthic diatom species). In addition, larval density effect was tested on crustose coralline algae substrate. Larval settlement was highest on CCA followed by *U. lens.* Settlement was very low on conspecific mucus and γ-aminobutyric acid, varying between 1% and 2%. *U. lens* inoculated with the diatom *Navicula incerta* induced a reduced settlement of 9% compared to the 22% obtained on films of *U. lens* alone. The settlement induction efficiency of all the benthic diatoms tested (*Amphora* sp., *N. incerta*, *Proschkinia* sp. and *Nitzschia* sp.) was very low and not significantly different among diatom species. Larval settlement at 48h post-addition and survival after one month were higher for the lower larval density, whereas post-larval growth rates were not influenced by larval density.

Key words: Abalone larvae, crustose coralline algae, *Ulvella lens*, diatoms, GABA, mucus, settlement, early growth, survival, *Haliotis tuberculata coccinea*

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Introduction

The induction of larval settlement is a critical stage in abalone seed production. Abalone larvae require specific cues for attachment and stimulation of metamorphosis (Morse & Morse, 1984; Roberts 2001). The absence of such cues leads to low settlement and survival rates in early post larvae (Searcy-Bernal et al., 1992; Slattery, 1992; Roberts et al., 1999; Daume et al., 1999; Takami et al., 2002).

Substances that have been reported to induce settlement in abalone larvae include: crustose coralline algae (CCA), benthic diatom biofilms, spores of the green algae *Ulvella lens*, chemicals like GABA (gamma-amino butyric acid), abalone mucus trails, or bacterial films (Morse & Morse, 1984; Searcy-Bernal et al., 1992; Slattery, 1992; Seki, 1997; Takami et al., 1997; Bryan & Qian, 1998; Roberts, 2001; Gordon et al., 2004; Daume, 2006). Published literature have documented many contradictions and wide variation in abalone settlement rates, on the settlement cues previously cited, depending on the abalone species or the methodology selected for each study.

On one hand benthic diatom biofilms have been traditionally used in abalone hatcheries worldwide to induce larval settlement, but its effectiveness is unpredictable and larval settlement rates are frequently low (1% to 10% of larvae) (Daume, 2000). On the other hand, abalone hatcheries in Japan successfully settle abalone larvae (*Haliotis discus hannai*) on the green algae *U. lens* (Takahashi & Koganezawa 1988) and its use has been lately spread to other countries.

Besides, abalone settlement rates and early growth and survival have been recently suggested to be markedly affected by larval and post-larval density (Daume et al. 2004).

Since *Haliotis tuberculata coccinea* Reeve, 1846, is a new candidate species for aquaculture, studies related to that species are scarce and have focused on spawning techniques (Peña, 1986; Viera et al., 2003), larval development (Courtois de Viçose et al., 2007), ecology (Pérez & Moreno, 1991) as well as culture techniques and nutrition (Toledo et al., 2000; Viera et al., 2005). To date, there is no information available neither about the settlement process of its larvae, nor on the adequate settlement cues and larval densities and their effect on post-larval growth and survival

Hence, a better understanding of *H. tuberculata coccinea* settlement processes and the identification of a reliable approach for efficient recruitment and early growth, are therefore of great importance for furthering the development of culture techniques and the production

of this species; both arousing public interest considering they would contribute to the recovery of wild *H. tuberculata coccinea* populations

The present study investigates the settlement induction efficiency of substrates, reported to induce settlement in other abalone species, on *H. tuberculata coccinea* larvae to identify suitable settlement substrates for this abalone species. The objectives of the present study were to:

1) Evaluate the effectiveness of GABA, abalone mucus, CCA, *U. lens*, *N. incerta*, and its combination with *U. lens*, on larval settlement of *H. tuberculata coccinea*

2) Assess the efficiency of four strains of diatoms for larval settlement of H. *tuberculata coccinea*

This study also examines whether larval density influences overall settlement rate and later post-larval performance.

Material and methods

Experimental protocol

Captive Haliotis tuberculata coccinea broodstock were kept in 60-l tanks with a flow through system at the Instituto Canario de Ciencias Marinas (Canary Islands, Spain). Ripe *H. tuberculata coccinea*, were induced to spawn (male to female ratio 1:2) by the hydrogen peroxide technique (Morse et al., 1977) and all the larval experiments were run at the Instituto Canario de Ciencias Marinas, (Canary Islands, Spain) in 2008. Larvae used in each experiment were from the same batch and obtained from fertilised gametes from various males and females.

They were judged to be competent for settlement when the third tubule of the cephalic tentacle could be observed (Hahn, 1989; Courtois de Viçose et al., 2007).

Settlement plates consisted of 50 cm^2 plastic squares colonised by settlement cues to be tested for experimental plates, while control plates were not colonised. Each replicate consisted of four settlement plates placed vertically in 12 L containers filled with 1µm filtered seawater, supplied with low aeration. After 24 h of introduction of the larvae flow was initiated at a rate of 1% exchange rate per hour and was increased up to 20% exchange rate per hour after 72h, seawater outlet being fitted with 125-µm mesh screens to prevent the loss of larvae. Seawater temperature in the rearing containers was 19±0.5 ºC, with an artificial

photoperiod 12:12 hours L: D provided at a light intensity of 2000 Lux measured using a Digital Light Meter, (HT170N, HT ITALIA, Italy).

Settled larvae were counted, on every settlement plate of each replicate (∑ 12/treatment), under a dissecting microscope 48 hours after the larvae were added. Plates were kept immersed at all times during observation. Larvae were considered as settled once permanently attached to the substrate after shedding the velum to complete metamorphosis.

Algal cultures

Four species of diatoms *N. incerta*, *Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp. were grown in f/2 medium (Guillard, 1975) plus silicate, at ambient temperature and under continuous light of 62 ± 8 μmol photon m^{-2} s^{-1}. Photon flux density (irradiance) was measured using a Digital light meter (HT170N, HT ITALIA, Italy). The cultures were not axenic.

In each diatom treatment, individual diatom species were cultured with initial inoculums of 10^5 cells ml^{-1} for 5 days and allowed to attach to settlement plates until they reached a density of approximately 10^5 cells cm^{-2}.

In CCA treatment, settlement plates were left to be colonised by CCA in nursery tanks until reaching approximately 57% percent cover.

10-day-old *U. lens* germlings, that colonised the settlement plates with 27% percentage cover, were obtained from mature (spore producing) *U. lens* according to the methods for spore collection of Takahshi & Koganezawa, (1988).

In order to colonise the plates with *N. incerta* in combination with *U. lens*, settlement plates with germlings of *U. lens* were placed in *N. incerta* culture and grown for 5 days such that they were inoculated with the diatom at a density of approximately 10^5 cells cm^{-2}.

Larval settlement

Four different experiments were conducted in order to test, respectively, GABA, abalone mucus, CCA, *U. lens*, the combination of *U. lens* and *N. incerta*, four different diatoms and, finally, two larval densities on larval settlement of *H. tuberculata coccinea*. Algal colonisation of experimental plates is described above and regarding conspecific mucus treatment, specimens of H. *tuberculata coccinea* (3-4 cm shell length) were allowed to crawl on the settlement plates for 24h before the introduction of the larvae.

In the first experiment the larval response to 1 μM GABA (Morse et al., 1979), conspecific mucus, *U. lens* and CCA, was tested. Each type of substrate was tested in triplicate at a larval density of 2000 larvae 12 L^{-1} container, representing 10 larvae cm^{-2} of substrate.

The following treatments were tested in the second experiment: a negative bare control, U. *lens*, diatom film of *N. incerta, U. lens* in combination with *N. incerta* and as a positive control the CCA. Each type of substrate was tested in triplicate with a larval density of 2000 larvae 12 L^{-1} containers representing 10 larvae cm^{-2} of substrate.

In the third experiment settlement plates colonised by CCA were tested against settlement plates colonised by monospecific diatom films of *N. incerta*, *Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp. Each type of substrate was tested in triplicate at a larval density of 2000 larvae 12 l^{-1}, representing 10 larvae cm^{-2} of substrate.

In the fourth experiment two larval densities were tested: 2000 and 200 larvae 12 L^{-1} representing 10 larvae and 1 larva cm^{-2} of CCA substrate, respectively.

Post larval growth and survival

Post-larvae from the fourth larval settlement experiment were weekly fed with 200ml of mix diatoms inoculum (10^6 cells ml^{-1}) cultured as described above, their growth and survival being followed during 4 weeks after settlement. The number of live post-larvae was counted, on every settlement plate of each replicate ($\sum$ 12/treatment), under a dissecting microscope at weekly intervals. The shell length of 10 randomly selected post-larvae per treatment was measured weekly with a profile and measuring projector MITUTOYO model PJ-H3000 (Japan). Plates were kept immersed at all times and replaced immediately after observation and measurement.

Daily growth rate (DGR) was calculated according to the formula: Lf-Li / t, where Lf=final shell length (μm), Li=initial shell length (μm) and t =time in days.

Estimates of cell density and percentage cover of algae

Ten randomly chosen fields of view of the settlement plates were photographed at a magnification of 400 x. Number of diatoms cells cm^{-2} as well as percentage cover of CCA and *U. lens* were calculated by processing the images with Image J Software (National Institutes of Health, USA, Image J 1.42q)

Data analysis

Statistical analysis was performed using the Statgraphics Plus 5.1. Software. Analyses of variance (one-way ANOVAs) were performed for settlement rate, survival rate and daily growth rate (DGR). Data showing significant differences (P<0.05) were analyzed by paired comparisons using Tukey's HSD test. Equality of variance was assessed with Bartlett's test. When data were not normally distributed, a non-parametric one-way ANOVA on Kruskal–Wallis ranks was tested.

Results

Larval settlement

The number of settled larvae was significantly higher on the CCA than on *U. lens* ($F_{4,10}$=142.97, P<0.0001) and both treatments induced a significantly higher settlement (P<0.05) of larvae than mucus film, GABA and the control (Fig. 1). After 48h, a settlement rate of 31% was achieved on CCA, followed by a settlement rate of 22% on *U. lens* and less than 2% on mucus film, GABA and the control. There was no significant difference between the mucus film, the GABA treatment and the control (P>0.05).

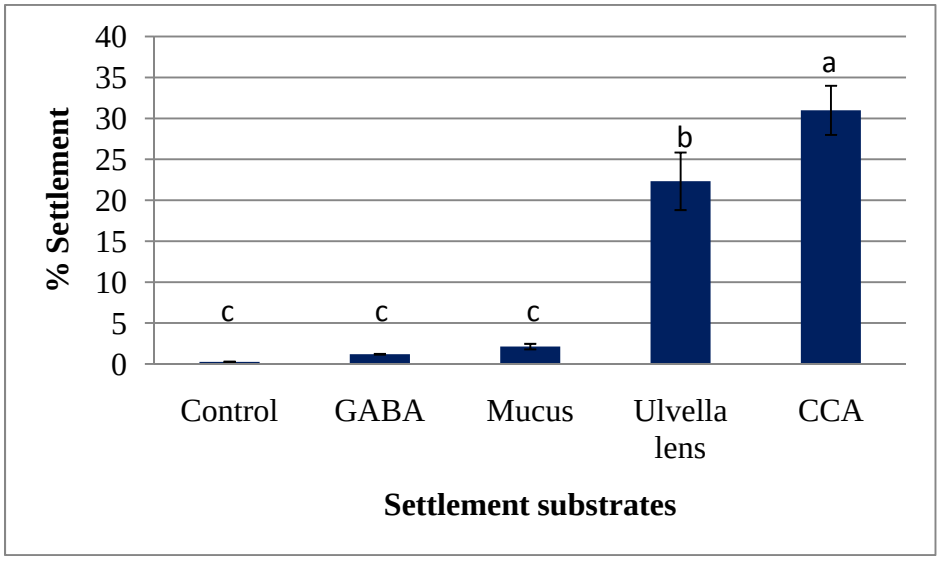

Figure. 1. Percentage settlement of *H. tuberculata coccinea* after 48h on crustose coralline algae (CCA), *Ulvella lens*, conspecific mucus and induce by GABA. Vertical bars indicate the standard deviation. Values with different letters are significantly different (P <0.05) (n=3)

A similar result was obtained when larval settlement was compared between CCA, *U. lens*, *U. lens* combined with *N. incerta* and a film of *N. incerta*. A significantly higher number of larvae settled on CCA ($F_{4,10}$ =29.57, P<0.0001) compared to *U. lens* and both treatments induced a significantly higher settlement (P<0.05) of larvae than *U. lens* inoculated with *N. incerta*, a film of *N. incerta* and the control (Fig. 2). Settlement was significantly higher (P=0.000) on plates with *U. lens* combined with *N. incerta* than on film of *N. incerta* alone, and the control. Settlement rates of 34% on CCA, 22% on *U .lens*, 10% on *U .lens* inoculated with the diatom *N. incerta*, and 1% on a film of *N. incerta* were determined after 48h.

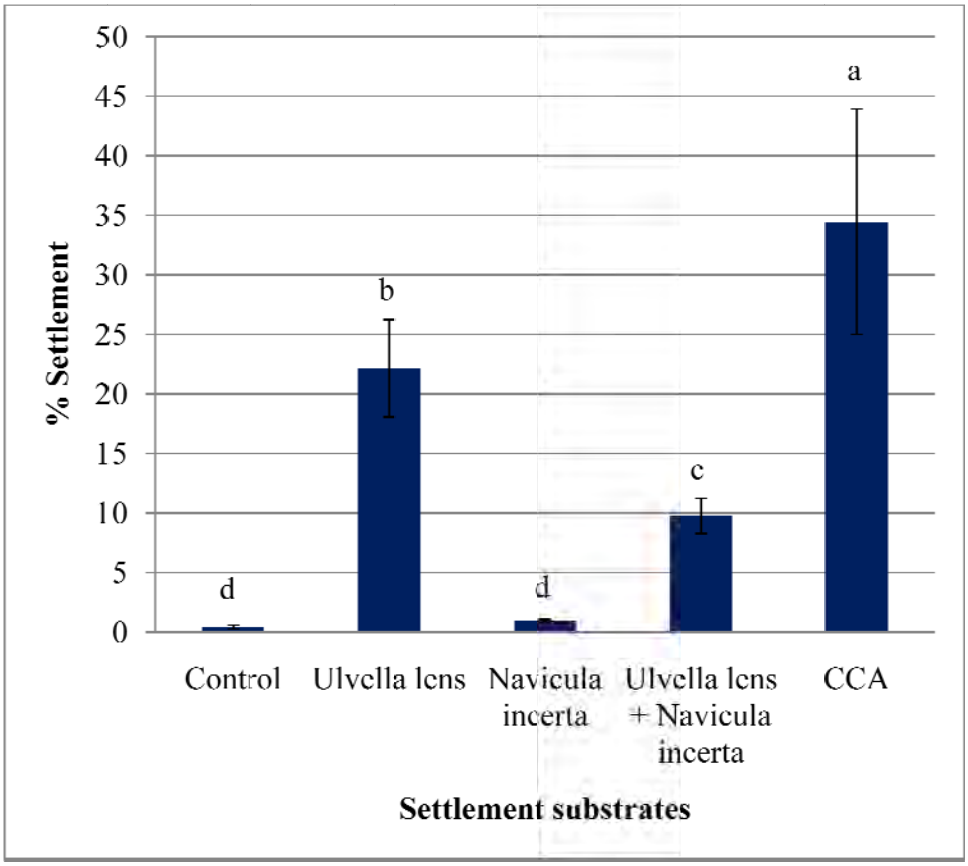

Figure. 2. Percentage settlement of *H. tuberculata coccinea* after 48h on *Ulvella lens*, the diatom *Navicula incerta*, *Ulvella lens* combined with *Navicula incerta* and crustose coralline algae (CCA). Vertical bars indicate the standard deviation. Values with different letters are significantly different (P <0.05) (n=3)

In the third experiment, significantly more larvae settled on CCA than on films of *N. incerta*, *Proschkinia* sp., *Nitzschia* sp., or *Amphora* sp. ($F_{5,12}$=215.71, P<0.0001). Indeed, a settlement rate of 30% was calculated on CCA after 48h whereas settlement on the monospecific diatom films remained lower than 1%. There was no significant difference, in the settlement rates, between the monospecific diatom films and the control (P>0.05) (Fig. 3).

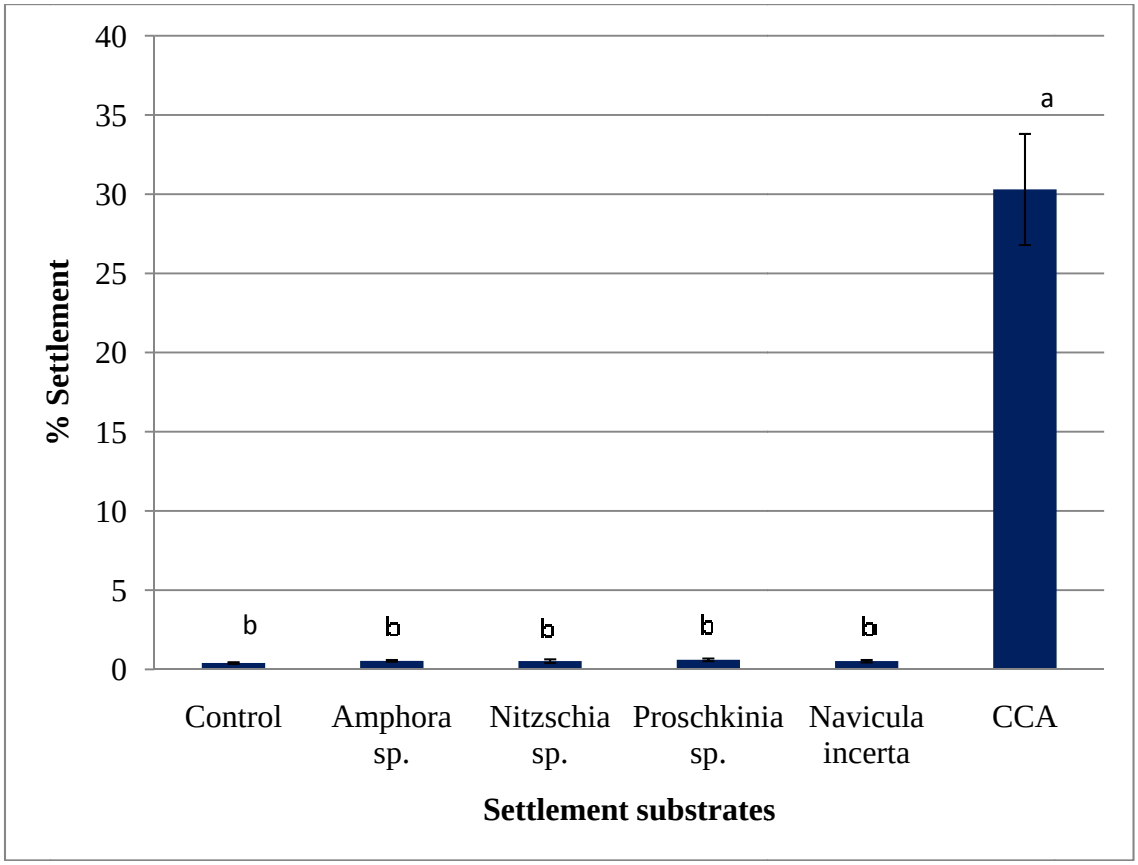

Figure 3. Percentage settlement of *H. tuberculata coccinea* after 48h on monospecific diatom films and crustose coralline algae (CCA). Vertical bars indicate the standard deviation. Values with different letters are significantly different (P <0.05) (n=3)

Settlement of *H. tuberculata coccinea* larvae was significantly higher on the low density treatment when induced to settle on CCA with 57% average percentage cover ($F_{1,4}$=17.61, *P*=0.013) (Fig. 4). Thus after 48h 49% of larvae settled in the low density treatment whereas only 30% of larvae settled in the high density treatment.

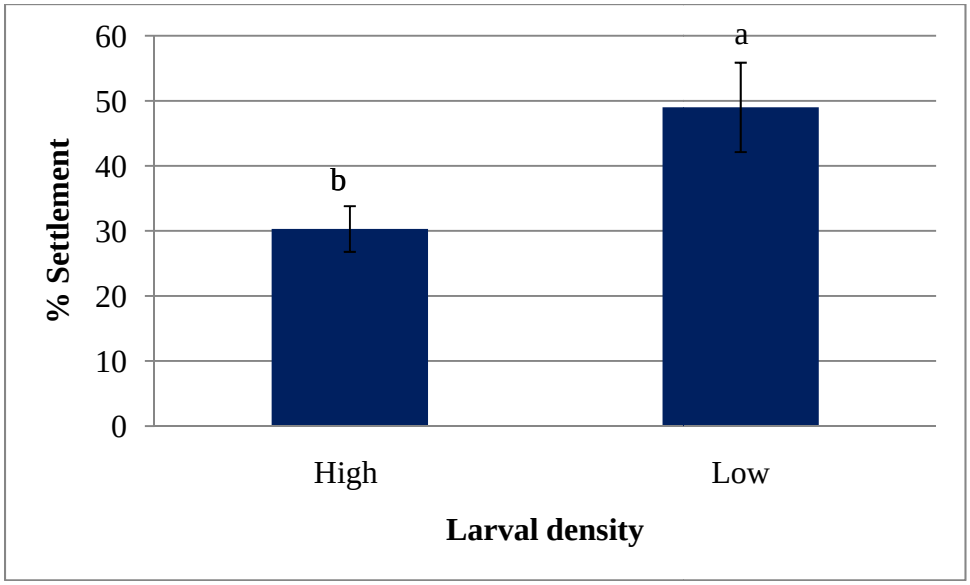

Figure 4. Percentage settlement of *H. tuberculata coccinea* after 48h on crustose coralline algae (CCA) at high and low larval density. Vertical bars indicate the standard deviation. Values with different letters are significantly different (P <0.05) (n=3)

Post larval growth and survival

There was no significant difference in survival ($F_{1,4}$=2.75, *P*=0.17) between high and low larval density treatments on CCA, although post-larval survival was slightly higher at the lower larval density with 73.94± 3.42 % survival 4 weeks after settlement (Table 1) (Fig.5)

Table 1. Mean daily growth and survival rates of *H. tuberculata coccinea* postlarvae on crustose coralline algae (CCA), at high and low larval density (n=3 and n=10)

Larval Density	Survival rate at week 4 (%±SE)	DGR at week 4 (μm day^{-1}, ± SE)
High	58.64 ± 8.58	19.58±1.67
Low	73.94± 3.42	23.04±4.47

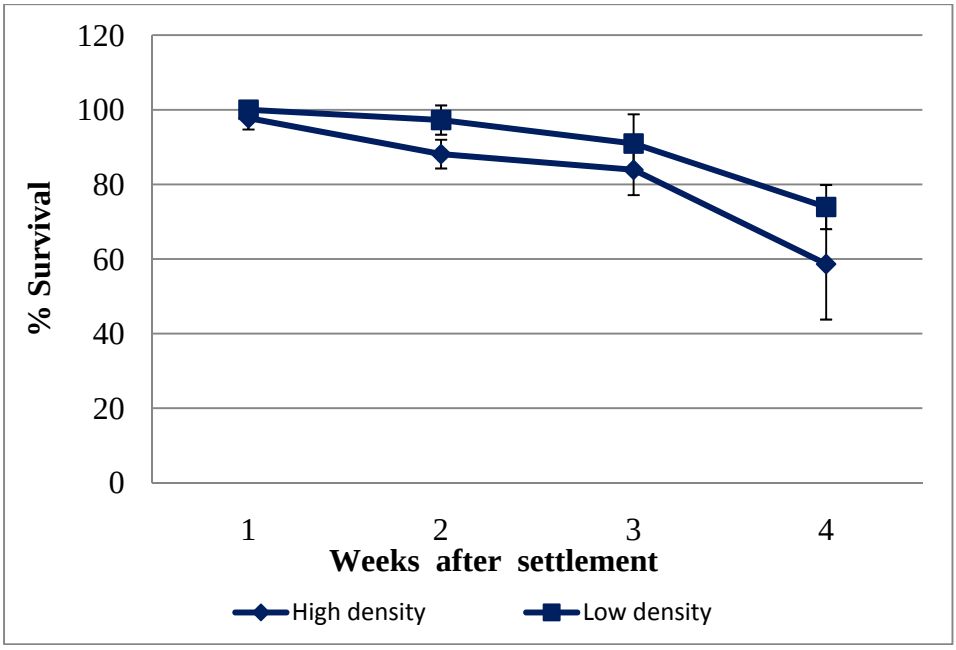

Figure 5. Survival of *H. tuberculata coccinea* postlarvae, during 4 weeks post settlement, at high and low larval density on crustose coralline algae. Vertical bars indicate the standard error (n=3)

After 4 weeks of experiment, post-larvae grown on CCA substrate, at low and high density, and fed weekly 200ml of 10^6 cells ml^{-1} diatom mix (*N. incerta*, *Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp.) reached an average of 891µm and 758µm in shell length, respectively (Fig. 6). When daily growth rates were analysed, there was no significant difference between the low and high density treatment during the 4 weeks following settlement (($F_{1,18}$=1.13, *P*=0.30) (Table 1).

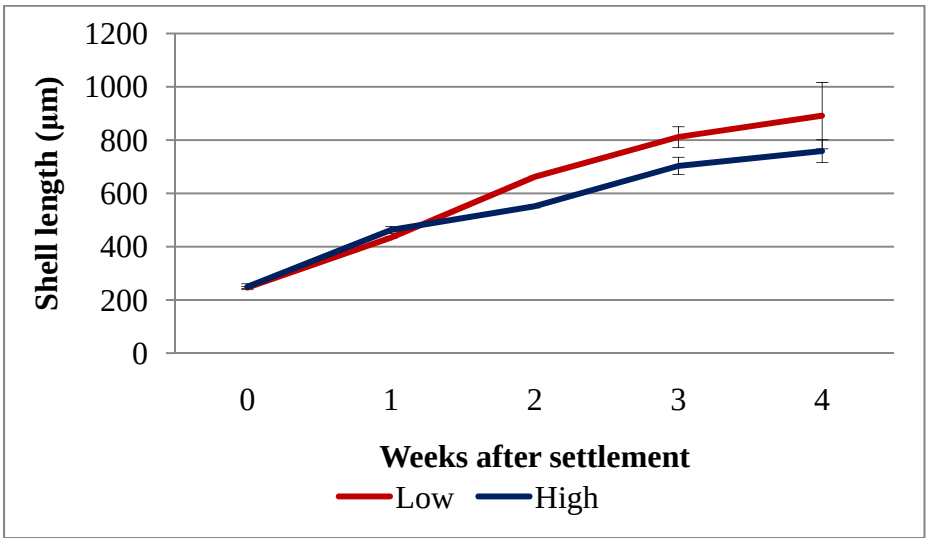

Figure 6. Early growth of *H. tuberculata coccinea* postlarvae, during 4 weeks post settlement, at high and low larval density on crustose coralline algae. Vertical bars indicate the standard error (n=10)

Discussion

The present study was performed to test, for the first time, the settlement induction efficiency of substrates, reported to induce settlement in other abalone species, on *H. tuberculata coccinea* larvae and determine their suitability for this abalone species.

The results obtained demonstrate that larval *H. tuberculata coccinea* will settle at a higher rate on CCA than all other substrates tested, confirming that CCA are among the best settlement inducing substrates (Roberts, 2001). The settlement rate on CCA (30-34%) is similar to that reported by Daume et al., (1999) for *H. rubra* larvae (25%).

Larvae of the same batch, and across experiments, have also settled well on *U. lens* these results being in concordance with the settlement rates obtained for *H. rubra*, on *U. lens*, by Daume et al., (2000) (20%). The settlement rates observed on *U. lens* that was inoculated with *N. incerta* (10%) are consistent with the results of Daume et al., (2000) (9%), lending support to the conclusion that diatom colonization on *U. lens* reduces settlement induction potential of this alga.

The settlement trials on conspecific mucus led to low settlement rates. Similarly, Bryan & Qian, (1998) also reported low settlement rates (12%) on conspecific mucus alone whereas they observed increased settlement rates (> 50%), as Slaterry, (1992) did, on a diatom plus conspecific mucus treatment. Hence, the absence of diatoms, in the conspecific mucus treatment of the present study, could probably explain the low settlement rates obtained.

The controversial efficiency of GABA as settlement inducer (Roberts, 2001) is reflected in this study given that the low settlement rates observed, with 1 μM GABA in this study, are consistent with the poor results from GABA reported by Slattery, (1992) but contradictory to the ones of Searcy-Bernal et al., (1992)

In all cases the experiments were conducted in the absence of antibiotics, at 1 μM GABA concentration and with settlement plates previously colonised by diatoms. According to Searcy-Bernal et al., (1998) these last two factors reduce GABA's efficiency as an inducer of larval settlement. Besides, GABA's efficiency could be species dependent as reported by Roberts & Nicholson, (1997) for *H. iris* and *H. virginea*.

Not many diatoms are known to be consistently suitable for abalone larval recruitment (Roberts, 2001). The low settlement observed on monospecific diatom films across experiments, a system traditionally used in abalone hatcheries, is consistent with the one reported by Daume et al., (1999) for *H. rubra* and is unlikely to be related to diatom

abundance, as densities of 10^5 cell cm^{-2} were reached. More likely explanations involve the age, or growth phase, of the diatom biofilm, as cells were only allowed to grow for 5 days to reach this density. On the contrary, Kawamura & Kikuchi, (1992) obtained a successful settlement of *H. discus hannai* post-larvae with *N. ramosissima*. Nevertheless, other types of diatom could be successful for the settlement requirements of *H. tuberculata coccinea*.

In the present study, survival was not significantly higher at the low larval density but was higher than that obtained by Gordon et al., (2006) using much higher larval densities on mixes of diatoms. The effect of larval density on settlement was tested on CCA substrate and this experiment also showed significantly higher settlement for the low density treatment. These results are bolstered by those of Daume et al., (2004) who showed higher settlement of *H. rubra* at low larval density on *U. lens*.

Growth rates obtained in the present study were within the range reported in earlier studies for other abalone post-larvae conducted by Takami et al., (1997) and Gordon et al., (2006), but lower than the ones obtained by Strain et al., (2006) and Viana et al., (2007). These differences could be explained considering that the settlement substrates, diatom species as well as their densities, and finally the larval densities tested were varying between all these experiments. Moreover, the sloughing of surface layers observed for most crustose species (Giraud & Cabioch, 1976) could be contributing to the reduction of attached diatom cells on the CCA substrate and might therefore affect growth rates of post-larvae, given that corallines present a low food value for abalone post-larvae (Roberts, 2001).

This study demonstrates that larvae of *H. tuberculata coccinea* will settle at a high rate on CCA and suggests that *U. lens*, which is easier to manage than CCA, can be used as an efficient and consistent settlement substrate for *H. tuberculata coccinea* larvae. On the contrary, none of the diatom species tested were successful in the induction of *H. tuberculata coccinea* larval settlement. In addition this study shows that larval density has an effect on settlement rates and post-larval survival of *H. tuberculata coccinea*.

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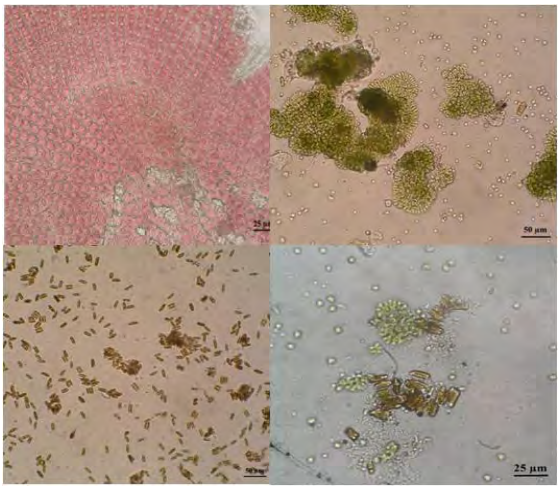
6. STUDY III: LARVAL SETTLEMENT, EARLY GROWTH AND SURVIVAL OF *HALIOTIS TUBERCULATA COCCINEA* USING SEVERAL ALGAL CUES



Larval settlement, early growth and survival of *Haliotis tuberculata coccinea* using several algal cues

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Abstract

Settlement and metamorphosis of *Haliotis tuberculata coccinea* larvae were examined in the presence of different algal species: crustose coralline algae (CCA), *Ulvella lens* and *Ulva rigida*. The age, enrichment level and combination of algal species were tested as settlement cues. Larval settlement was the highest on CCA (61%) and on a mix of 45 day-old *U. lens* and *U. rigida* (52%) and followed by 45 day-old *U. rigida* (46%). Settlement was the lowest (about 3%) on a mix of 4 day-old *U. lens and U. rigida* and 45 day-old enriched or non-enriched *U. lens.* Within all treatments post-larvae were fed during four weeks with a mix of diatoms (*Amphora* sp., *Proschkinia* sp., *Nitzschia* sp and *Navicula incerta*). The best post-larval growth was obtained on the 45 day-old mix of *U. lens* and *U. rigida.* This substrate was also the best of the green macroalgae germlings substrates tested for settlement induction and provided good survival rates. The substrates biochemical's composition showed that protein content had a significant effect on settlement and survival. The algal cues were differentiated using their fatty acids composition. 18:1n-7, 18:2n-6, 16:4n-3, arachidonic acid (ARA), and eicosapentaenoic acid (EPA), were suggested to play important roles for settlement and survival. Different fatty acids were correlated with settlement and survival rates, but only EPA levels were correlated with both. The results of this study denote the high value of *U. rigida* for settlement and growth of *H. tuberculata,* as well as the influence of substrate age on settlement success.

Keywords: Abalone larvae, crustose coralline algae, *Ulvella lens*, *Ulva rigida*, settlement, early growth, survival, biochemical composition, fatty acid composition, *Haliotis tuberculata coccinea*

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Introduction:

Knowledge relevant to settlement cues is critical for abalone culture (Roberts, 2001), since adequacy of the selected cues will directly determine settlement rates and early post larval survival (Daume et al., 1999a; Roberts, 2001; Searcy-Bernal et al., 1992; Slattery, 1992; Takami et al., 2002). Cues from a wide range of sources are reported to induce settlement of *Haliotis* larvae, these include crustose coralline algae (CCA) (Daume et al., 1999b; Daume et al., 2000; Morse & Morse, 1984; Morse et al., 1980c; Moss & Tong, 1992a; Roberts et al., 2004; Takami et al., 1997a), films of benthic diatoms (Daume et al., 1999b; Kawamura & Kikuchi, 1992; Roberts et al., 2007; Seki & Kan-No, 1981b), abalone mucus trails (Bryan & Qian, 1998; Searcy-Bernal et al., 1992; Seki, 1997; Seki & Taniguchi, 1996; Slattery, 1992), bacterial films (Bryan & Qian, 1998; Roberts, 2001) or several purified chemicals like gamma-amino butyric acid (GABA) (Bryan & Qian, 1998; Morse & Morse, 1984; Morse, 1985; Morse et al., 1980c; Searcy-Bernal et al., 1992; Trapido-Rosenthal & Morse, 1986).

Despite the established use of benthic biofilms, consisting of bacteria and mixed diatom species, in abalone hatcheries worldwide (Daume, 2006), larval settlement rates on such substrates can be very low (1% to 10% of larvae) (Daume et al., 2000). Besides, the germlings of the green alga *Ulvella lens* have been successfully used to induce abalone settlement (Daume et al., 2004; Daume et al., 2000; Daume & Ryan, 2004a; Takahashi & Koganezawa, 1988). Daume (2006) raises the hypothesis that age and biochemical characteristics of *U. lens* influences abalone larval settlement based on the clearer preference of *Haliotis rubra* and *Haliotis laevigata* larvae for older rather than for younger *U. lens*. However this hypothesis has not been tested on other abalone species.

Other green macroalgae have been also studied for their potential for settlement induction and post-larval nutrition. For instance, Seki (1997) reported that the foliose green algae *Ulva* sp. induced metamorphosis of *Haliotis discus hannai* and (Strain et al., 2006) suggested that germlings of *Ulva* sp. could provide a suitable food source for abalone juveniles. *H. rubra* larvae showed high settlement response to the green algae *Ulva australis* and *Ulva compressa* (Huggett et al., 2005). However, to the best of the authors' knowledge, the use of germlings of *Ulva spp.* or macroalgae other than *U. lens* as abalone settlement inducers has not been investigated until now.

Since each abalone species responds differently to settlement cues (Daume et al., 1999b), in previous studies a range of cues were tested for *Haliotis tuberculata coccinea* and

showed that *U. lens* induced higher settlement rates than diatom biofilms (Courtois de Viçose et al., 2010).

In order to improve economical efficiency of *Haliotis tuberculata* culture techniques and production, and more specifically of *H. tuberculata coccinea,* further research was needed to improve settlement induction and post-larval growth. The present study investigates the effects of age as well as proximate biochemical and fatty acid composition of *U. lens, Ulva rigida* germlings, and crustose coralline algae, on larval settlement, and post-larval survival and growth of *H. tuberculata coccinea*.

Material and methods

Algal cultures

Four and forty five-days-old *U. lens* and *U. rigida* germlings were obtained from mature (spore producing) *U. lens* and *U. rigida* thalli. Plates with mature *U. lens* were left in the dark during 2 weeks and *U. rigida* thalli were subjected to 7 days of cold treatment (4ºC) prior to starting the conditioning of the experimental plates. Both were then placed in tanks, holding the experimental plates, maintained with no water flow, low aeration and a complete f/2 mix. The methods for germlings production and their culture were adapted from Daume et al., (2004) and Strain et al., (2006). The cultures of old germlings (45 days old) from *U. lens*, *U. rigida* and the mix of both were maintained aerated under natural photoperiod during the 45 days and the f/2 culture medium was renewed weekly. Enriched, 45-days-old, *U. lens* germlings differentiate themselves from the old *U. lens* germlings by the fact that the f/2 mix was applied at two times the concentration during the entire period of culture. In CCA treatment, settlement plates were left to be colonised by CCA in nursery tanks until reaching 46.67±7.88 percent cover.

Four species of diatoms *N. incerta*, *Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp. were cultured with initial inoculums of 10^5 cells ml^{-1} and grown for 5 days in f/2 medium plus silicate (1 mgL^{-1}) (Guillard, 1975) at ambient temperature and under continuous light of 62 ± 8 µmol photon m^{-2} s^{-1}. Photon flux density (irradiance) was measured using a Digital light meter (HT170N, HT ITALIA, Italy). The cultures were not axenic. Diatom cell concentration was evaluated with a Neubauer hemocytometer. Prior to the counts ultrasound (1 or 3 min) was applied to the diatoms samples to avoid possible cell aggregations.

Estimates of algal cover

Ten randomly chosen fields of view of the settlement plates, from each treatment, were photographed at a magnification of 400 x at the time of larval settlement. Percentage cover of CCA, *U. lens* and *U. rigida* were then calculated by processing the images with Image J Software.

Larval rearing

Captive *Haliotis tuberculata coccinea* broodstock were kept in 60-l tanks placed in a flow through system at the Instituto Canario de Ciencias Marinas (Canary Islands, Spain). Ripe *H. tuberculata coccinea*, were induced to spawn (male to female ratio 1:2) by ultraviolet irradiated sea water (Kikuchi & Uki, 1974) and larvae were obtained from fertilized gametes from various males and females. The study was conducted at the Instituto Canario de Ciencias Marinas, (Canary Islands, Spain) in 2009 using larvae from one single mixed batch obtained from gametes of 12 males and 14 females. Competent larvae for settlement were obtained when the third tubule of the cephalic tentacle could be observed (Courtois de Vicose et al., 2007; Hahn, 1989).

Larval settlement experimental protocol

Settlement plates consisted of 50 cm^2 plastic squares. Each replicate consisted of four settlement plates placed vertically in 12 L containers filled with 1μm filtered seawater, supplied with low aeration. 24 h after the introduction of the larvae, water was renewed a rate of 1% per hour and was increased up to 20% exchange rate per hour after 72h. Outlets were fitted with 125-μm mesh screens to prevent the loss of larvae. Seawater temperature in the rearing containers was 21±0.5 ºC, with an artificial photoperiod 12:12 hours L: D provided at a light intensity of 2000 Lux measured using a Digital Light Meter, (HT170N, HT ITALIA, Italy).

Settled larvae were counted, on every plates of each replicate ($\sum$ 12/treatment), under a dissecting microscope 48 hours after the larvae were introduced to the tanks. Plates were kept immersed at all times during observation. Larvae were considered as settled once permanently attached to the substrate after complete metamorphosis. The settlement study aimed at evaluating the effect of algal type, as well as its age and biochemical composition on *H. tuberculata coccinea* settlement rate. Eight treatments were tested: young (4 days old) and old

(45 days old) *U. lens*, enriched old *U. lens*, young (4 days old) and old (45 days old) *U. rigida*, the combination of young and old *U. lens* and *U. rigida* and finally CCA as the control treatment. Each type of substrate was tested in triplicate at a larval density of 200 larvae per 12 litre container, representing 1 larvae cm^{-2} of substrate. Number of larvae was estimated by counting them in three 3-ml subsamples drawn from the entire larval batch.

Post larval growth and survival

Post-larvae were fed weekly with 200ml inoculums of diatoms mixture (10^6 cells ml^{-1}), their growth and survival being followed during 4 weeks after settlement. The number of live post-larvae was counted, on every settlement plate of each replicate ($\sum$ 12/treatment), under a dissecting microscope at weekly intervals. The shell length of 10 randomly selected post-larvae per replicate was measured weekly with a profile and measuring projector MITUTOYO model PJ-H3000 (Japan). Plates were kept immersed at all times and replaced immediately after observation and measurement.

Daily growth rate (DGR) was calculated according to the formula:

$$\frac{Lf - Li}{t}$$

where Lf=final shell length (μm), Li=initial shell length (μm) and t =time in days.

Analytical methods

Triplicate samples of algae, from each treatment, were collected by scraping the surface of settlement plates at the start of the settlement experiments. The settlement plates used for the biochemical analysis of the substrates were colonized and cultured in the same conditions together with the ones used for the study of larval settlement rate. The samples were stored at -80ºC prior to be analyzed for total lipids, protein, carbohydrate, ash and total fatty acids. Before analysis all samples where homogenized with mortar and pestle before being weighted for further analysis. Total lipids were analyzed gravimetrically after extraction with chloroform-methanol (2:1) (Folch et al., 1957). Total protein was calculated from total Kjeldahl nitrogen according to AOAC (2005) standard methods. Ash content was determined gravimetrically after incinerating the samples at 600ºC during 24h. Fatty acids in the lipid extracts were transesterified to methyl esters (FAMEs) with 1% sulphuric acid: methanol complex (Christie, 1982). FAMEs samples were extracted into hexane and stored at -80ºC. Fatty acids were analyzed in a Thermo Finnigan- GC Focus gas chromatograph equipped with

a flame ionization detector (260ºC). FAMEs were separated with capillary column (Supercowax 28m x 0.32mm x 0.25 i.d.) using helium as the carrier gas under the conditions described by Izquierdo et al., (1989).

Data analysis

Statistical analysis was performed using the Statgraphics Plus 5.1. Software. Analyses of variance (one-way ANOVAs) were performed to compare proximate biochemical composition, as well as settlement, survival and daily growth rate (DGR) between treatments. Data showing significant differences (P<0.05) were analyzed by paired comparisons using Tukey's HSD test. Multiple regression analyses were carried out to explain the variation in settlement, growth and survival between treatments. The proximate biochemical composition of the algal cues; proteins, lipids, ash and carbohydrates contents, were the factors selected for the analyses. Principal component analysis was performed on the data of fatty acids composition of the algal cues and principal components were identified. Kendall rank correlation between the main principal components and settlement, growth and survival data was then performed in order to identify the effects of fatty acids composition determinant among treatments. Assumption of normality and homogeneity of variance were assessed with standardized skewness and kurtosis and Bartlett's test.

Results

Estimates of algal cover

The percentage cover of the different substrate, independently of age and algal species, ranged between 39.85±7.43 and 46.67±7.88, at the time of larval settlement. The differences in algal percent cover between treatments was not significant ($F_{7, 72}$=1.40, P=0.22).

Larval settlement

The number of settled larvae was significantly higher on CCA and the combination of old *U. lens* and *U. rigida* treatment ($F_{7, 16}$=207.51, P<0.001) (Fig. 1), while this last one did not induce significantly higher larval settlement than the old *U. rigida* treatment (P>0.05). Forty eight hours after the introduction of the larvae, an average settlement rate of 61±6% was achieved with CCA (Fig. 1), followed by settlement rates of 52±0.6 % and 46±7 % with the combination of old *U. lens* and *U. rigida* and old *U. rigida* treatments, respectively. Larval

settlement rates on young *U. lens* and young *U. rigida* reached 14±2 % and 10±0.6%, respectively, and were not significantly different (P>0.05). Both treatments induced a significantly lower larval settlement than CCA, the combination of old *U. lens* and *U. rigida* and old *U. rigida* treatments (P<0.01). Larval settlement rates were not significantly different between the combination of young *U. lens* and *U. rigida*, old *U. lens* and enriched old *U. lens* treatments (P>0.05) and were significantly lower than the ones obtained on the treatments previously cited ($F_{7,16}$=207.51, P<0.001) with values of 3±1.5%, 3±0.6% and 3±0.7% respectively (Fig. 1).

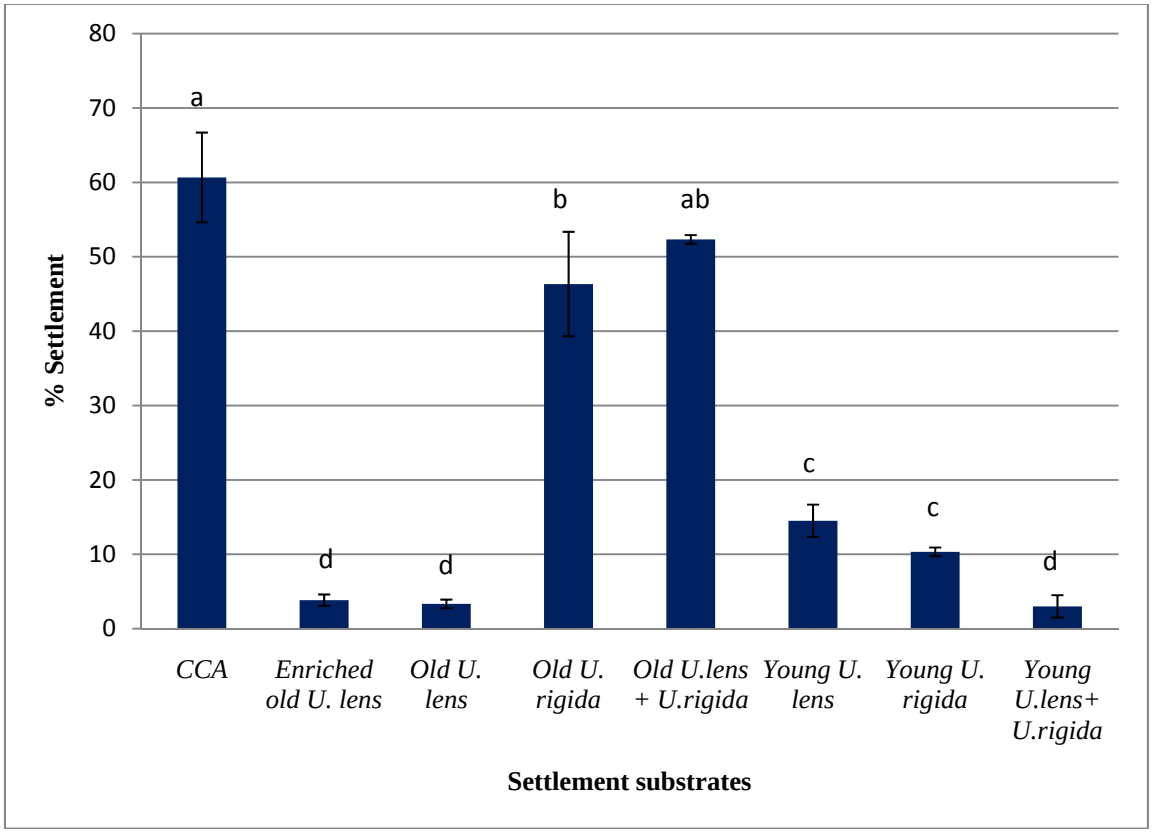

Figure 1. Percentage settlement of *H. tuberculata coccinea* after 48h on CCA, enriched old *U. lens*, old and young *U. lens*, old and young *U. rigida*, as well as old and young *U. lens* and *U. rigida*. Vertical bars indicate the standard deviation. Values with different letters are significantly different (P <0.05) (n=3).

Post larval growth and survival

After 4 weeks of experiment, the young and old combinations of *U. lens* and *U. rigida* produced the largest post-larvae with an average of 1144 µm and 1249 µm in shell length, respectively (Fig. 2).

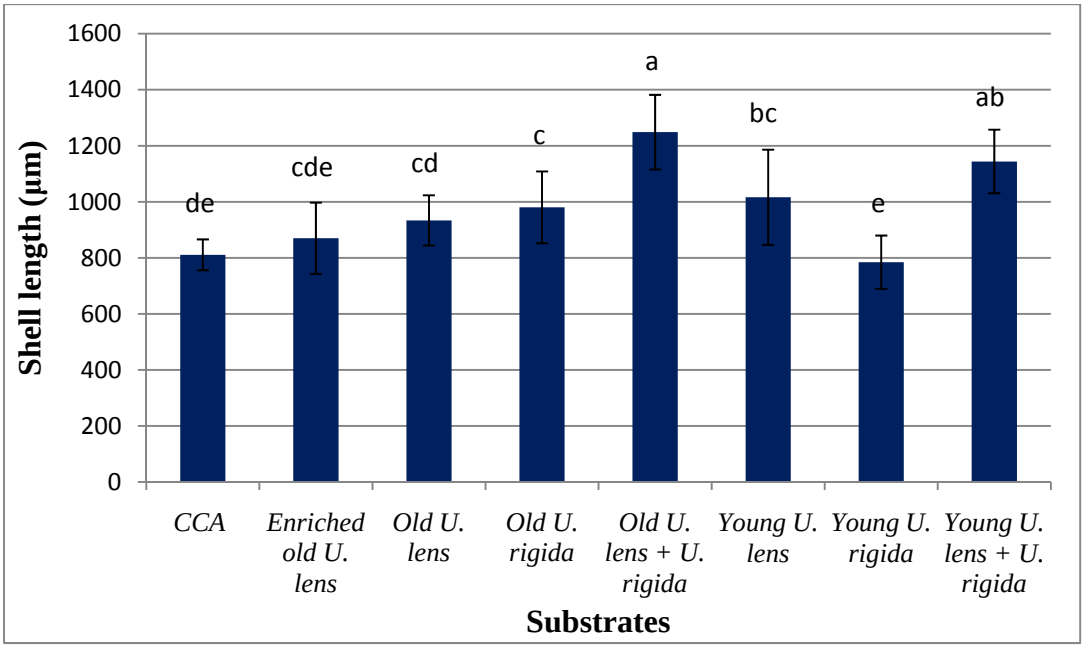

Figure 2. Early growth of *H. tuberculata coccinea* post-larvae, at 4 weeks post settlement, on the eight algal cues tested. Vertical bars indicate the standard deviation (n=10)

Daily growth rates of post-larvae fed weekly 200ml of 10^6 cells ml^{-1} diatom mix (*N. incerta*, *Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp.) during 4 weeks, were significantly higher on the young and old combinations of *U. lens* and *U. rigida* ($F_{7,72}$=22.77, P<0.001). Post-larvae exhibited the lowest DGR on young *U. lens* treatment with 23±4µm; this value being significantly lower than the ones observed on the young and old combinations of *U. lens* and *U. rigida* ($F_{7,72}$=22.77, P<0.001) (Table 1).

Table 1. Mean daily growth and survival rates of *H. tuberculata coccinea* post-larvae on the eight algal cues tested, (n=10 and n=3).

Treatment	Survival rate at week 4 (%±SD)	DGR at week 4 (µm day^{-1}, ± SD)
CCA	$63±0.8^{c}$	$25±4.3^{de}$
Enriched old *U. lens*	$41±3.5^{d}$	$27±3.7^{cde}$
Old *U. lens*	$35±2.4^{d}$	$30±3.7^{cd}$
Old *U. rigida*	$68±1.7^{bc}$	$32±5.6^{c}$
Old *U. lens* + *U. rigida*	$76±1.0^{b}$	$44±5.5^{a}$
Young *U. lens*	$70±0.7^{bc}$	$33±4.5^{bc}$
Young *U. rigida*	$36±3.3^{d}$	$23±4.4^{e}$
Young *U. lens* + *U. rigida*	$92±7^{a}$	$39±4.8^{ab}$

Different superscripts in each column indicate means which differ significantly at 95% level (ANOVA Tukey's test; P<0.05)

Post-larval survival, 4 weeks after settlement, was significantly higher ($F_{7, 16}$=127.16, P<0.001) on young combination of *U. lens* and *U. rigida*, with 92±7%. In contrast, survival rates were significantly lower ($F_{7, 16}$=127.16, P<0.001) on old *U. lens*, young *U. rigida* and enriched old *U. lens* but not significantly different between the three substrates (P>0.05) (Table 1).

Biochemical composition of algae and outcome on settlement, survival and growth

The proximate biochemical composition of the different settlement cues is shown in Table 2. Overall, CCA significantly showed the lowest protein ($F_{7, 16}$=360.3, P<0.001) and lipid contents ($F_{7, 16}$=146.87, P<0.001), whereas enriched old *U. lens* had the highest protein content ($F_{7, 16}$=360.3, P<0.001), as well as the highest lipid content together with old *U. lens.* Accordingly, CCA had the highest ash content whereas enriched and unenriched old *U. lens* had the lowest ash levels ($F_{7,16}$=670.83, P<0.001). The highest carbohydrate content was obtained in the combined old *U. lens* and *U. rigida* treatment, without significant differences from old *U. lens or* old *U. rigida* as opposed to the enriched old *U. lens* treatment showing the significantly lowest carbohydrate content ($F_{7,16}$=227.91, P<0.001).

Table 2: Proximate chemical analysis (% dry weight) of the eight settlement substrates tested at the starting date of the settlement experiments (mean±SD, n=3)

Substrates	**% Proteins (DW)**	**% Lipids (DW)**	**% Ash (DW)**	**% Carbohydrates (DW)**
CCA	4.01±0.27^{e}	0.30±0.06^{e}	54.40±0.86^{a}	41.29±0.73^{c}
Enriched old *U. lens*	29.14±0.92^{a}	7.71±0.19^{a}	32.14±0.16^{f}	31.00±1.14^{e}
Old *U. lens*	14.97±0.34^{c}	7.03±0.67^{a}	32.85±0.25^{f}	45.14±0.30^{ab}
Old *U. rigida*	16.33±0.76^{c}	4.35±0.32^{c}	34.62±0.41^{e}	44.70±1.17^{ab}
Old *U. lens* + *U. rigida*	11±0.63^{d}	5.79±0.64^{b}	36.02±0.42^{e}	47.18±1.03^{a}
Young *U. lens*	10.70±0.45^{d}	1.82±0.28^{d}	43.43±0.45^{d}	44.04±1.18^{b}
Young *U. rigida*	26.32±0.92^{b}	1.65±0.15^{d}	48.53±0.25^{c}	23.48±0.92^{f}
Young *U. lens* + *U. rigida*	10.17±1.32^{d}	1.56±0.44^{d}	51.23±1.17^{b}	37.04±0.73^{d}

Different superscripts in each column indicate means which differ significantly from others at 95% level (ANOVA Tukey's test; P<0.05).

Table 3: Fatty acid composition (% of total fatty acid) in the eight settlement substrates tested at the starting date of the settlement experiments.

Fatty acids		CCA	Enriched old *U. lens*	Old *U. lens*	Old *U. rigida*	Old *U. lens + U. rigida*	Young *U. lens*	Young *U. rigida*	Young *U. lens+ U. rigida*
	14:0	0.88	0.96	2.42	0.92	2.24	3.50	2.57	4.55
	15:0	0.40	0.25	0.30	0.18	0.20	0.53	0.32	0.76
Palmitic	**16:0**	24.33	19.35	29.05	31.30	30.16	33.95	21.48	34.03
	16: 0ISO	0.04	0.28	0.23	0.22	0.05	0.03	0.04	0.08
	17:0	0.24	0.32	0.21	0.38	0.13	0.45	0.54	0.28
Stearic	**18:0**	2.98	0.75	1.49	1.37	1.25	1.83	2.69	1.99
	20:0	0.39	0.14	0.27	0.13	0.22	0.49	0.27	0.40
∑ Saturated fatty acids		**29.26**	**22.06**	**33.98**	**34.48**	**34.26**	**40.77**	**27.92**	**42.09**
	14:1n-7	0.13	1.74	0.57	0.63	0.30	0.88	0.68	0.56
	14:1n-5	0.34	0.47	0.48	0.18	0.10	0.19	0.19	0.73
	15:1n-5	0.09	0.03	0.01	0.01	0.01	0.10	0.07	0.09
Palmitoleic	**16:1n-7**	2.56	16.60	4.63	6.16	5.14	3.08	7.37	2.42
	16:1n-5	0.23	0.67	0.79	0.49	0.23	0.37	0.46	0.39
Oleic	**18:1n-9**	4.79	8.67	18.31	21.91	27.25	15.51	9.69	18.48
	18:1n-7	2.58	7.68	7.09	5.99	3.81	5.18	6.06	4.56
	18:1n-5	0.25	0.25	0.23	0.14	0.12	0.17	0.21	0.21
	20:1n-9+n-7	0.73	0.65	0.48	0.97	1.15	0.71	0.81	0.81
	20:1n-5	0.45	0.13	0.14	0.28	0.34	0.08	0.34	0.10
	22:1n-11	0.32	0.17	0.30	0.52	0.20	0.23	0.22	0.29
	22:1n-9	0.72	0.07	0.07	0.53	1.45	0.24	0.29	0.72
∑ Monounsaturated fatty acids		**13.19**	**37.13**	**33.10**	**37.80**	**40.11**	**26.74**	**26.41**	**29.36**
	16:2n-6	0.00	1.81	0.85	0.98	0.85	0.71	0.92	0.55
	16:2n-4	0.24	0.11	0.24	0.15	0.08	0.39	0.36	0.37
	16:3n-4	0.13	0.74	0.45	0.55	0.50	0.76	1.11	0.51
	16:3n-3	0.15	0.00	0.00	0.05	0.00	0.00	0.00	0.00
	16:3n-1	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	16:4n-3	0.00	6.84	1.54	2.62	2.05	3.04	4.21	2.48
	16:4n-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	18:2n-9	0.09	0.08	0.41	0.00	0.03	0.00	0.05	0.08
Linoleic	**18:2n-6**	5.68	9.24	6.13	6.96	6.56	7.13	9.26	7.49
	18:2n-4	0.04	0.03	0.09	0.03	0.00	0.00	0.06	0.05
	18:3n-6	0.04	0.97	0.34	0.22	0.37	0.67	0.35	0.58
	18:3n-4	0.11	0.00	0.00	0.00	0.00	0.03	0.04	0.03
Linolenic	**18:3n-3**	0.68	9.57	5.44	6.99	7.70	10.38	10.73	5.42
	18:3n-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	18:4n-3	0.08	2.99	1.88	0.98	0.96	1.39	4.02	0.97
	18:4n-1	0.09	0.00	0.00	0.01	0.00	0.00	0.05	0.03
	20:2n-9	0.00	0.06	0.01	0.02	0.31	0.06	0.00	0.06
	20:2n-6	1.00	0.29	0.43	0.83	0.17	0.26	0.42	0.18
	20:3n-9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	20:3n-6	0.21	0.13	0.72	0.22	0.06	0.12	0.16	0.18
	20:3n-3	0.00	0.18	0.04	0.31	0.30	0.22	0.38	0.18
ARA	**20:4n-6**	30.80	2.19	2.55	3.18	0.92	1.25	2.00	1.34
	20:4n-3	0.04	0.16	0.56	0.15	0.12	0.18	0.38	0.24
EPA	**20:5n-3**	15.53	3.99	6.91	2.06	1.50	3.63	6.11	3.88
	22:4n-6	0.15	0.05	0.05	0.08	0.17	0.02	0.06	0.04
	22:5n-6	0.10	0.44	1.33	0.15	0.38	0.49	0.51	0.36
	22:5n-3	0.18	0.11	0.29	0.07	0.23	0.25	0.94	0.82
DHA	**22:6n-3**	2.14	0.81	2.64	1.09	2.37	1.51	3.54	2.71
∑ Polyunsaturated fatty acids		**57.55**	**40.82**	**32.92**	**27.72**	**25.63**	**32.49**	**45.67**	**28.55**

The mean amounts of fatty acids for each algal cue tested are given in Table 3. The fatty acids found in highest proportion in all treatments were the saturated fatty acid (SAT), particularly 16:0 (palmitic acid), the monounsaturated fatty acids (MUFA) including 16:1n-7 (palmitoleic acid), 18:1n-9 (oleic acid), 18:1n-7 and the polyunsaturated fatty acids (PUFA) 18:2n-6 (linoleic acid), 18:3n-3 (linolenic acid), 20:4n-6 (arachidonic acid (ARA)), 20:5n-3 (eicosapentaenoic acid (EPA)) and 22:6n-3 (docosahexaenoic acid (DHA)). Fatty acid profile varied among the treatments highlighting the difference of the CCA treatment in comparison to the green algae by presenting the lowest amount of MUFA, particularly 18:1n-9 and 18:1n-7 and the highest amount of PUFA, principally, 18:2n-6, 18:3n-3, ARA and EPA. Old *U. lens* and Young *U. rigida* followed CCA in EPA content and were higher than the other cues. Besides, the enriched old *U. lens* treatment presented the lowest amount of SAT and DHA as well as the highest amount of 16:1n-7, 18:1n-7, 16:4n-3, 18:3n-4. In general the species *U. rigida*, both at young and old stages, was higher than *U. lens* in n-6 fatty acids contents, particularly ARA and 18:2n-6. In both species, the older the algal culture the higher the ARA content and the lower the 18:2n-6. On the contrary, nutrient enrichment of rearing seawater increased 18:2n-6 in enriched old *U. lens* in comparison to old *U. lens* but reduced other polyunsaturated fatty acid contents such as ARA, EPA or DHA. Finally old *U. rigida* was also characterized by the high 18:1n-9 and 22:1n-9 contents, which were also reflected in the fatty acid composition of the mixture of old *U. rigida* and old *U. lens.*

Fig. 3 shows the results of the principal component analysis of the fatty acids composition from the different algal substrates. Based on the Eigen values, seven components were extracted to describe the data. The first two components accounted for 50% of the total variance found in the different fatty acids composition of the algal cues, the first component being the most significant and representing 33% of the total variance. Based on the first two components, Fig. 3 shows that the fatty acids profiles of CCA, enriched old *U. lens*, old *U. lens* in combination with old *U. rigida* and old *U. lens* treatments are the furthest from each others. The first component presented the highest correlation level with the following fatty acids: 14:1n-7, 16:2n-6, 16:3n-1, 16:3n-3, 16:4n-3, 18:1n-7, 18:2n-6, 18:3n-3, 18:4n-3, ARA and EPA, denoting that they were the ones likely to be involved in differentiating CCA from enriched old *U. lens* treatment. The second component presented the highest correlation level with the following fatty acids: 18:1n-5, 18:1n-9, 18:2n-4, 18:2n-9, 20:1n-9+n-7, 20:2n-9, 22:1n-9, 20:3n-6 and EPA, suggesting they are likely to be involved in differentiating old *U. lens* in combination with old *U. rigida* from old *U. lens* treatment

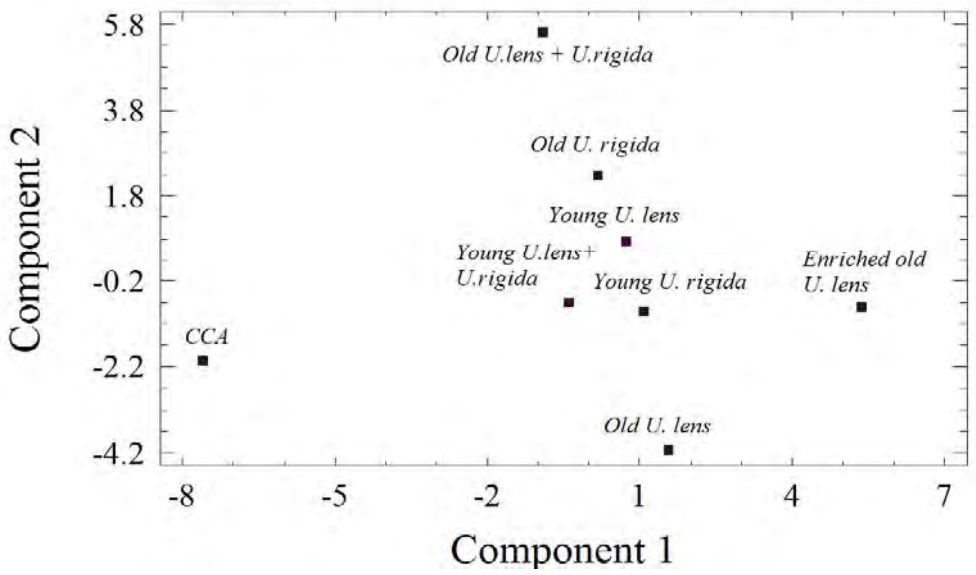

Figure 3. Graphic representation of the principal component analysis carried out on the fatty acid profiles of the different algal treatments used as settlement substrates.

The protein content of the algal substrate was negatively correlated to settlement rate (Fig. 4). The amount of protein was the only variable retained in the multiple regression model to explain the variation in settlement rates between the 8 experimental algal cues tested.

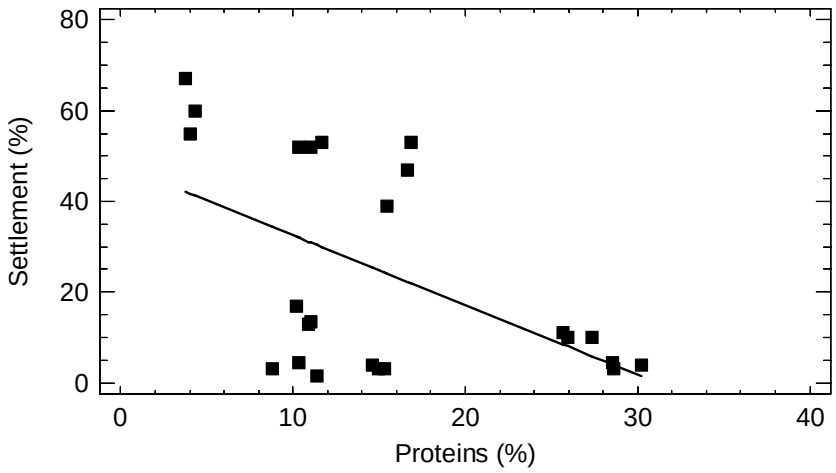

Figure 4: Relation between protein content of algal cues and settlement rates on the 8 experimental substrates tested (n=24). The regression is significant (Adjusted R^2=0.25, F=8.62, df=1, P <0.01).

A Kendall rank correlation was carried out between larval settlement, and the first components of the principal component analysis (Fig. 3). The first component, differentiating the CCA from enriched old *U. lens*, was significantly correlated (P<0.05) with settlement indicating that composition of the substrate in the following fatty acids: 14:1n-7, 16:2n-6,

16:3n-1, 16:3n-3, 16:4n-3, 18:1n-7, 18:2n-6, 18:3n-3, 18:4n-3, ARA and EPA, could play a role in the settlement process.

Post-larvae survival rate was negatively correlated to the protein content of the algal substrate (Fig. 5). The amount of protein, present in the algal cues, being the parameter presenting the highest correlation with survival was the one retained in the model to explain the variation in survival rates between the 8 experimental algal cues tested. The relationship between protein content and post-larvae survival rate was found to be significant (Adjusted R^2=0.39, F=16.04, P <0.01).

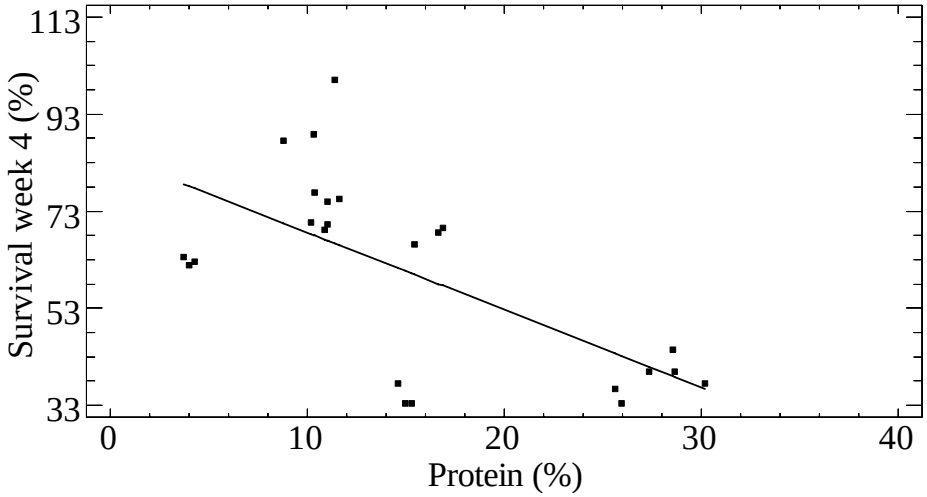

Figure 5. Parameters affecting post-larvae survival in the first four weeks post-settlement, for the 8 experimental substrates tested. Relation between protein content of algal substrates and survival rates (n=24). (R^2=0.39, df=1, F=16.04, P <0.01).

The Kendall rank correlation carried out between growth and survival and the first components of the principal component analysis of the cues fatty acid composition, showed that component 2 had a significant correlation (P<0.05) with survival reflecting the fact that survival could be depending principally on the following fatty acids profile: 18:1n-5, 18:1n-9, 18:2n-4, 18:2n-9, 20:1n-9+n-7, 20:2n-9, 22:1n-9, 20:3n-6 and EPA of the substrate.

However the moderate correlation of growth with components 1 and 2 indicates that the fatty acid composition of the algal substrate is not significantly affecting post-larval growth.

Discussion

The present study on *H. tuberculata coccinea* showed the good potential of germlings of the green macroalgae, *U. rigida*, on settlement. Besides, the study provide interesting information about the link between settlement attractiveness of a substrate and its potential for

ensuring post-larval growth and survival as suggested by (Daume et al., 1999b; Gordon et al., 2006)

Biochemical and fatty acid composition of algae

Algae proximate biochemical and fatty acid compositions differed between species, culture conditions and algal developmental stages, in agreement with previous studies (Fabregas et al., 1996; Shpigel et al., 2000; Thompson et al., 1993). The differences in proximate biochemical composition of *U. lens* and *U. rigida* observed here provide a good illustration of this phenomenon. Protein contents of both old and young *U. lens*, were lower than the ones observed for the same species by Daume & Ryan, (2004), whereas lipid contents were higher in old *U. lens* in the present study. These differences can be related to variations in the culture conditions between both studies or in the algal developmental stage, since in our study older algal cultures were associated to increased lipid and reduced ash contents. The increased protein and lipid contents observed for enriched old *U. lens* compared to the non enriched old *U. lens* are illustrating the effects of the culture conditions on the algal biochemical composition. *U. rigida* presented lower protein and lipid content than the ones reported by Strain et al., (2006) for another *Ulva* species, denoting species specific or genetic differences, as well as differences in environmental conditions. Accordingly, the protein contents of young and old *U. rigida* were higher than the ones of young and old *U. lens*, in agreement with a higher protein content found also for *Ulva sp.* (Strain et al., 2006). The fatty acids composition of the substrates is further evaluated in regard to larval settlement and post-larval growth and survival.

Larval settlement

Larval settlement of *H. tuberculata coccinea* was the highest on CCA as shown in previous studies (Courtois de Viçose et al., 2010) and in other abalones species (Daume et al., 1999a, b; Daume et al., 2000). Larvae settled particularly well on cues containing old *U. rigida*, including both the mixture of germlings of *U. lens* and *U. rigida*, grown for 45 days, and germlings of *U. rigida* alone. Despite Huggett et al., (2005) reporting that *H. rubra* are able to settle on *U. australis* and *U. compresa* plants, the present study reports for the first time the high potential of *U. rigida* germlings as settlement inducers. *Ulvaceae* species present great potential, they are ubiquitous, fast growing and easily cultured, in contrast to the slower growing CCA.

Settlement on *U. lens* was influenced by the algal maturity as suggested by Daume & Ryan (2004). In the present study, young *U. lens* and young *U. rigida* treatments presented a high number of small individual patches while the corresponding older treatments presented fewer larger patches, both resulting in similar algal percent cover, in agreement with other studies (Daume et al., 2004). These observations illustrate the statement of Daume & Ryan (2004) suggesting that the developmental stage of *U. lens* was more important for settlement induction than its percent cover. Moreover, settlement success with young *U. lens* was the same as in other abalone species (Daume & Ryan, 2004). However, increased in algal age from 4 days to 6 weeks in old *U. lens* did not improve settlement ratio, whereas increased from 6 weeks to 8 weeks old raised settlement ratio in *H. laevigata* (Daume & Ryan, 2004b). This suggests that the age of the substrate might not be the only factor involved in settlement differences but also its proximate biochemical composition known to be affected by age and culture conditions. This is further illustrated by the higher settlement rates found for old *U. rigida* than for young *U. rigida* and the changes in its biochemical composition and morphological development.

The result of the multiple regression analysis of the settlement suggested that the protein content of the substrate may explain some of the variation in settlement rates, possibly associated to a variation in the amino acids content and proportions which are well known attractants (Kolkovski et al., 2009) which will also affect growth and survival of juveniles (Daume et al., 2003)

Settlement rates on old and old enriched *U. lens* were lower compared to the ones reported in other studies (Daume et al., 2004; Daume et al., 2000; Daume & Ryan, 2004a) and were correlated to high protein and lipid content, this last one being thought to be detrimental for abalone growth (Britz & Hecht, 1997). Besides, changes in lipid content of the substrate will also affect its fatty acids profile. A well-balanced biochemical composition, rather than high components contents seem to be necessary to obtain good settlement rates as suggested for older abalone stages (Strain et al., 2006).

Fatty acids play an important role as membrane constituents (Jensen et al., 1990) and were reported to play a role in induction of larval metamorphosis by perturbing the cell membranes. In the present study, the algal substrates were be differentiated by their fatty acids composition and only a few fatty acids were correlated to settlement processes. Studies on sea urchin settlement have isolated fatty acids from coralline red algae and *U. lens* responsible of settlement induction and highlighted the positive effect of certain fatty acids (Kitamura et al., 1993; Takahashi et al., 2002). In the present study, 18:1n-7, 18:2n-6, 16:4n-3, 18:3n-3, ARA

and EPA contents in CCA, *U. lens* and *U. rigida* were correlated with settlement and were common to the ones identified by Kitamura et al., (1993) and Takahashi et al., (2002) for settlement induction of sea urchin. Particularly, ARA was highest in algae leading to the highest settlement rates, CCA and old *U. rigida*, whereas it was lower in young *U. rigida*, leading to lower settlement rates. It would be interesting to further investigate these fatty acids and their effect on settlement induction taking into account that 18:2n-6, and EPA have been correlated with *H. tuberculata* growth (Mai et al., 1996) and that EPA was found to accumulate in abalone foot muscle independently of species age and diet (Dunstan et al., 1996).

This study showed that *H. tuberculata coccinea* larvae may distinguish different developmental stages and composition of the algae and that germlings of the green algae *U. rigida* are suitable to improve larval settlement.

Post larval growth and survival

Settlement inducing algal substrates were also considered for their ability to support early post-larval growth and survival, concurring with other studies, where conditions that induced a good larval settlement were usually followed by high growth rates and survival of the settled post-larvae (Daume et al., 1999b).

The fact that post-larval growth could not be correlated with proximate biochemical composition of the substrate nor its fatty acid composition could be explained by the fact that at 4 weeks post-settlement post-larvae growth is mainly dependent on diatom ingestion and it is likely that post-larvae are not yet capable of accessing larger particles such as macroalgae germlings of the substrate as food source. Daume, (2006) stated that *Ulvella lens* is not ingested effectively by abalone under 3 mm of shell length, this could also be applied to *U. rigida.* Daume et al., (2000) showed that the green alga *U. lens* is not sufficient for the initial growth of *H. rubra* post-larvae with a growth rate of 13 μm day^{-1} on *U. lens*. Growth rates obtained on *U. lens* and *U. rigida* in the present study are within the range of the ones reported on *U. lens* by Daume & Ryan (2004) and Daume et al., (2004). Growth rates are also in agreement with the ones obtained by Daume et al., (2000) and Gordon et al., (2006) and within the range of the ones (25-37 μm day^{-1}) reported by Daume et al., (1999) and (Kawamura & Takami, 1995) for post-larvae feeding on diatoms. As suggested above, these results indicate that post-larval growth in this experiment was sustained by diatoms and not by the macro-algal substrate.

The high growth and survival rates observed on combination of old *U. lens* and *U. rigida* treatment, also yielding high settlement rate are reminiscent of Daume et al., (1999b) observations, revealing that settlement patterns are reflected and manifested in growth and survival of *Haliotis* larvae. A dual combination of mature algal cues seems to be beneficial for *H tuberculata coccinea* larval settlement, growth and survival indicating that larval requirements, not satisfied by a mono-algal substrate could be covered by a mixture of them probably due to the multitude of factors involved in these processes.

Protein content of the substrate explains most of the variation in survival rates as it was also the case for settlement rates and as found by Daume et al (2003). A range of fatty acids, differentiating the various algal substrates and correlated with *H. tuberculata coccinea* post-larval survival were identified; out if these 18:2n-9 as well as 20:1n-9+1n-7 may be playing a role as a source of energy and therefore affecting survival. The fatty acids correlated with survival were different from the ones associated with settlement with the exception of EPA. This PUFA is reported to promote fast growth in *H. discus hannai* juveniles (Dunstan et al., 1996; Mai et al., 1996, 1995b); its concentration is correlated with the food value of macroalgae species for *H. tuberculata* and *H. discus hannai* and was found in abalone foot muscle independently of species age and diet (Dunstan et al., 1996). It would be interesting to further investigate the effect of this PUFA on survival, especially when green algae substrates are used to induce settlement and to sustain juvenile growth as they are among the best diets identified for several abalone species (Daume et al., 2004; Mai et al., 1996; Strain et al., 2006), and could thus have a major impact on survival.

Conclusions

The effects of germlings of the green macroalgae, *U. rigida*, on settlement, growth and survival of *H. tuberculata coccinea* were evaluated for the first time and were found suitable. Larvae showed a high settlement rate on CCA, on a combination of mature green algae *U. lens* and *U. rigida* and on mature *U. rigida* alone. Post-larval growth and survival rates were best on combination of mature *U. lens* and *U. rigida.* Our study also showed that the developmental stages, as well as the culture conditions of the algae used as settlement substrates, affect the proximate biochemical composition including the fatty acid profile of these substrates and that these parameters are likely to impact settlement and survival

These results suggest that green macroalgae play an important role in the early life of abalone and a better understanding of their role are required to improve the performances of

abalone culture. *Ulvaceae* species are found worldwide and can be easily cultured, in contrast to the slower growing CCA. Besides they are both good as settlement substrates and juvenile diet. Further research investigating development techniques for *Ulvaceae* spores culture may provide reliable wordwidely applicable techniques for *Haliotis* spp settlement and nursery rearing, consequently improving the sustainability of abalone post-larvae production.

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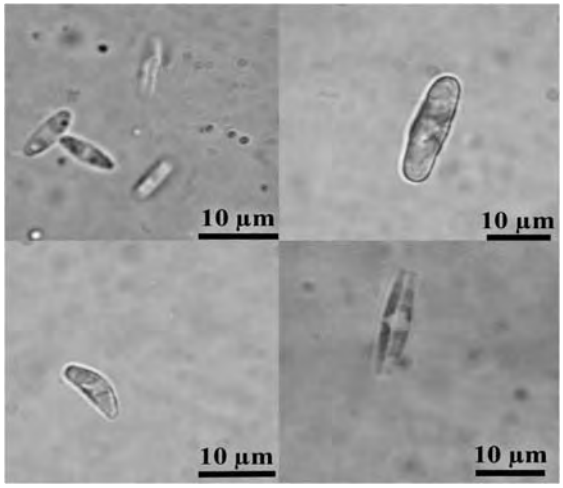
7. STUDY IV: POTENTIAL VALUE OF *NAVICULA INCERTA*, *PROSCHKINIA* SP., *NITZSCHIA* SP., AND *AMPHORA* SP. AS FEED FOR *HALIOTIS TUBERCULATA COCCINEA* POST-LARVAE: EFFECT OF DENSITY ON ALGAL GROWTH RATES



Potential value of *Navicula incerta, Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp. as feed for *Haliotis tuberculata coccinea* post-larvae: Effect of density on algal growth rates.

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Abstract

Benthic diatoms are the main source of food of post-larval abalone up to juveniles of 6-8 mm shell length. Diatom cells quantity and their biochemical composition are therefore influencing juvenile's growth rates and survival but they are variable among species and greatly affected by harvest stage or culture conditions (temperature 28.5±1.4 degree C, light intensity 62 ± 8 μmol photon m^{-2} s^{-1}, and F/2 medium). This study compares growth pattern, cell attachment, and biochemical composition of four diatoms: *Navicula incerta*, *Proschkinia* sp., *Nitzschia* sp. and *Amphora* sp., grown at different original inoculating densities (0.05 x 10^6 cells mL^{-1}, 0.10 x 10^6 cells mL^{-1} and 0.25 x 10^6 cells mL^{-1}) and harvested in log-phase and stationary phase of growth for biochemical analysis. The total protein, carbohydrate, lipid and ash composition as well as the fatty acid composition, of the four species were studied. The results showed that all diatoms species presented better growth densities when inoculated at 0.10 x 10^6 cell mL^{-1} with *Proschkinia* sp. being significantly the highest reaching 6.56 10^6 cells mL^{-1} in log-phase of growth. *Amphora* sp., had the highest cell attachment capacity when inoculated at 0.10 x 10^6 cell mL^{-1} (11580 cells mm^{-2}), whereas *Navicula incerta* had the lowest one (7750 cells mm^{-2}). Protein and lipids (% DW) contents were generally highest in cells during log-phase of growth; *Amphora* sp. in log-phase of growth presented the highest lipid content (9.74% DW); whereas significant differences in carbohydrate between the two growth phases were only observed for *Proschkinia* sp. Both species, presented higher energy contents in log-phase of growth as well as appropriate protein and lipid contents to feed abalone. There were no significant differences in ash content among the four diatoms species. Polyunsaturated fatty acids (PUFA) content ranged from 23.25% to 38.62% of the total fatty acids (TFA) and

the four diatoms tested were richer in n-3 PUFA than in n-6 PUFA. All the analyzed diatoms had significant quantities of 20:5n-3 (EPA) (between 12.69% and 17.68% of TFA) and *Proschkinia* sp., in log-phase of growth, had the highest quantity of 20:4n-6 arachidonic acid (ARA). The results indicate the adequacy of the selected diatoms species for abalone post-larvae, in terms of algal cells biometric parameters, production, attachment capacity and nutritive value. They suggest the importance of culture conditions and harvest protocols for diatoms large scale production in order to respond to abalone post-larval nutritional requirements.

Keywords: Diatoms; Growth; Cell attachment; Biochemical composition; Fatty acids; Abalone; *Haliotis tuberculata coccinea*

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Introduction

Cultured microalgae are the main food source for the first larval stages of most species cultured in aquatic systems and the improvement of their nutritional value, to adapt them to the necessities of different species, is required (De Pauw & Persoone, 1988). The differences in nutritive values of microalgae are frequently due to differences in their biochemical composition (Fernández-Reiriz et al., 1989), which is known to be influenced by their growth rates, phases of their life cycle and culture conditions (Brown et al., 1996; Otero & Fábregas, 1997; Richmond, 1986). Benthic diatoms are the principal food source for post-larval abalone (Hahn, 1989; Kawamura, 1996; Kawamura et al., 1998b), thus biofilms of natural mixed benthic diatoms are used in abalone hatcheries worldwide (Daume et al., 1997; Daume et al., 2000; Hahn, 1989; Roberts, 2001). However, the quality of these natural biofilms is challenging to sustain (Hahn, 1989) and imply uncontrolled species and densities. Various studies found a relation between benthic diatom density and post-larval abalone settlement, growth and survival (Daume et al., 2004; Gorrostieta-Hurtado & Searcy-Bernal, 2004; Roberts et al., 2007; Searcy-Bernal et al., 2003; Searcy-Bernal et al., 2001; Watson et al., 2004). Besides, post-larvae of various abalone species presented a better growth rate when fed with monospecific cultured benthic diatoms (Carbajal-Miranda et al., 2005; Daume et al., 2000; Gordon et al., 2006; Kawamura & Takami, 1995). However, only few studies have focused on their nutritional quality and the influence of algal biochemical composition on newly settled abalone (Daume et al., 2003; Gordon et al., 2006; Uriarte et al., 2006; Viana et al., 2007a). Hence, maintaining a suitable diatom film, in terms of density and nutritional quality, is a key factor to abalone hatcheries success. Benthic diatoms can be cultured and their growth manipulated under controlled conditions (Dunstan et al., 1994). However, studies on the effects of the environmental conditions on the cultures characteristics as well as the changes in the fatty acid composition of benthic diatoms are scarce (Liang et al., 2002; Liang et al., 2001; Mercado et al., 2004) in comparison to studies carried out on phytoplanktonic microalgae (Brown et al., 1996; Leonardos & Lucas, 2000; Milke et al., 2008; Pernet et al., 2003; Renaud et al., 2002; Sánchez-Saavedra & Voltolina, 2006).

Since *Haliotis tuberculata coccinea* is a new candidate species for aquaculture in the Canary Islands there is no information available yet about diatoms to be used as feed for its post-larvae. Hence, there is a need to determine the optimum benthic diatom culture techniques to provide this abalone species with a highly valuable and nutritious food which would cover its nutritional requirements during the first stages of its life cycle. The present study examines

the biometric parameters of four benthic diatoms species and the effect of density on their growth response as well as their proximate and fatty acid composition, with the purpose of adjusting diatom's culture conditions for future production to be used as feed supply tailored to the nutritional requirements of abalone post-larvae.

Material and methods

Diatom cultures:

Four species of diatoms *Navicula incerta*, *Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp. obtained from the Instituto de Ciencias Marinas de Andalucía (ICMAN, CSIC) in Cádiz and from Universidad Autónoma del estado de Baja California, were selected considering they are frequently used for feeding trials with other species of abalone post-larvae (Carbajal-Miranda et al., 2005; Daume et al., 2000; Gordon et al., 2006; Kawamura et al., 1998b). Non-axenic batch cultures of each strain were maintained at the Instituto Canario de Ciencias Marinas in 1L Erlenmeyer flasks under continuous illumination and in f/2 medium plus silicate (1 mg L^{-1}) (Guillard, 1975). The cultures were maintained neither aerated nor agitated and the medium was weekly renewed by reinoculation into fresh medium.

For each species, three separate experiments were performed to determine: 1.-The growth curve as a function of different inoculating densities, 2.-The cell attachment capacity at different inoculating densities and 3. - The biochemical and fatty acid composition in function of the harvesting period at the best inoculated density, determined in the two previous experiments.

During the growth curve experiment 10 mL test tubes were inoculated in triplicate at 0.05 x 10^6 cells mL^{-1}, 0.10 x 10^6 cells mL^{-1} and 0.25 x 10^6 cells mL^{-1}, for each species, and grown during 7 days (21 test tubes/ species/ density). Cell attachment capacity was monitored in 20 mL Petri dishes containing one glass cover slip. Attachment capacity of each species was tested in triplicate at three inoculums densities 0.05 x 10^6 cells mL^{-1}, 0.10 x 10^6 cells mL^{-1} and 0.25 x 10^6 cells mL^{-1}, and growth of attached cells was monitored daily on glass cover slip during 7 days (21 glass cover slips/ species/ density). Finally, biochemical analysis was performed on samples from 10 L bags. The bags, for each species, were inoculated in six replicates at 0.10 x 10^6 cells mL^{-1} with three of them harvested in log-phase of growth and the other three in stationary phase of growth. Cultures were harvested by filtration on GF/A Whatman® filters, and stored at - 80°C prior to biochemical analysis.

In all experiments, cultures were grown in f/2 medium plus silicate (1 mgL^{-1}) (Guillard, 1975) under continuous light of 62 ± 8 μmol photon m^{-2} s^{-1} and 28.5±1.4ºC. Photon flux density (irradiance) was measured using a Digital light meter (HT170N, HT ITALIA, Italy).

Cell counts and growth:

Cells were counted in a Neubauer haemocytometer. To avoid possible cell aggregations, ultrasound (1 or 3 min) was applied to the samples prior to evaluation of cell concentration.

Cultures were monitored daily, triplicate aliquots were removed from each of the three test tubes for each species; these test tubes were then discarded. Average growth rates were estimated, during logarithmic phase of growth, using the following formulae:

$$\mu=Ln\ (N_1/N_0)/\ (t_1-t_0)$$

Where N_1 = cell density at time t_1 and N_0 = cell density at time t_0 (Guillard, 1973).

The number of attached cells was estimated, in log-phase of growth, on glass cover slips, inserted horizontally in Petri dishes, after unattached cells had been removed by gently flushing the cover slips with water jets from a pipette. Ten randomly chosen fields of view were photographed under a microscope at 400 x and cells were then counted using the software Image Pro Plus (Media Cybernetics, Inc, Silver Spring, Maryland, USA). The number of cells mm^{-2} was calculated.

Analytical methods:

Triplicate samples of frozen cells, collected in both log-phase and stationary phase of growth, from each of the experimental bags, and were analyzed in triplicates for total lipids, protein, carbohydrate, ash and total fatty acids. Before analysis all samples where homogenized with mortar and pestle prior weighting for further analysis. Total lipids were gravimetrically analyzed after extraction with chloroform-methanol (2:1) (Folch et al., 1957). Total protein was calculated from total Kjeldahl nitrogen according to AOAC, (1995) standard methods. Ash content was gravimetrically determined after incinerating the samples at 600ºC for 24h. Fatty acids in the lipid extracts were transesterified to methyl esters (FAMEs) with 1% sulphuric

acid: methanol complex (Christie, 1982). FAMEs samples were extracted into hexane and stored at -80ºC. Fatty acids were analyzed in a Thermo Finnigan- GC Focus gas chromatograph equipped with a flame ionization detector (260ºC). FAMEs were separated with capillary column (Supercowax 28m x 0.32mm x 0.25 i.d.) using helium as the carrier gas under the conditions described by (Izquierdo et al., 1989).

Data analysis:

Statistical analysis was performed using statistical program, Statgraphics Plus 5.1. (Manugistics, Rockville, Maryland, USA).

A two-way ANOVA was applied to assess the diatom species and inoculums density effects, and the possible interaction between them, on growth rates, cell production and cell attachment capacity. An analysis of variance (one-way ANOVA) was performed for analysis of proximate and fatty acids composition between phases of growth and species. Data showing significant differences ($P<0.05$) were analyzed by paired comparisons using Tukey's HSD test. Equality of variance was assessed with Bartlett's test.

Results

Biometric parameters and growth rates

The four species studied showed quite different cell size as estimated from the length of the apical and transapical cell axis, *N. incerta* and *Nitzschia* sp. being double the length of *Amphora* sp. and *Proschkinia* sp. (Fig. 1).

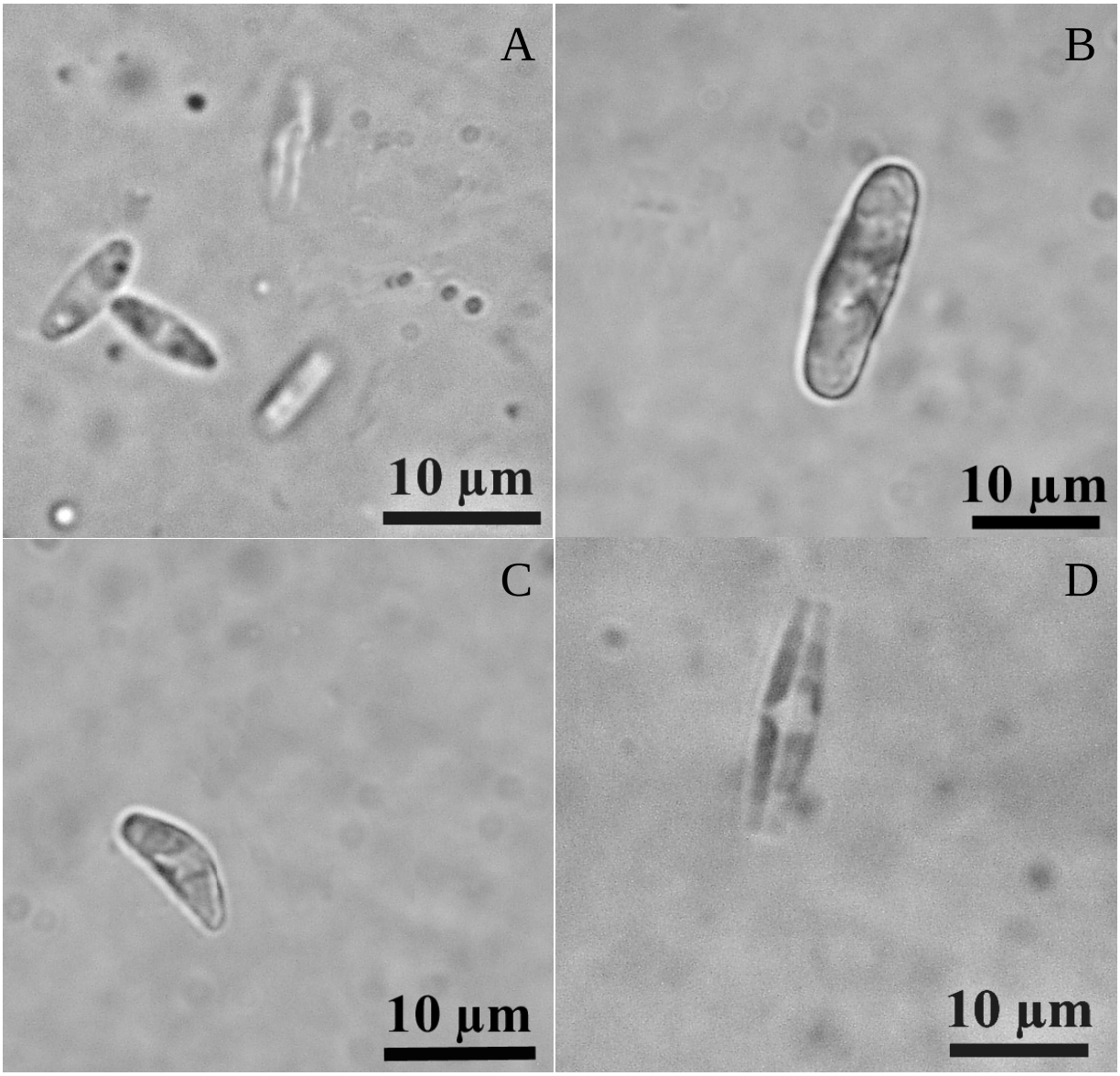

Figure. 1 Benthic diatoms species characteristics, length and width in µm. A: *Proschkinia* sp. 8.16±0.41, 2.99±0.12; B: *Navicula incerta* 18.01±0.95, 4.07±0.36; C: *Amphora* sp.: 10.57±0.20, 3.67±0.11; D: *Nitzschia* sp. 18.23±0.55, 3.00±0.20

Growth rates (GR) ranged from 0.50±0.09 day^{-1} to 1.31±0.13 day^{-1} in all density treatments (Table 1). Two-way ANOVA showed that GR and cell counts were significantly affected by inoculums density, diatom species and the interaction between both of them (P<0.05). GR were significantly lower (P<0.05) for all species when inoculated at 0.25 x 10^6 cells mL^{-1} and were the highest at 0.05 x 10^6 cells mL^{-1} and 0.10 x 10^6 cells mL^{-1}; not being significantly different (P>0.05) between the two densities at the exception of *N. incerta*, showing the significantly highest GR at 0.05 x 10^6 cells mL-1 (1.31±0.13 day^{-1}). Significant different GR (P<0.05) were observed between species at 0.05 x 10^6 cells mL^{-1} and 0.25 x 10^6

cells mL^{-1} inoculums whereas no significant difference in GR (P>0.05) was noticed when inoculated at 0.10 x 10^6 cells mL^{-1}.

Cell counts at log-phase of growth was significantly different between species (P<0.05) independently of inoculums' density and was significantly lower (P<0.05), for all species, when inoculated at 0.05 x 10^6 cells mL^{-1} while no significant differences in cell counts were observed when inoculated at 0.10 x 10^6 cells mL^{-1} and 0.25 x 10^6 cells mL^{-1} (Table 1). The cell counts at log-phase of growth of *Proschkinia* sp. were significantly higher (P<0.05) than the ones of the other species, ranging from 3.76 x 10^6 to 6.56 x 10^6 cells mL^{-1} for the three density treatments.

Table 1. Growth rate and cell count at log-phase of growth of four species of diatoms grown at three different densities, (mean±SD, n=3)

Species	**Densities (10^6 cells mL^{-1})**		
	0.05	**0.10**	**0.25**
Growth rate μ (day^{-1})			
***Amphora* sp**	0.98±0.06 $^{B, a}$	0.94±0.01 $^{A, a}$	0.61±0.04 $^{AB, b}$
***Nitzschia* sp**	0.94±0.03 $^{B, a}$	0.87±0.07 $^{A, a}$	0.60±0.02 $^{AB, b}$
***Proschkinia* sp**	0.86±0.01 $^{B, a}$	0.84±0.01 $^{A, a}$	0.65±0.01 $^{A, b}$
Navicula incerta	1.31±0.13 $^{A, a}$	0.86±0.09 $^{A, b}$	0.50±0.09 $^{B, c}$
Cell count x10^6 (mL^{-1})			
***Amphora* sp**	0.97±0.17 $^{B, b}$	1.66±0.08 $^{B, a}$	1.56±0.15 $^{B, a}$
***Nitzschia* sp**	0.69±0.15 $^{BC, b}$	1.72±0.15 $^{B, a}$	1.53±0.10 $^{B, a}$
***Proschkinia* sp**	3.76±0.01 $^{A, b}$	6.56±0.49 $^{A, a}$	6.31±0.06 $^{A, a}$
Navicula incerta	0.57±0.10 $^{C, b}$	0.70±0.19 $^{C, a}$	0.69±0.12 $^{C, a}$

Different lower case superscripts across a row indicate difference between densities for the same species at 95% level
Different upper case superscripts within a column indicate difference between species for the same density at 95% level (ANOVA Tukey's, test; P<0.05)

The log-phases of growth were similar between *Amphora* sp. and *Nitzschia* sp. while they were shorter, in all densities treatments, for *N. incerta* and *Proschkinia* sp. presented extended ones (Fig. 2).

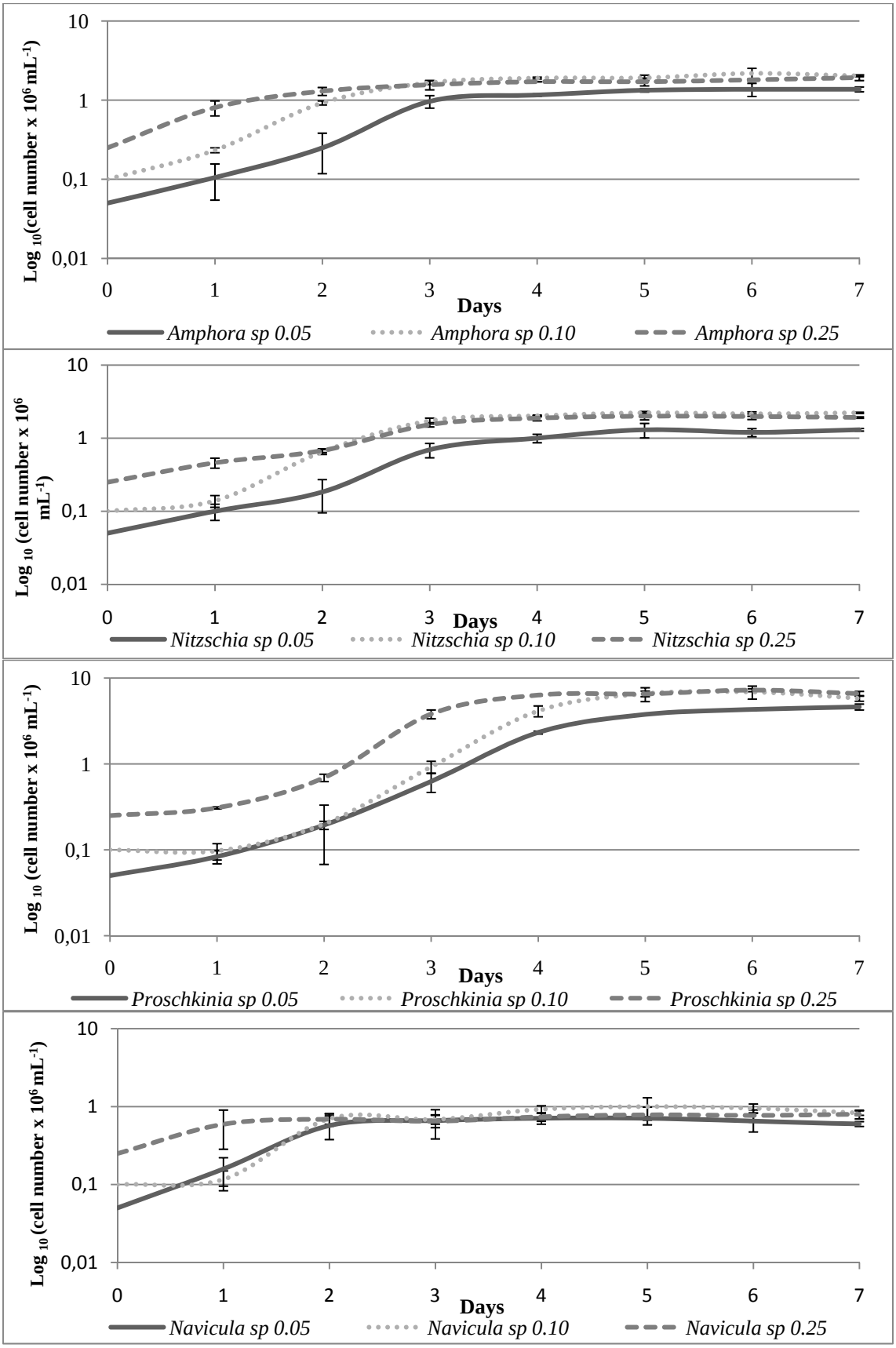

Figure. 2 Growth curve of four species of diatoms grown at three different densities. Vertical bars indicate the standard deviation (n=3)

Two-way ANOVA denoted a significant effect of inoculums density, diatom species and the interaction between both of them (P<0.05) on cell attachment capacity. All the diatom species tested generally presented, in log-phase of growth, a higher cell attachment capacity when inoculated at 0.10 x 10^6 cells mL^{-1}, however *Amphora* sp., with the overall highest cell attachment capacity, was the only species which did not present significant differences (P>0.05) in cell attachment capacity between the three density treatments. *Nitzschia* sp. and *N. incerta* did not present significant differences (P>0.05) in cell attachment capacity between the 0.10 x 10^6 and 0.25 x 10^6 cells mL^{-1} treatments and the 0.10 x 10^6 and 0.25 x 10^6 cells mL^{-1} treatments respectively (Table 2). Significant differences (P<0.05) in cell attachment capacities of the different diatoms species were observed across the three density treatments, however *Amphora* sp. and *Nitzschia* sp. generally presented higher cell attachment capacities across density treatments. The number of attached cells of *Amphora* sp. and *Nitzschia* sp. was significantly higher (P<0.05) when inoculated at 0.25 x 10^6 cells mL^{-1} (9.80 10^3±1.12 10^3 and 9.65 10^3± 1.3610^3 respectively) than the one of *Proschkinia* sp. and *N. incerta* (3.17 10^3±0.64 10^3 and 3.67 10^3± 1.03 10^3 respectively) while significant difference (P<0.05) in the number of attached cells was only observed between *Amphora* sp. and *N. incerta* in the 0.10 x 10^6 cells mL^{-1} treatment (Table 2).

Table 2. Attached cells at log-phase of growth of four species of diatoms grown at three different densities (mean±SD, n=3)

Species	**Densities (10^6 cells mL^{-1})**		
	0.05	**0.10**	**0.25**
Attached cell count mm $^{-2}$			
***Amphora* sp**	11.05 10^3 ±1.30 10^3 **A, a**	11.58 10^3±1.16 10^3 **A, a**	9.80 10^3±1.12 10^3 **A, a**
***Nitzschia* sp**	7.06 10^3±0.90 10^3 **B, b**	10.80 10^3±0.2 10^3 **AB, a**	9.65 10^3±1.36 10^3 **A, a**
***Proschkinia* sp**	1.64 10^3±0.7 10^3 **C, b**	8.48 10^3±0.83 10^3 **BC, a**	3.17 10^3±0.64 10^3 **B, b**
Navicula incerta	5.51 10^3±0.43 10^3 **B, ab**	7.75 10^3±1.00 10^3 **C, a**	3.67 10^3±1.03 10^3 **B, b**

Different upper case superscripts down a column indicate means which differ significantly, within the same density, at 95% level

Different lower case superscripts across a row indicate means which differ significantly, within the same species, at 95% level (ANOVA Tukey's test; P<0.05)

Production of nutrients

The biochemical composition showed (Table 3) that for all diatoms, except for *N. incerta*, the lipid content was significantly (P<0.05) higher during the log-phase than during the stationary phase of growth. The highest (P<0.05) lipid content (9.74±1.70 % DW) was

found in *Amphora* sp., in log-phase of growth, and the lowest one ($P< 0.05$) in *Nitzschia* sp. (3.11±0.24 % DW) during stationary phase of growth. As opposed to the other species, *N. incerta* showed a highest lipid content, not significantly different ($P>0.05$) from the one of *Amphora* sp. in log-phase of growth, and *Proschkinia* sp., experienced the biggest drop in lipid content, being reduced by half, between the two phases of its culture. Protein content followed a similar trend, dropping at during stationary phase in the case of *Amphora* sp. and *Proschkinia* sp. (19.70±0.95 and 20.72±1.40 % DW, respectively). Both presented the highest protein contents and high energy content during log-phase of growth and experienced the highest drop in protein contents between both growth phases, whereas protein contents in *N. incerta* and *Nitzschia* sp. did not show statistical differences ($P>0.05$) between growth phases. Carbohydrate content followed a trend inverse to that of protein content, increasing during the stationary phase of growth except for *N. incerta.* The highest carbohydrate content was found in *N. incerta,* in log-phase of growth, (27.23±3.77 % DW) followed by *Proschkinia* sp., in stationary phase of growth (23.73±5.03 % DW), which showed the most pronounced difference in carbohydrate content between its two phases of growth. No significant differences ($P> 0.05$) were found in the ash content among all diatoms cultures.

Table 3. Proximate chemical analysis (% dry weight) and gross energy (GE) of four species of diatoms grown at an initial inoculum of 0.10x 10^6 cells mL^{-1} and harvested in exponential (Exp) (Day 2) and stationary (Sta) (Day 7) phase of growth, (mean±SD, n=3)

Species	Growth Phase	Lipid (%)	Protein (%)	Ash (%)	Carbohydrates (%)	GE (kJ g^{-1}) DM
Amphora sp.	Exp	9.74±1.70^{a}	19.70±0.95^{a}	57.10±3.40	13.50±5.01^{cd}	10.82
	Sta	7.31±0.68^{cd}	13.07±0.71^{bc}	60.50±0.90	19.15±1.77^{bc}	9.24
Navicula incerta	Exp	6.11±0.17^{de}	13.00±0.60^{bc}	53.70±3.80	27.23±3.77^{a}	10.16
	Sta	8.88±0.63^{ab}	13.00±1.07^{bc}	58.00±0.43	20.20±0.70^{abc}	10.00
Nitzschia sp.	Exp	4.90±0.55^{ef}	14.50±0.40^{b}	62.00±1.50	18.70±1.70^{bc}	8.56
	Sta	3.11±0.24^{g}	14.20±1.22^{b}	61.00±0.00	21.80±1.21^{ab}	8.33
Proschkinia sp.	Exp	7.82±0.72^{bc}	20.72±1.40^{a}	61.40±8.02	10.05±6.70^{d}	9.68
	Sta	4.00±0.56^{fg}	11.60±1.93^{c}	60.80±2.80	23.73±5.03^{ab}	8.39

Different superscripts down a column indicate means which differ significantly at 95% level (ANOVA Tukey's test; $P<0.05$)

Fatty acid composition

Levels of saturated fatty acids (SAT), with 14:0 and 16:0 as dominating saturated fatty acids, did not differ significantly ($P > 0.05$) among the four diatoms tested at any of the growth phases studied. Polyunsaturated fatty acids (PUFA) constituted the largest fraction of the total fatty acids (TFA) (23.25% to 38.62% of TFA) except in the case of *N. incerta*, in both phases of growth, and *Proschkinia* sp., during its stationary phase of growth, where the monounsaturated fatty acids (MUFA) were highest. The proportion of the various PUFA varied among the diatom species and between growth phases, and *Amphora* sp. is the only species presenting an increase of both PUFA and n-3 PUFA between log-phase and stationary phase of growth reaching 38.29±5.72% and 25.19±4.76 %, respectively.

Unlike the other diatoms, *Amphora* sp. contained high levels of 18:4n-3. All of the analyzed diatoms were richer in n-3 PUFA than in n-6 PUFA and had significant quantities of 20:5n-3, eicosapentaenoic acid (EPA) (between 12.69% and 17.68% of TFA). *Proschkinia* sp., in log-phase, had the highest quantity of 20:4n-6 arachidonic acid (ARA), whereas *N. incerta* had a significantly lower content ($P < 0.05$) of this fatty acid as well as n-6 PUFA. *Proschkinia* sp., *Nitzschia* sp. and *Navicula incerta* showed a decrease in EPA and ARA between the log-phase and the stationary phase of growth, while *Amphora* sp. followed an increase. Levels of 22:6n-3, docosahexaenoic acid (DHA) (0.19%-1.90% TFA) increased between log-phase and stationary phase of growth and were generally low among the diatoms tested with the significantly lowest value ($P < 0.05$) being identified in *N. incerta* (Table 4).

Table 4. Fatty acid composition (% of total fatty acid) in *Amphora* sp., *Navicula incerta*, *Nitzschia* sp. and *Proschkinia* sp. during logarithmic and stationary phase of growth (mean±SD, n=3

	FAMES	*Amphora*.sp (Log)	*Amphora* .sp (Sta)	*Navicula incerta*(Log)	*Navicula incerta*(Sta)	*Nitzschia.* sp (Log)	*Nitzschia.* sp (Sta)	*Proschkinia* . sp (Log)	*Proschkinia.* sp (Sta)
	12:0	0.75±0.42	1.35±0.76	0.21±0.15	1.47±0.61	1.41±0.53	2.42±1.14	0.53±0.38	0.16±0.03
*	**14:0**	10.97^{a}±1.93	8.71^{b}±0.37	5.12^{c}±0.56	5.68^{c}±1.10	9.49^{ab}±0.54	8.11^{b}±1.40	3.10^{d}±0.48	3.31^{d}±0.23
	15:0	0.64±0.07	0.52±0.06	0.37±0.07	0.33±0.01	2.75±1.79	2.71±0.08	2.65±1.77	1.29±0.13
*** PALMIITIC**	**16:0**	15.31^{b}±1.64	19.59^{ab}±5.08	20.80^{ab}±2.51	17.48^{ab}±2.69	17.41^{ab}±0.43	15.13^{ab}±1.53	20.25^{ab}±1.64	22.61^{a}±2.55
	17:0	3.01±0.53	2.71±0.16	3.11±0.87	2.46±0.60	2.37±0.29	2.06±1.20	2.13±1.70	2.20±0.04
*** STEARIC**	**18:0**	1.11^{b}±0.34	1.32^{b}±0.67	0.90^{b}±0.20	1.41^{ab}±0.24	1.21^{b}±1.06	2.23^{a}±0.61	0.79^{b}±0.11	1.02^{b}±0.23
	20:0	0.27±0.05	0.22±0.02	0.05±0.00	0.24±0.33	0.11±0.01	0.18±0.05	0.09±0.01	0.45±0.71
*** ∑ Saturated fatty acids**		**32.05±0.08**	**34.41±5.88**	**29.73±2.28**	**29.06±2.68**	**34.29±0.50**	**32.84±3.68**	**29.28±1.95**	**31.05±1.60**
	14:1n-7	0.39±0.32	0.38±0.16	0.46±0.06	0.27±0.13	0.71±0.19	0.96±0.12	0.58±0.18	0.47±0.04
	14:1n-5	0.06±0.02	0.05±0.00	0.02±0.00	0.03±0.01	0.42±0.27	1.06±0.54	0.27±0.23	0.09±0.01
	15:1n-5	0.03±0.00	0.03±0.01	0.03±0.01	0.03±0.01	0.24±0.09	0.23±0.05	0.18±0.04	0.20±0.04
*** PALMITOLEI**	**16:1n-7**	25.57^{c}±4.18	21.56^{d}±0.77	38.72^{a}±3.20	38.07^{a}±2.28	19.62^{d}±1.01	18.33^{d}±1.39	25.41^{c}±0.43	31.92^{a}±3.81
	16:1n-5	0.26±0.01	0.23±0.01	1.36±2.25	1.20±2.00	0.91±0.40	1.20±0.05	2.01±2.22	2.72±0.07
*** OLEIC**	**18:1n-9**	3.30^{a}±0.13	3.20^{a}±0.34	0.86^{c}±0.32	1.72^{c}±0.56	3.11^{a}±0.37	3.82^{a}±0.81	1.16^{bc}±0.21	1.13^{bc}±0.27
	18:1n-7	0.58±0.04	0.54±0.04	2.37±0.31	2.80±0.50	1.05±0.07	2.03±0.18	1.33±0.05	2.44±0.30
	18:1n-5	0.07±0.01	0.07±0.01	0.56±0.03	0.71±0.03	0.08±0.01	0.12±0.01	0.07±0.00	0.06±0.01
	20:1n-9	0.00±0.00	0.01±0.00	0.19±0.03	0.25±0.06	0.12±0.05	0.40±0.47	0.08±0.03	0.14±0.07
	20:1n-7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.41±0.24	0.00±0.00	0.04±0.04
	20:1n-5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.03±0.05	0.00±0.00	0.15±0.11	0.00±0.00
	22:1n-11	0.08±0.07	0.01±0.01	0.50±0.44	0.61±0.53	0.14±0.05	0.21±0.02	0.20±0.20	0.09±0.04
	22:1n-9	0.33±0.03	0.34±0.05	0.28±0.31	0.45±0.23	0.21±0.12	0.30±0.20	0.23±0.16	0.12±0.01
*** ∑ Monounsaturated fatty acids**		**30.69^{c}±4.01**	**26.40^{d}±0.51**	**45.34^{a}±0.25**	**46.15^{a}±1.26**	**26.49^{d}±1.11**	**28.69^{cd}±0.85**	**31.46^{c}±1.33**	**39.43^{b}±3.69**
	16:2n-6	2.03±0.19	1.75±0.03	1.18±0.14	1.00±0.08	1.78±0.09	1.60±0.33	0.70±0.38	0.67±0.09
	16:2n-4	0.10±0.00	0.08±0.02	0.05±0.01	0.07±0.01	0.49±0.54	0.28±0.11	1.47±1.96	0.16±0.08
	16:3n-4	0.00±0.00	0.10±0.06	0.07±0.03	0.03±0.01	0.06±0.05	0.03±0.00	0.99±1.18	0.09±0.05
	16:3n-3	0.07±0.08	0.03±0.01	0.02±0.01	0.02±0.00	0.78±0.17	1.57±0.26	0.73±0.36	0.71±0.03
	16:3n-1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.15±0.09	0.00±0.00	0.12±0.08	0.00±0.00
	16:4n-3	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00	0.08±0.05	0.00±0.00	1.07±0.76	0.00±0.00
	16:4n-1	0.03±0.01	0.03±0.02	0.15±0.05	0.14±0.05	2.19±0.82	1.92±1.07	1.33±0.94	0.95±0.47
	18:2n-9	0.29±0.09	0.47±0.20	0.02±0.01	0.02±0.01	0.05±0.01	0.03±0.02	0.05±0.00	0.02±0.02
*** LINOLEIC**	**18:2n-6**	3.90^{ab}±0.21	3.86^{ab}±1.04	1.01^{c}±0.29	2.92^{b}±1.09	4.13^{ab}±1.23	6.43^{a}±1.95	1.14^{c}±0.23	0.51^{d}±0.06
	18:2n-4	0.00±0.00	0.01±0.01	0.31±0.05	0.27±0.05	0.09±0.05	0.14±0.05	0.23±0.11	0.40±0.51
	18:3n-6	1.74±0.58	1.49±0.17	0.50±0.18	0.39±0.19	0.49±0.10	0.44±0.09	1.42±0.09	0.47±0.05
	18:3n-4	0.00±0.00	0.02±0.01	0.02±0.01	0.02±0.01	0.03±0.03	0.00±0.00	0.07±0.01	0.05±0.03
*** LINOLENIC**	**18:3n-3**	0.28^{b}±0.07	0.34^{b}±0.11	0.24^{b}±0.10	0.37^{b}±0.15	1.66^{a}±0.13	1.58^{a}±0.25	0.19^{bc}±0.10	0.11^{c}±0.06
	18:3n1	0.06±0.01	0.08±0.02	0.33±0.03	0.30±0.03	0.10±0.01	0.15±0.08	0.35±0.09	0.29±0.01
	18:4n-3	6.31±2.68	5.48±0.63	0.52±0.00	0.48±0.08	1.22±0.21	0.78±0.20	0.05±0.01	0.08±0.03
	18:4n-1	0.01±0.01	0.03±0.01	0.00±0.00	0.00±0.00	0.07±0.03	0.06±0.04	0.04±0.02	0.02±0.01
	20:2n-9	0.00±0.00	0.08±0.05	0.02±0.01	0.02±0.01	0.03±0.02	0.14±0.11	0.03±0.02	0.09±0.07
	20:2n-6	0.00±0.00	0.04±0.02	0.01±0.01	0.02±0.00	0.04±0.00	0.09±0.07	0.21±0.15	0.15±0.00
	20:3n-9	0.14±0.04	0.13±0.01	0.11±0.03	0.07±0.02	0.07±0.02	0.08±0.05	0.05±0.06	1.09±1.86
	20:3n-6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.10±0.06	0.09±0.05	0.77±0.02	0.77±0.92
	20:3n-3	0.00±0.00	0.00±0.00	0.00±0.00	0.05±0.03	0.08±0.10	0.30±0.32	0.40±0.28	0.00±0.00
*** ARA**	**20:4n-6**	4.15^{b}±1.31	4.83^{b}±1.29	1.96^{c}±0.34	1.76^{c}±0.86	5.71^{ab}±0.86	4.24^{b}±1.12	10.16^{a}±1.29	6.04^{ab}±0.46
	20:4n-3	0.22±0.08	0.23±0.05	0.24±0.02	0.20±0.01	0.22±0.09	0.20±0.17	0.10±0.02	0.03±0.02
*** EPA**	**20:5n-3**	15.44^{ab}±5.89	17.68^{a}±4.46	15.97^{ab}±1.70	15.61^{ab}±1.32	16.55^{ab}±0.81	14.11^{ab}±1.78	15.20^{ab}±2.11	12.69^{b}±1.41
	22:4n-6	0.12±0.03	0.13±0.02	0.00±0.00	0.02±0.01	0.09±0.02	0.73±1.06	0.05±0.00	0.21±0.15
	22:5n-6	0.06±0.02	0.06±0.00	0.19±0.11	0.14±0.07	0.14±0.07	0.33±0.33	0.21±0.00	0.82±1.07
	22:5n-3	0.36±0.05	0.55±0.37	0.15±0.09	0.06±0.02	0.74±0.15	0.74±0.12	1.38±0.13	1.11±0.04
*** DHA**	**22:6n-3**	0.86^{a}±0.25	0.89^{a}±0.05	0.19^{b}±0.15	0.29^{b}±0.26	1.69^{a}±0.53	1.90^{a}±1.26	1.25^{a}±0.03	1.61^{a}±0.33
*** ∑ Polyunsaturated fatty acids**		**36.16^{ab}±11.56**	**38.29^{a}±5.72**	**23.25^{c}±2.27**	**24.23^{c}±1.45**	**38.62^{a}±1.97**	**37.64^{a}±3.52**	**38.19^{a}±0.72**	**29.04^{bc}±5.29**
Total FA		98.91±0.10	99.09±0.08	99.14±0.14	99.43±0.09	99.40±0.36	99.17±0.16	98.94±0.11	99.52±0.01
*** n-3 PUFA**		23.54^{ab}±9.09	25.19^{a}±4.76	17.33^{c}±2.06	17.05^{c}±1.31	22.96^{ab}±2.13	21.02^{abc}±1.62	19.63^{bc}±1.61	16.33^{c}±1.24
*** n-6 PUFA**		11.99^{b}±2.35	12.13^{b}±0.90	4.86^{d}±0.37	6.24^{d}±0.24	12.42^{ab}±1.68	13.86^{ab}±1.92	14.57^{a}±1.48	9.64^{c}±1.58
*** n-3/n-6**		1.93^{c}±0.38	2.07^{c}±0.33	3.58^{a}±0.46	2.74^{b}±0.24	1.89^{c}±0.46	1.54^{cd}±0.28	1.35^{d}±0.03	1.71^{cd}±0.19
*** n-3 HUFA**		16.89±6.27	19.34±4.19	16.55±1.96	16.18±1.22	19.28±1.64	17.08±1.52	18.13±1.91	15.43±1.18
*** EPA/DHA**		17.65^{b}±1.76	19.81^{b}±4.94	113.88^{a}±56.52	92.37^{a}±67.14	10.27^{b}±2.36	9.66^{b}±5.13	12.16^{b}±1.96	8.20^{b}±2.49

Different superscripts across a row indicate means which differ significantly at 95% level (ANOVA Tukey's test; P<0.05). Only fatty acids marked with * were statistically compared

Discussion

Biometric parameters and growth rate

Based on the sizes of diatoms (9.4±0.1µm-76.3±1.6µm) previously reported to be used to feed abalone post-larvae (Kawamura et al., 1998b; Kawamura & Takami, 1995) the four diatom species investigated in this study showed adequate size range, to be potentially used for *H. tuberculata coccinea* post-larvae. The length of *N. incerta* cells reported in the present study is larger than the ones reported by Simental-Trinidad et al., (2001) and Correa-Reyes et al., (2001) whereas *Amphora*'s dimensions correspond to those found by De la Peña, (2007). In view of the biometric parameters of the diatom species studied, *Proschkinia* sp. and *Amphora* sp. match the criteria of Hahn, (1989) who suggested species smaller than 10µm as good candidates for abalone post-larvae. The differences observed, in terms of cells dimensions, between the diatoms species suggest each of them may be suited to feed post-larvae at different stages throughout the nursery stage of production as the radula was found to be more capable of handling larger food particles as post-larvae grew older (Roberts et al., 1999a).

Under the culture conditions of this study cell counts of *Amphora* sp. are of the same order as that reported for *Amphora. coffeaformis* (1.43 x 10^6 cells mL^{-1}) by Renaud et al., (1999) while *Nitzschia* sp. presented lower cell productions than *Nitzschia. frustulum* and *Nitzschia* sp. (2.24-3.12 x 10^6 cells mL^{-1}) (Renaud et al., 1999). However, *Amphora* sp. and *Nitzschia* sp. GR during log-phase of growth varied in function of inoculums densities and the best GR obtained were higher than the ones of *Amphora. coffeaformis* (0.38), *Nitzschia. Frustulum* (0.52), *Nitzschia* sp. (0.57) and *Nitzschia laevis* reported by Renaud et al., (1999) and Correa-Reyes et al., (2001). Due to the differences in algae species and culture settings across trials, the comparison of GR and cell counts values between studies are subjective, however the GR obtained in the present study, being found to be intermediate between the ones of tropical (0.38-0.57) and temperate, planktonic diatoms/microalgae species (1.7-3.8) (Brown et al., 1996; Renaud et al., 1999; Renaud et al., 2002) suggest that these diatoms species may be suitable as feed for abalone post-larvae production in subtropical region.

N. incerta cell numbers mL^{-1} in log-phase of growth, at all original inoculating densities tested, were higher than the ones of *Navicula jeffreyi*, (0.16-0.315 x 10^6 cells mL^{-1}) found by Watson et al., (2004) after 1 week of growth at different lower light intensities and the ones of *N. incerta* (0.25 x 10^6 cells mL^{-1}) reported by Carbajal-Miranda et al., (2005) under a constant light intensity of 150 µmol photon m^{-2} s^{-1}. Moreover a higher number of *N. incerta* cells had

attached to the substrate in comparison to the one found by Searcy-Bernal et al., (2003) under lower light intensity. For all the species tested GR were higher than the ones of benthic diatoms grown at higher (150 μmol photon m^{-2} s^{-1}) (Mercado et al., 2004) and lower (11.4-31.3 μmol photon m^{-2} s^{-1}) light intensities (De la Peña, 2007). Although varying culture methodologies employed between the different studies set hurdles for comparisons, the results obtained in these studies suggest optimized microalgae's growth within margins of light intensities and the fact that these may be different among diatom species as observed in several studies (Correa-Reyes et al., 2001; Mercado et al., 2004; Sánchez-Saavedra & Voltolina, 2006).

The high GR found for all species at 0.05 x 10^6cells mL^{-1} and 0.10 x 10^6cells mL^{-1} together with their decrease when inoculating density was increased to 0.25 x 10^6cells mL^{-1} suggest a nutrient limitation and/or the attenuation of average irradiance in the cultures due to the increase in cell density for all species. Significantly higher cell concentration obtained in *Proschkinia* sp. cultures could be related to differences in cell size since the higher area to volume ratio of smaller cells facilitates nutrient assimilation (Richmond, 1986). Based on the values obtained in the present study it seems that the growth and production capacities are species dependant and can be enhanced by manipulating the cell densities conditions, with inoculums densities of 0.10 x 10^6cells mL^{-1} being the best suited to sustain both increased GR and cell production.

Based on their growth form and adhesion strength, the four species used in the present study fall into the categories A and B described by Kawamura & Hirano (1992). Type A diatoms are described as cells with gliding movement and very low adhesive strength, while type B diatom cells are secreting high amounts of mucus to form a film firmly attaching the cells to the substrate. This was the case of *Amphora* sp. (Type B) which in turn showed the highest cell attachment capacity, across all treatments, of all the species tested while the other diatom species presented differences in attachment capacity between densities treatments.

Nevertheless, attachment capacity values were studied on horizontal substrates, whereas they may differ when vertical substrates are used in larger production scale conditions (Daume et al., 2000).

Production of nutrients

Decreases of cell protein contents and increase in lipid and carbohydrate between log-phase and stationary phase of growth were described in previous studies (Brown et al., 1996; Brown et al., 1997) and reported as typical of cultures becoming nutrient limited (D'Souza &

Kelly, 2000; Enright et al., 1986). In the present study all diatoms, except *N. incerta,* presented a decrease in protein and lipid content and an increase in carbohydrate between log-phase and stationary phase of growth. *N. incerta,* increased its storage products as lipid instead of carbohydrate and kept unchanged protein content between log-phase and stationary phase of growth; that could be explained by its significantly higher GR at low density and lower cell production in all density treatments or it could be an indication of silicon deficiency prior to the one of nitrogen and phosphorous. In support of these hypotheses, Zhu et al., (1997) stated that, once stationary phase is reached, rapidly growing cells present more carbohydrates and/or lipids. Besides, an accumulation of lipids was described by Coombs et al., (1967) in silicon deficient diatom cultures. Protein has been found to be the main chemical component in nutrient sufficient cells (Darley, 1977). This trend was only observed in the present study for *Amphora* sp. and *Proschkinia* sp. during log-phase of growth, while carbohydrate content was dominant for *Nitzschia* sp., indicating that this last one could become nutrient limited very early in its culture cycle. The marked differences in lipid and carbohydrate content between the two growth phases for *Proschkinia* sp. might be attributed to a more pronounced nutrient limitation of the culture explained by its high cell production

The average protein and lipid contents of the diatoms studied were lower than the ones (22.5-30%) described by Brown et al., (1997) and Renaud et al., (1999), whereas the levels of carbohydrate were higher. Similarly, Brown et al., (1997), observed lower protein level in static cultures compared to aerated ones as well as higher levels of carbohydrate in cultures of *Thalassiosira pseudonana* grown under continuous light whereas cultures grown under a 12:12h light: dark regime had higher protein contents. On the contrary, the protein levels found in the present study were similar to the ones (15-16%) found by Uriarte et al., (2006) and Viana et al., (2007a) for *Nitzschia* sp., *N. incerta* and *Amphora* sp. With respect to the average lipid levels, differences in lipid contents were observed between planktonic and benthic diatoms cultures as the lipids contents observed in the present study, were similar to the ones reported by Viana et al., (2007a) while they were lower than the ones reported for *Chaetoceros* sp. (11.9-13.1%) by Renaud et al., (2002). Ash values were within the range (41-57%) reported by Viana et al., (2007a) but higher than the ones (16-40%) reported by Uriarte et al., (2006).

According to the studies establishing the optimum requirements for protein, carbohydrate and lipid in the diet of post-larval and juvenile abalone, (Daume et al., 2003; Gordon et al., 2006; Mai et al., 1995b; Viana et al., 2007a) the diatoms from the present study that would best fit the abalone post-larvae nutritional requirements would be *Amphora* sp. and *Proschkinia* sp. in their log-phase of growth, as they present the highest protein and lipid

content, and carbohydrate content within the range needed for juvenile abalone as well as good energy contents.

Fatty acid composition

Fatty acids play an important role in animal nutrition as energy sources, membrane constituents, and metabolic intermediates. The long chain PUFA, specially DHA and EPA essential fatty acids are of major importance during early development (Marty et al., 1992; Sargent et al., 1999) and cannot be synthesized in sufficient quantities to maintain growth and survival (Watanabe et al., 1983).

The fatty acid profiles of the diatoms tested were characteristic of most diatoms: high proportions of 16:1n-7 and 16:0, with variable, but usually high, proportions of 14:0 and EPA, being the predominant n-3PUFA in the diatoms studied (Brown et al., 1996; Brown et al., 1997). EPA is reported to promote a fast growth in *H. discus hannai* juveniles (Dunstan et al., 1996; Mai et al., 1996). EPA might be of special relevance during early abalone post-larvae feeding (Viana et al., 2007a) as it seems that this fatty acid could readily be absorbed from the diatoms (Gordon et al., 2006). N-6 PUFA and particularly ARA are important for larval stages of fish and could also be for abalone as Viana et al., (2007a) noted its increase in post-larvae tissues once these last ones started grazing. EPA and ARA contents of the diatoms tested were within average values (4-10% and 13-17%) reported for other diatoms (Gordon et al., 2006; Liang et al., 2001; Renaud et al., 1999). As typically reported for diatoms, the proportion of DHA was low in all diatoms tested, being the lowest for *N. incerta*. DHA is also low in abalone tissues and therefore presumed to be of a lesser quantitative importance in abalone nutrition (Dunstan et al., 1996; Mai et al., 1996). Based on previous nutritional studies realized on various abalone species (Daume et al., 2003; Gordon et al., 2006; Mai et al., 1996; Viana et al., 2007a), the levels of PUFA, n-3 PUFA and more specifically EPA and DHA found in this study suggest the diatoms tested could be suited for *H. tuberculata coccinea* post-larvae and fulfil their nutritional requirements.

All diatoms in the present study showed high levels of saturated fats, especially 14:0 and 16:0, which were comparable to the ones (21-37%) found for *Amphora* sp. cultured outdoor (De la Peña, 2007). This feature could be beneficial for abalone post-larvae since higher levels of saturated fatty acids was suggested to be beneficial for rapidly growing larvae because energy is released more efficiently (Thompson et al., 1993). Moreover, they were found in high proportion (25%) in tissues of post-larval *Haliotis fulgens* (Viana et al., 2007a).

In summary, adjusting species, culture conditions and harvest period for large scale diatom production in abalone hatcheries would allow a better adaptation of microalgae feed production to abalone post-larval nutritional requirements, subsequently leading to the improvement of post-larvae growth and survival and therefore to the sustainability of abalone post-larvae production. The results indicate the adequacy of the selected diatoms species, commonly used for other abalone species, for *H. tuberculata coccinea* post-larvae, in terms of, algal cells biometric parameters, production, attachment capacity and nutritive value. Further studies based on post-larvae feeding experiments would be useful to better estimate the nutritional value of these diatoms and the nutritional requirements, in terms of biochemical compounds, of *H. tuberculata coccinea* post-larvae.

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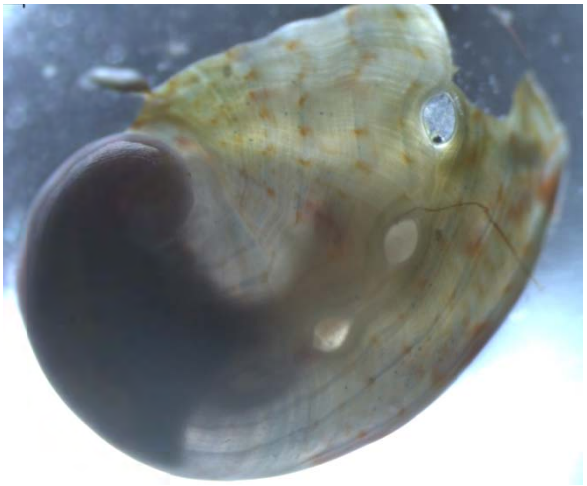
8. STUDY V: IMPROVING NURSERY PERFORMANCES OF *HALIOTIS TUBERCULATA COCCINEA*: NUTRITIONAL VALUE OF FOUR SPECIES OF BENTHIC DIATOMS



Improving nursery performances of *Haliotis tuberculata coccinea*: Nutritional value of four species of benthic diatoms

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Abstract

Four diatoms species *Amphora* sp., *Navicula incerta*, *Nitzschia* sp. and *Proschkinia* sp., were tested as food for *Haliotis tuberculata coccinea* post-larvae. Larvae were settled on *Ulvella lens* and *Ulva rigida* germlings at a density of 0.5 larvae cm^2 of substrate. Growth and survival, in each dietary treatment, were monitored during ten weeks. Experimental nursery tanks were weekly inoculated with 2L of the different diatoms inoculums (10^5-10^6 cells ml^{-1}). Proximate biochemical compositions were significantly different for the four diatom species. *Amphora* sp. presented a significantly higher lipid and protein contents with 7.11±0.29% DW and 18.50±0.97% DW respectively, while *Proschkinia* sp. presented the lowest protein content and the highest ash content. The fatty acid profiles of the diatoms were variable but remained typical of the diatom family. Larvae contained significantly more lipids (25.30±0.93% DW) than juveniles. The different fatty acid compositions found for the various diatom species were dissimilar to the ones of larvae, juveniles and substrates, the fatty acid compositions of these last two not being different among treatments. 16:4n-3, 20:4n-6 (arachidonic acid, ARA) and 22:5n-3 (docosapentaenoic acid, DPA) were the most abundant fatty acids in juveniles while 20:5n-3 (ecosapentaenoic acid, EPA) was the dominant polyunsaturated fatty acid (PUFA), present at a comparable level in all diatoms. This suggests that the post-larvae and juveniles are capable to absorb a selection of fatty acids from the food source or that they are able to synthesise them from precursor dietary fatty acids. Growth rates obtained in the present study are presenting different phases and diatoms diets made little difference to growth rate of post-larvae until reaching around 0.8-1 mm SL, after which divergence in growth rate among diets became significant. Post-larvae fed the diatom with the highest protein and lipid contents (*Amphora* sp.) showed the best daily growth rate (DGR) of 50± 3µm over the feeding trial, highlighting the joint influence of diatom protein and lipid content on growth. Around 2 mm

SL, juveniles exhibited increased growth rates, in all treatments, reaching 94 μm day^{-1}, indicating a possible shift in nutrition towards green algae germlings and suggesting that *U. lens* and *U. rigida* are used as a complement of the diatom diet at this stage. 18:2n-6 and 18:3n-3 found in high amount in all juveniles as well as in all substrates could be an indication of that nutritional shift and could be involved in growth enhancement.

Keywords*: Haliotis tuberculata coccinea;* Abalone*;* Post-larvae; Juveniles; Nutrition; Diatoms; Early growth*; Ulvella lens; Ulva rigida*

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Introduction

Benthic diatoms are the main food source of abalone post-larvae once metamorphosis has been completed (Hahn, 1989; Kawamura & Takami, 1995; Seki & Kan-No, 1981). The access to adequate nutrition is crucial for growth and survival of abalone post-larvae and juveniles (Hahn, 1989; Kawamura et al., 1998a). Studies on abalone post-larval feeding have focused on the effect of different diatoms and other algae on post-larval growth and survival and on the factors affecting their food value (Courtois de Vicose et al., Submitted-a; Daume et al., 2000; Ishida et al., 1995; Kawamura et al., 1998b; Kawamura et al., 1998a; Kawamura et al., 1995; Kawamura & Takami, 1995; Ohgai et al., 1991; Roberts et al., 1999). The dietary value of benthic diatoms has been found to depend on their physical characteristics such as size or attachment strength as well as their digestibility, itself influenced by the physical characteristics of the cells (Kawamura et al., 1998a; Kawamura et al., 1995; Matthews & Cook, 1995; Seki & Kan-No, 1981). In turn, dietary value of diatoms, affecting post-larval growth and survival, also depends on the stage of abalone post-larvae development as ingestion and digestion capacities improve during development, widening the range of diatoms species consumed (Kawamura et al., 1998b; Roberts et al., 1999). Three major transitions have been reported in post-larval and juvenile feeding: the transition from lecitotrophy to particle feeding, the beginning of the effective ingestion and digestion of diatoms cells, appearing around 600-800µm shell length, and the nutritional shift from diatoms to macroalgae (Kawamura et al., 1998a). Differences in growth rates in response to different algae have been observed only from the second feeding stage and onwards, since the first transition is influenced by the residual benefit of the yolk supply. (Daume et al., 2000; Kawamura et al., 1998a; Roberts et al., 1999).

Both quality and quantity of the diatoms provided are determinant key factors for survival and growth of abalone post-larvae and juveniles (Kawamura et al., 1998a; Kawamura et al., 1995; Roberts et al., 1999). Changes in the biofilm on which post-larvae feed, such as, cell density, extracellular polysaccharides and associated bacteria also affect the dietary value of diatoms as well as species or strain, culture conditions and developmental stage of the culture (Daume, 2006; Daume et al., 2000; Kawamura et al., 1998a; Kawamura et al., 1995; Kawamura & Takami, 1995; Searcy-Bernal et al., 2001). Besides, biochemical and fatty acid composition of algae is known to be influenced by the culture conditions and developmental stage of the culture (Brown et al., 1996; Courtois de Vicose et al., Submitted-a; Courtois de Vicose et al., Submitted-b; Liang et al., 2002; Liang et al., 2001; Mercado et al., 2004;

Simental-Trinidad et al., 2001). The effect of the biochemical and fatty acid composition of algae has been studied on juvenile abalone (Dunstan et al., 1996; Mai et al., 1996, 1995b, a, 1994). Nevertheless, studies on post-larvae are scarce and none of them deals with Haliotis tuberculata despite its growing interest in European aquaculture (Daume et al., 2003; Gordon et al., 2006; Uriarte et al., 2006; Viana et al., 2007).

Hence, in an attempt to improve the performances of *Haliotis tuberculata coccinea* post-larvae and juveniles, in the nursery, the effects of different benthic diatoms, and their biochemical composition, were evaluated, in terms of growth and survival.

Material and methods

Abalone rearing

Broodstock *H. tuberculata coccinea* were induced to spawn by the UV irradiated seawater technique (Kikuchi & Uki, 1974) at a ratio of 2:1 females: male. Larvae were hatched and reared in 1μm filtered, UV treated, seawater (22 ± 0.5 ºC) at the Instituto Canario de Ciencias Marinas (Canary Islands, Spain). Larvae used to conduct this study came from the same batch and were considered competent for settlement when the third tubule of the cephalic tentacle became visible (Courtois de Vicose et al., 2007; Hahn, 1989).

Algal cultures

A combination of 45-days-old *Ulvella lens* and *Ulva rigida* germlings, found to induce successful settlement of *H. tuberculata coccinea* (Courtois de Vicose et al., Submitted-b) were obtained from mature, spore producing thalli. *U. lens* and *U. rigida* germlings were obtained according to methods previously described (Daume et al., 2004; Strain et al., 2006). The mix cultures of *U. lens* and *U. rigida* germlings on all settlement plates were maintained aerated under natural photoperiod during 45 days, weekly renewing the f/2 culture medium used.

Four species of diatoms *Navicula incerta*, *Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp. were supplemented as diets to the post-larvae. All diatoms were cultured, in 40 L horizontally laid algal bags, at initial inoculums of 10^5 cells ml^{-1} and grown for 5 days in f/2 medium supplemented with silicate (1 mgL^{-1}) (Guillard, 1975) at ambient temperature and under continuous light of 62 ± 8 μmol photon m^{-2} s^{-1}. Photon flux density (irradiance) was measured using a Digital light meter (HT170N, HT ITALIA, Italy). Unialgal but not axenic

cultures were employed. Diatom cell concentration was weekly evaluated with a Neubauer hemocytometer. Prior to the counts, ultrasound (1 to 3 min) was applied to the diatoms samples to avoid possible cell aggregations.

Post-larval and juvenile feeding trial

Competent larvae were allowed to settle on plates colonized with the described mix of *U. lens* and *U. rigida* germlings. Each, 100 L, settlement tank contained four colonized settlement plates (60 x30 cm) plus four 50 cm^2 platelets placed vertically. Settlement tanks were filled with 1μm filtered seawater at 21±1 ºC, gently aerated. Competent larvae were introduced at a density of 7200 larvae 100 L^{-1} tank (0.5 larvae cm^{-2} of settlement substrate). Number of larvae was estimated by counting them in three 3-ml subsamples drawn from the entire larval batch. Twenty four hours after the introduction of the larvae, water flow was initiated at a rate of 1% exchange rate per hour and increased to 20% exchange rate per hour after 72h. Seawater outlet was provided with a 125-μm mesh screens to prevent the loss of larvae.

Three days after the introduction of the larvae, diatoms were added as food to the 100 L tanks by the weekly inoculation of 2 L of the respective diatom inoculums (10^5-10^6 cells ml^{-1}), previously described. Each diatom treatment was tested in triplicate. Post-larvae were reared under a photoperiod of 12:12 L: D at a light intensity of 2000 Lux (Digital Light Meter, HT170N, HT ITALIA, Italy).

Post-larval growth and survival were recorded for ten weeks after settlement. The number of live post-larvae was counted, on every platelet of each replicate, under a dissecting microscope at fortnightly intervals. The shell length of 8 randomly selected post-larvae per replicate was measured fortnightly with a profile and measuring projector (MITUTOYO, PJ-H3000, Tokyo, Japan). Plates were kept immersed at all times and replaced immediately after observation and measurement.

Daily growth rate (DGR) was calculated according to the formula:

$$\frac{Lf - Li}{t}$$

where *Lf* is final shell length in micrometers, *Li* is initial shell length in micrometers, and *t* is time in days.

In order to estimate the percentage of algal cover, ten randomly chosen fields of view of the settlement plates, from each treatment, were photographed at a magnification of 400 x at the time of settlement and at the end of the experiment. Percentage cover of mix *U. lens* and *U. rigida* germlings were then calculated by processing the images with Image J Software (National Institutes of Health, USA, Image J 1.42q).

Sampling and biochemical analysis

Triplicate samples of substrate, from each treatment, were collected by scraping the surface of settlement plates at the end of the feeding trial. Triplicate samples of diatoms cells were collected from the algal bags along the trial. Both were analysed for total lipids, protein, carbohydrate, ash and total fatty acids. Triplicate samples from the initial larval batch were collected at the beginning of the experiment, and triplicate samples of juveniles, from each treatment, at the end of the experiment. All samples were stored at -80ºC until analyzed. Total lipids content was determined gravimetrically after extraction by chloroform: methanol (2:1) (Folch et al., 1957). Total protein was calculated from total Kjeldahl nitrogen according to standard methods (AOAC., 2005). Ash content was determined gravimetrically after incinerating the samples at 600ºC during 24h. Fatty acids in the lipid extracts were transesterified to methyl esters (FAMEs) with 1% sulphuric acid: methanol (Christie, 1982). FAMEs were separated with capillary column (Supercowax 28m x 0.32mm x 0.25 i.d.) using helium as the carrier gas under the conditions described by Izquierdo et al. (1989). Fatty acids were analyzed in a Thermo Finnigan- GC Focus gas chromatograph equipped with a flame ionization detector (260ºC).

Statistical analysis

Statistical analysis was performed using the Statgraphics Plus 5.1. Software. Analyses of variance (one-way ANOVAs) were performed to compare proximate biochemical composition, as well as survival and DGR between treatments. Data showing significant differences ($P<0.05$) were analyzed by paired comparisons using Tukey's HSD test. Cluster analysis was carried out on the larvae, juveniles, diatoms and substrates fatty acid composition data in order to establish groups by proportion of similarity. Multiple regression analyses were carried out to explain the variation in the average growth between treatments. The average proximate biochemical composition of diatoms; proteins, lipids, ash and carbohydrates

contents, were the factors selected for the analyses. Principal component analysis was performed on the data of fatty acids composition of diatoms and substrates and principal components were identified. Kendall rank correlation between the main principal components and growth data was then performed in order to identify the effects of fatty acids composition determinant among treatments. Assumption of normality and homogeneity of variance were assessed with standardized skewness and kurtosis and Bartlett's test.

Results

Post-larval and juvenile feeding trial

After ten weeks of feeding trial, the shell length of post-larvae fed *Amphora* sp. was significantly highest (3.7±0.3mm) ($F_{3,\ 28}$=24.00, P<0.001) (Fig 1.). Post-larvae from the *Amphora* sp. treatment also showed a significantly highest average daily growth rate (DGR) (50.19± 3.31μm) along the feeding trial ($F_{3,\ 28}$=24.29, P<0.001) (Table 1). Growth rate during the first four weeks of the trial was not significantly different between diets (P>0.05) although on the fourth week the post-larvae's shell length of abalone fed *Amphora* sp. was significantly higher than the one of the post-larvae fed *N. incerta*, with 0.86 mm and 0.73 mm average shell length respectively ($F_{3,\ 28}$=4.12, P=0.01). After the fourth week of trial, growth rates varied significantly between diets (Table 1.). On the sixth week after settlement, DGR of the abalone fed *Amphora* sp. and *Nitzschia* sp. were significantly higher than the ones of abalone fed *Proschkinia* sp ($F_{3,\ 28}$=3.75, P=0.02) while the growth rates of the ones fed *N. incerta* were not significantly different from those of *Amphora* sp. and *Proschkinia* sp. fed specimens (P>0.05). On the eight week a decrease in post-larval growth rate was observed in the *Nitzschia* sp. fed juveniles as well as an increase in growth of those fed *N. incerta*. This resulted in an overall significantly higher growth rates in the *Amphora* sp. and *N. incerta* fed juveniles ($F_{3,\ 28}$=5.47, P<0.01) while the ones fed *Proschkinia* sp. presented an intermediate growth rate not significantly different from *Amphora* sp. or *Nitzschia* sp. fed juveniles (P>0.05). From week eight growth rate markedly increased, regardless of the diatom treatment and at week ten no significant differences were found among the different treatments (P>0.05). Post-larval survival was not significantly different among treatments along the feeding trial (P>0.05), except for a significantly lower survival of post-larvae fed *N. incerta* on the second week of the experiment ($F_{3,\ 28}$=6.06, P=0.018).

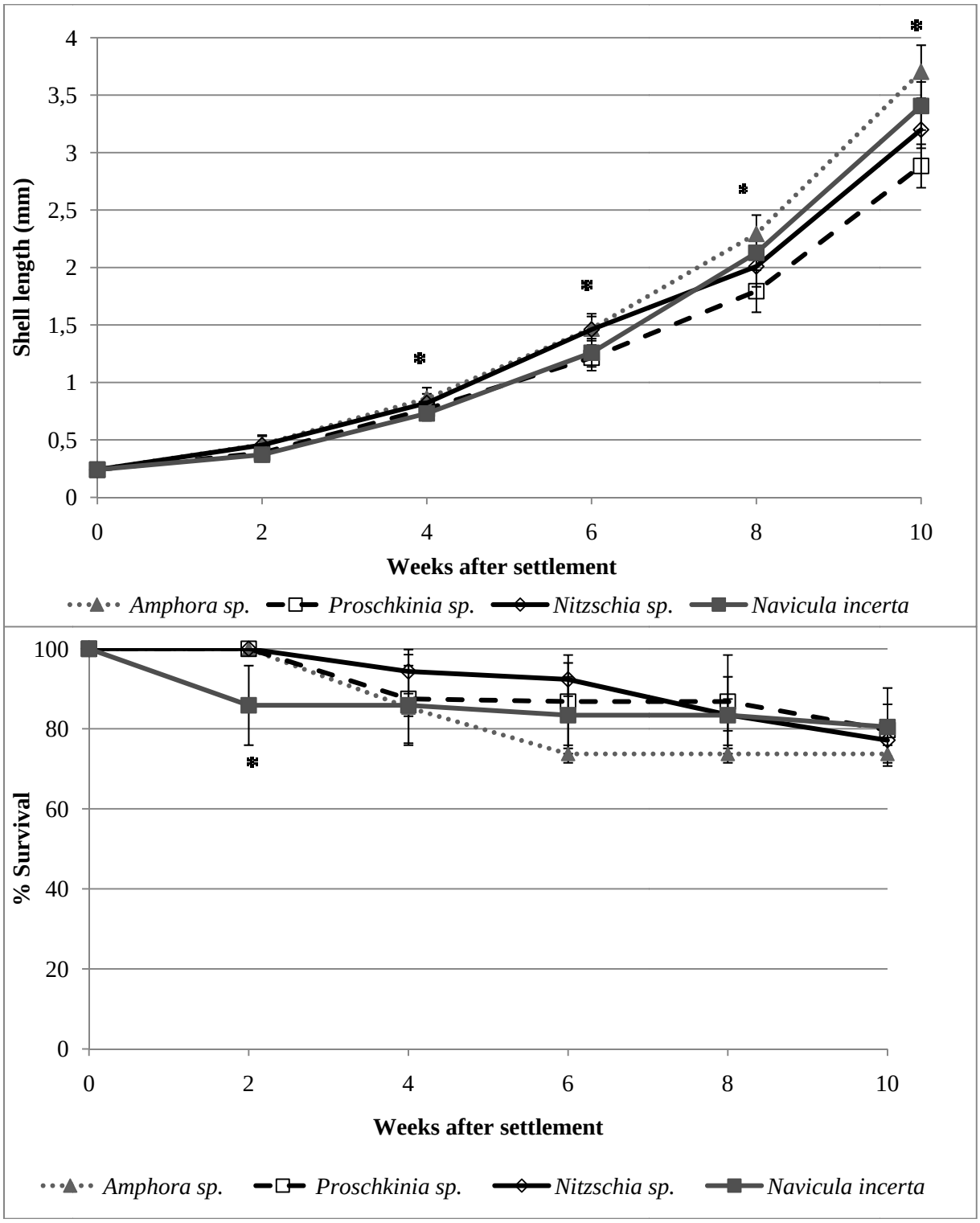

Figure. 1. Shell length (A) and survival (B) of *H. tuberculata coccinea* post-larvae over the ten week feeding trial on four different species of diatoms. Vertical bars indicate the standard deviation. (n=8 and n=3). Weeks marked with * presented statistical differences in growth and survival among diatoms treatments.

Table 1. Growth rates of *H. tuberculata coccinea* post-larvae fed four different diatoms diets (mean±SD, n=8)

Treatment	DGR (μm day^{-1}, ± SE)					
	Wks 0- 2	Wks 2-4	Wks 4-6	Wks 6-8	Wks 8-10	Average DGR
***Amphora* sp.**	18.15±7.18^{a}	28.71±10.56^{a}	43.40±10.36^{ab}	58.83±13.87^{ab}	94.14±11.97^{a}	50.19±3.31^{a}
N. incerta	10.84±4.79^{a}	25.62±4.56^{a}	37.76±9.63^{bc}	62.10±14.94^{a}	85.18±21.54^{a}	45.86±3.0^{b}
***Nitzschia* sp.**	17.83±6.81^{a}	26.15±9.42^{a}	45.68±7.52^{a}	39.21±16.08^{c}	79.35±17.44^{a}	42.88±2.33^{b}
***Proschkinia* sp.**	12.15±7.08^{a}	27.24±9.15^{a}	31.89±8.23^{c}	41.37±11.61^{bc}	72.67±19.51^{a}	38.30±2.33^{c}

Different superscripts down a column indicate means which differ significantly at 95% level (ANOVA Tukey's test; P<0.05)

Biochemical and fatty acid composition

The average proximate biochemical compositions of each diatom species are given in Table 2.

Table 2. Proximate chemical analysis (% dry weight) of the four diatom species fed to the post-larvae during the growth experiment (mean±SD, n=3)

Diatoms	% Lipids (DW)	% Proteins (DW)	% Ash (DW)	% Carbohydrates (DW)
***Amphora* sp.**	7.11±0.29^{a}	18.50±0.97^{a}	56.18±1.73^{b}	18.21±0.91^{b}
N. incerta	4.35±0.17^{b}	13.31±0.87^{b}	53.92±0.72^{b}	28.42±1.28^{a}
***Nitzschia* sp.**	3.52±0.26^{c}	13.58±0.33^{b}	58.49±1.59^{ab}	24.41±1.46^{a}
***Proschkinia* sp.**	3.60±0.16^{c}	8.41±0.75^{c}	63.43±3.73^{a}	24.56±3.60^{a}

Different superscripts down a column indicate means which differ significantly at 95% level (ANOVA Tukey's test; P<0.05)

Overall, *Amphora* sp. presented significantly highest lipid ($F_{3, 8}$=162.33, *P*<0.001) and protein contents ($F_{3, 8}$=85.87, *P*<0.001), (7.11±0.29% DW and 18.50±0.97% DW) respectively, while *Proschkinia* sp. had the lowest protein content ($F_{3, 8}$=85.87, *P*<0.001) and a significantly lower lipid content (3.60±0.16% DW) ($F_{3, 8}$=162.33, *P*<0.001), not being significantly different from the one of *Nitzschia* sp. (3.52±0.26% DW) (*P*> 0.05). The protein contents of *N. incerta* and *Nitzschia* sp. were not significantly different (*P*> 0.05). The ash content of *Proschkinia* sp. was the highest and was significantly higher ($F_{3, 8}$=9.95, *P*<0.05) than the ones of *Amphora* sp. and *N. incerta*, that did not significantly differed (P> 0.05). The carbohydrate content of *Amphora* sp. was significantly lower ($F_{3, 8}$=12.21, *P*<0.05) than the ones of *N. incerta*, *Nitzschia* sp. and *Proschkinia* sp.

Independently of the diatoms they had been colonised with, the substrates from each treatment, consisting in a mixture of *U. lens* and *U. rigida* germlings plus the corresponding diatom, did not show significant differences in their proximate biochemical analysis at the end

of the growth experiment (P> 0.05) (Table 3). Besides, the percentage cover of mix *U. lens* and *U. rigida* germlings had gone from 30±9 % at the beginning of the experiment to 84±20 % at the end of the experiment independently of the diatoms inoculated in each treatment.

Table 3. Proximate chemical analysis (% dry weight) of the substrates plus the four diatom species the post-larvae and juveniles have fed on during the growth experiment (mean±SD, n=3)

Substrates	% Lipids (DW)	% Proteins (DW)	% Ash (DW)	% Carbohydrates (DW)
Substrate + *Amphora* sp.	6.84±1.64	21.15±4.39	36.01±3.20	35.99±5.41
Substrate + *N. incerta*	6.16±0.12	22.03±0.55	33.62±2.32	38.17±2.33
Substrate + *Nitzschia* sp.	5.15±0.77	24.92±5.73	28.97±8.35	40.94±2.56
Substrate + *Proschkinia* sp.	6.70±0.56	20.97±4.64	36.12±3.14	36.19±4.88

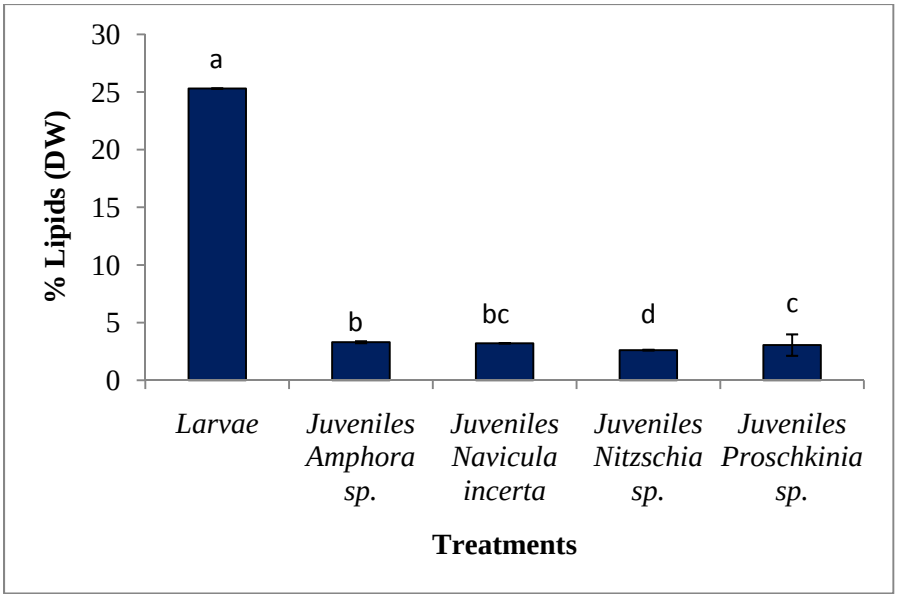

Figure. 2. Lipid contents (% dry weight) of the larvae and juveniles at the end of the feeding trial with the four diatom species. Vertical bars indicate the standard deviation. Values with different letters are significantly different (P<0.05) (n=3).

The initial larvae contained significantly ($F_{4,\ 10}$=5855.12, P<0.001) more lipids (25.30±0.93% DW) than the juveniles from all treatments (Fig.2). Lipid contents of abalone juveniles followed a similar trend than the diatoms. Thus, lipid content in juveniles fed *Amphora* sp. (3.29±0.03% DW) was significantly higher than those fed *Proschkinia* sp. (3.05±0.08% DW), in turn significantly higher than the juveniles fed *Nitzschia* sp. (2.61±0.01% DW) ($F_{4,\ 10}$=5855.12, P<0.001).

The mean amounts of fatty acids of larvae, juveniles, diatoms and substrates are summarised in Table 4.

Table 4. Fatty acid composition (% of total fatty acid) of: initial larvae, juveniles at the end of the feeding trial, diatoms tested throughout the experiment and substrates at the ending date of the feeding trial.

		Larvae	**Juveniles**				**Diatoms**				**Substrates +**			
		Larvae	*Amphora* sp.	*Navicula incerta*	*Nitzschia* sp.	*Proschkinia* sp.	*Amphora* sp.	*Navicula incerta*	*Nitzschia* sp.	*Proschkinia* sp.	*Amphora* sp.	*Navicula incerta*	*Nitzschia* sp.	*Proschkinia* sp.
	14:0	9.12	3.10	4.57	4.54	5.04	7.37	5.83	10.00	2.15	6.64	5.03	7.82	5.86
	15:0	0.57	0.39	0.23	0.21	0.26	0.47	0.16	0.42	0.57	0.36	0.21	0.23	0.30
Palmitic	16:0	34.83	28.33	22.11	21.34	22.34	20.54	18.06	10.74	21.85	46.68	33.10	24.46	32.53
	16:0ISO	0.03	0.25	0.18	0.18	0.16	0.00	0.00	0.00	0.00	0.10	0.10	0.07	0.09
	17:0	0.00	0.31	0.26	0.20	0.22	2.47	6.56	2.63	3.03	0.67	1.35	1.11	1.05
Stearic	18:0	2.67	8.71	6.11	5.79	5.82	0.97	0.88	2.18	0.90	1.63	1.28	0.80	1.14
	20:0	0.04	0.73	0.95	0.88	0.80	0.71	0.03	0.11	0.13	0.51	0.19	0.19	0.28
∑ Saturated		**47.27**	**41.83**	**34.41**	**33.14**	**34.64**	**32.53**	**31.52**	**26.08**	**28.63**	**56.59**	**41.26**	**34.69**	**41.25**
	14:1n-7	0.25	0.23	0.25	0.22	0.26	0.00	0.08	0.02	0.45	0.00	0.04	0.01	0.01
	14:1n-5	0.01	0.12	0.17	0.15	0.19	0.82	0.60	0.57	0.41	2.71	1.44	1.17	1.51
	15:1n-5	0.00	0.07	0.05	0.02	0.06	0.04	0.05	0.05	0.03	0.05	0.04	0.02	0.05
Palmitoleic	16:1n-7	4.33	2.37	1.86	2.73	2.94	25.31	32.14	24.72	29.40	16.53	9.62	11.64	13.95
	16:1n-5	0.20	0.22	0.25	0.28	0.28	0.50	0.90	1.03	0.77	0.60	0.57	0.59	0.52
Oleic	18:1n-9	16.42	2.49	2.18	2.08	2.29	3.87	4.27	3.85	1.64	3.46	2.98	3.02	2.37
	18:1n-7	11.99	7.05	9.36	9.93	9.59	0.81	0.84	1.01	1.78	4.99	7.03	8.04	4.25
	18:1n-5	0.28	0.16	0.25	0.22	0.23	0.10	0.83	0.11	0.13	0.12	0.23	0.16	0.16
	20:1n-9+n-7	4.87	3.36	3.73	3.65	3.70	0.20	0.02	0.04	0.16	0.09	0.05	0.09	0.13
	20:1n-5	0.43	0.57	0.77	0.81	0.78	0.01	0.15	0.00	0.01	0.39	0.48	0.62	0.33
	22:1n-11	0.20	0.43	0.45	0.43	0.46	0.07	1.68	0.03	0.05	0.61	0.52	0.46	0.48
	22:1n-9	0.09	0.12	0.19	0.28	0.14	0.26	0.09	0.00	0.00	0.06	0.17	0.06	0.07
∑ Monounsaturated		**39.06**	**17.20**	**19.51**	**20.80**	**20.93**	**31.99**	**41.65**	**31.44**	**34.83**	**29.61**	**23.17**	**25.86**	**23.82**
	16:2n-6	0.16	0.15	0.11	0.17	0.17	1.16	1.73	1.32	0.40	0.48	0.46	0.79	0.56
	16:2n-4	0.61	1.79	0.99	0.71	0.96	1.94	2.82	5.84	1.40	0.60	0.60	0.45	0.27
	16:3n-4	0.00	0.06	0.10	0.11	0.08	0.00	0.00	1.47	0.75	0.28	0.29	0.27	0.27
	16:3n-3	0.00	0.05	0.03	0.05	0.02	0.07	0.60	0.28	0.00	0.00	0.03	0.02	0.01
	16:3n-1	0.04	0.74	0.66	0.71	0.80	0.07	0.13	0.16	0.10	0.07	0.01	0.00	0.00
hexadecatetraeno	16:4n-3	1.56	9.07	7.25	6.82	6.28	0.07	1.47	4.63	2.08	0.51	3.07	3.43	1.07
	16:4n-1	0.00	0.11	0.07	0.05	0.07	0.00	0.00	0.00	0.00	0.00	0.03	0.02	0.04
	18:2n-9	0.00	0.02	0.00	0.03	0.03	0.26	0.04	0.56	0.03	2.17	0.01	0.00	0.01
Linoleic	18:2n-6	0.70	3.48	6.34	5.68	6.03	2.84	0.97	1.87	0.93	3.90	10.45	7.80	8.73
	18:2n-4	0.05	0.15	0.13	0.12	0.12	0.03	0.23	0.33	0.05	0.06	0.03	0.03	0.02
	18:3n-6	0.02	0.13	0.12	0.19	0.20	1.06	0.38	0.19	0.68	0.19	0.45	0.44	0.38
	18:3n-4	0.12	0.09	0.05	0.06	0.07	0.03	0.01	0.32	0.05	0.00	0.00	0.00	0.00
Linolenic	18:3n-3	1.45	5.81	9.25	8.77	9.15	0.22	0.63	2.19	0.40	5.37	15.02	13.46	11.61
	18:3n-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.75	0.82	0.00	0.00	0.00	0.00
	18:4n-3	0.22	0.37	0.55	0.72	0.61	4.41	0.72	0.67	0.10	0.45	1.77	1.94	0.77
	18:4n-1	0.00	0.08	0.04	0.04	0.06	0.01	0.01	0.21	0.04	0.12	0.25	0.07	0.21
	20:2n-9	0.25	0.31	0.38	0.33	0.34	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	20:2n-6	0.54	0.49	0.77	0.71	0.73	0.03	0.01	0.15	0.05	0.11	0.14	0.17	0.20
	20:3n-6	0.18	0.15	0.18	0.20	0.19	0.18	0.02	0.23	0.36	0.05	0.07	0.11	0.09
	20:3n-3	0.06	0.09	0.15	0.07	0.09	0.00	0.00	0.42	0.04	0.10	0.34	0.18	0.26
ARA	20:4n-6	2.88	4.16	3.96	4.53	4.45	3.63	0.29	1.25	5.03	1.44	2.47	2.72	2.63
	20:4n-3	0.66	0.19	0.23	0.27	0.25	0.27	0.37	2.75	0.00	0.02	0.09	0.07	0.06
EPA	20:5n-3	1.95	6.09	5.86	5.99	5.33	16.46	21.80	18.87	19.52	1.08	3.30	3.00	2.63
	22:4n-6	0.00	0.81	1.45	1.51	1.32	0.00	0.00	0.00	0.00	0.18	0.86	0.97	0.33
	22:5n-6	0.74	0.64	1.20	1.38	1.23	0.12	0.00	0.15	0.44	0.19	0.78	0.90	0.34
DPA	22:5n-3	1.41	4.37	4.32	4.45	3.84	0.16	0.69	1.35	0.20	0.15	0.77	0.92	0.38
DHA	22:6n-3	0.07	1.57	1.88	2.40	2.01	1.64	0.07	2.34	3.81	0.98	2.53	1.93	1.48
∑ Polyunsaturated		**13.67**	**40.97**	**46.08**	**46.06**	**44.43**	**34.66**	**33.01**	**48.30**	**37.28**	**18.50**	**43.80**	**39.68**	**32.33**
∑ n-3 PUFA		**7.39**	**27.62**	**29.53**	**29.55**	**27.59**	**23.30**	**26.35**	**33.50**	**26.15**	**8.66**	**26.91**	**24.96**	**18.26**
∑ n-6 PUFA		**5.22**	**10.00**	**14.13**	**14.37**	**14.33**	**9.02**	**3.41**	**5.17**	**7.89**	**6.54**	**15.67**	**13.89**	**13.26**
n-3/n-6		**1.42**	**2.76**	**2.09**	**2.06**	**1.93**	**2.58**	**7.73**	**6.48**	**3.31**	**1.32**	**1.72**	**1.80**	**1.38**
18:3n-3/18:2n-6		**2.07**	**1.67**	**1.46**	**1.54**	**1.52**	**0.07**	**0.65**	**1.17**	**0.43**	**1.37**	**1.44**	**1.72**	**1.33**

Among the different diatoms tested, *Amphora sp.* fatty acid profile was characterized by the highest content in saturated fatty acids, particularly with high palmitic acid (16:0) and in linolenic acid (18:2n-6) and the lowest eicosapentaenoic acid (EPA, 20:5n-3). *Navicula incerta* was highest in palmitoleic (16:1n-9) and eicosapentaenoic acids, and lowest in linoleic acid (18:2n-6), whereas *Nitzschia sp.* was lowest in palmitic acid and highest in stearic (18:0) and linolenic (18:3n-3) acids. Finally, *Proschkinia sp*. was highest in palmitic acid, close to *Ampora sp*., and in eicosapentaenoic acid, close to *Navicula incerta*. All diatoms were relatively poor in 18:1n-7 and high in eicosapentaenoic acid. Despite the fatty acid composition of the diatoms markedly differing among species, at the end of the trial the substrate for the different feeding regimes did not greatly differed, denoting the influence of the *U. rigida* and *U. lens* germlings, which developed along the trial. The substrate of *Amphora sp.* feeding regime was highest in saturated and palmitic acids, reflecting the diatom content. The substrate of *Nitzschia sp.* regime was lowest in palmitic acid denoting the *Nitzschia sp* inclusion. Linoleic and linolenic acid contents differed among the substrates being lowest for *Amphora sp.* substrate and highest for *Nitzschia sp.* substrate. Fatty acid composition of the abalone at the end of the study reflected rather the fatty acid composition of the substrates than that of the diatoms, particularly in linoleic and linolenic acids. The abalone juveniles were also high in 16:4n-3, whereas the highest long chain polyunsaturated fatty acids were eicosapentaenoic, arachidonic acid (ARA, 20:4n-6) and docosapentaenoic acid (DPA, 22:5n-3), regardless the feeding regime. Generally, the n-3/n-6 ratio was higher in juveniles than in the substrates and the highest in diatoms. Abalone, both initial larvae and juveniles, were higher in 18:0 (stearic acid) and lower in 16:1n-7 (palmitoleic acid) than the substrates and diatoms. In comparison to the initial larvae, the final juveniles were higher in polyunsaturated fatty acids and lower in saturated and monounsaturated fatty acids, particularly oleic acid (18:1n-9). Initial larvae contained very low level of 22:6n-3 (docosahexaenoic acid, DHA) in comparison to juveniles, diatoms and substrates.

A cluster analysis was carried out on mean fatty acid composition data of larvae, juveniles, diatoms and substrates in order to better explain differences among treatments and the nature of the material. The results are shown in Fig. 3.

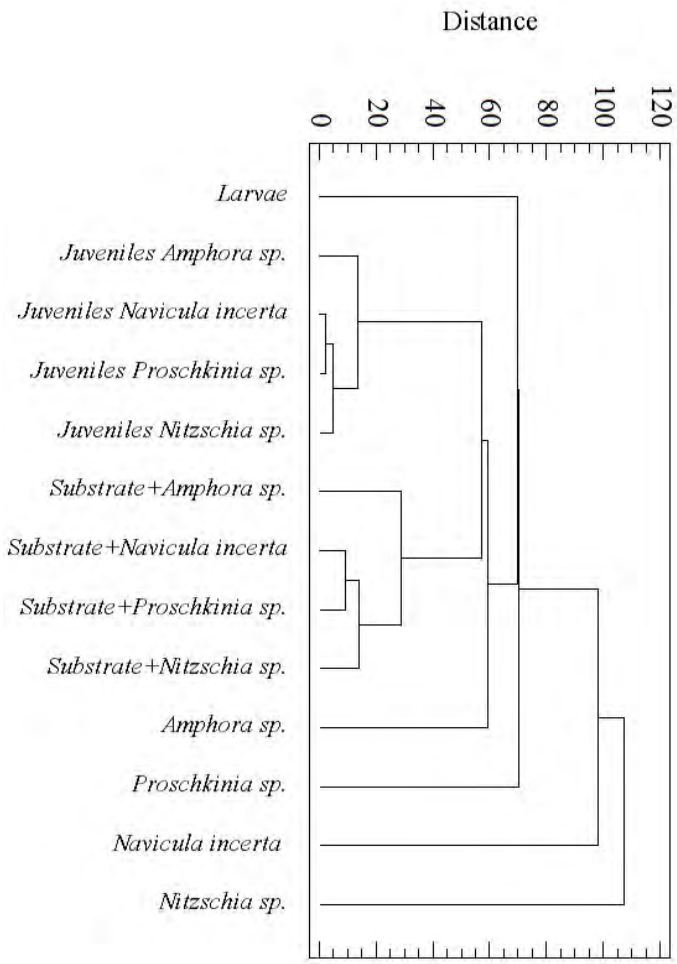

Figure. 3. Cluster analysis showing similarities in fatty acid compositions of larvae, juveniles, diatoms and substrates.

Two groups can be readily distinguished, one corresponding to juveniles regardless of the treatment and the other to the substrates. Each of the four diatoms are not showing similarities among their fatty acid composition, neither with the juveniles or larvae. Besides, the fatty acid composition of the larvae is dissimilar to the ones of juveniles, substrates or any of the four diatoms.

A principal components analysis of the fatty acids compositions of the diatoms and substrates was performed.

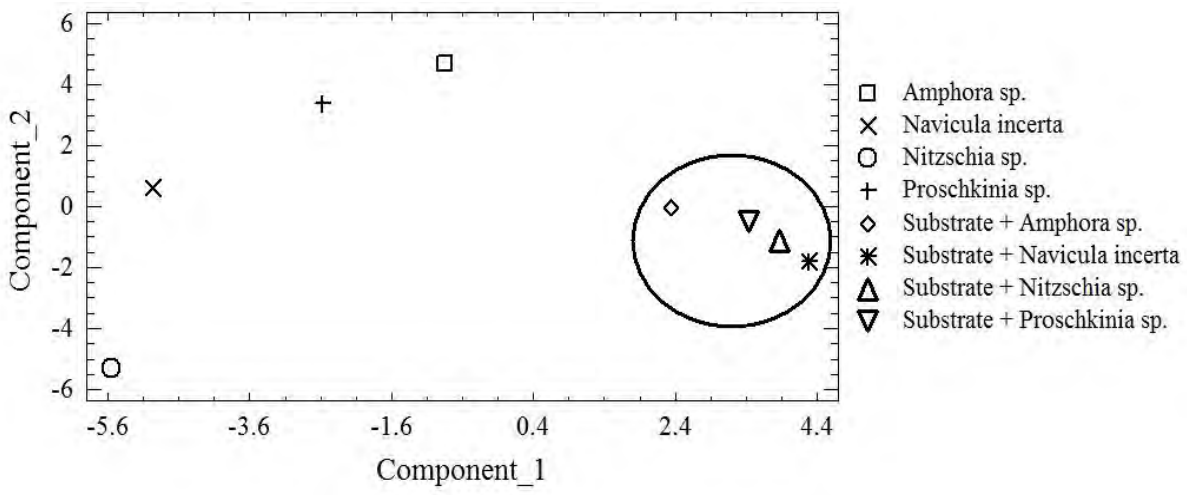

Figure. 4. Graphic presentation of the principal component analysis carried out on the fatty acid compositions of the four diatoms and substrates.

Based on the Eigen values, six components were extracted to describe the data. The first two components, presented in Fig. 4, accounted for 56% of the total variance found in the different fatty acids compositions, the first component explaining 30% of the total variance. Based on the first two components, we can observe the similarity in fatty acids compositions of the substrates (as in Fig.3.) as opposed to the differences recorded between the diatoms. *Nitzschia* sp and *Amphora sp*. were the diatoms showing the most dissimilarity in their fatty acids composition. The fatty acids found in the highest amounts and being the most likely involved in the differentiation of the diatoms between each other and from the substrates are: 14:0, 16:0, 14:1n-5, 16:1n-7, 18:1n-7, 16:2n-6, 16:2n-4, 16:4n-3, 18:2n-6, 18:3n-3, 20:4n-6 and 20:5n-3 as they are the ones on which the first two components are the most heavily weighed.

Biochemical composition significance on growth

Final shell length and average growth rates of juvenile abalone were significantly and positively correlated with the protein content of the diatoms (Fig. 5.) (Adjusted R^2= 0.83, F=48.51, P<0.001 and Adjusted R^2= 0.84, F=51.39, P<0.001 respectively).

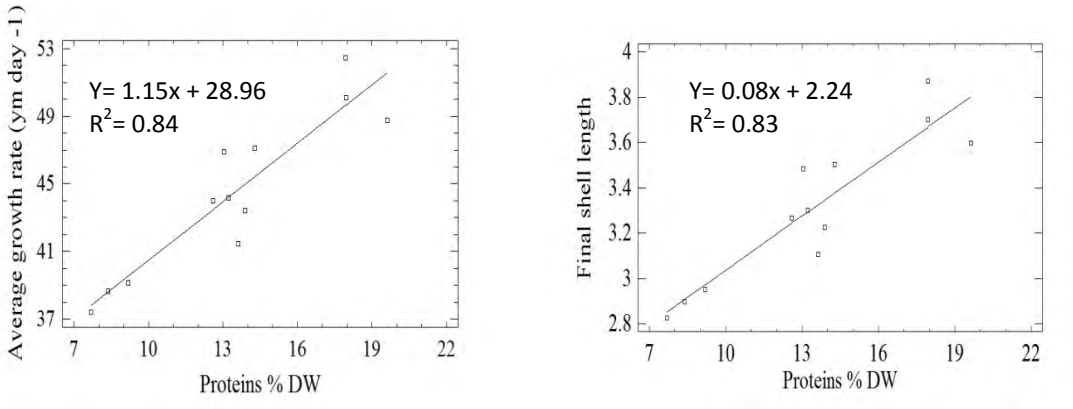

Figure. 5. Parameter affecting post-larval growth and final shell length during ten weeks feeding trial with four diatoms tested as feed. Relation between the protein content of diatoms and post-larval growth and shell length (n=12). Both regressions are significant (P<0.001)

A Kendall rank correlation was carried out between length and average growth rates and the first components of the principal component analysis of the fatty acids. However, neither the correlation of average growth nor the one of final shell length with the first components of the principal component were significant.

Discussion

The present study denoted the importance of *Amphora sp.* as a diatom for first feeding of post-larvae and early juveniles of *H. t. coccinea,* particularly during weeks 4 to 8 after settlement and in relation to its higher protein content. Along the study, post-larvae and early juvenile presented satisfying growth rates (up to 94 μm day^{-1}), being higher than that of *H. iris* cultured at 17.5^{o}C (up to 60 μm day^{-1}) (Roberts et al., 1999) or *H. discus hannai* at 20 ^{o}C (10-50 μm day^{-1}, from 4-6 wks) (Kawamura et al., 1998b). The importance of early diatom feeding has been observed for *H. discus hannai*, *H. iris* and *H. rubra* (Kawamura & Takami, 1995; Roberts et al. 1999; Daume et al., 2000). The present study also pointed out the importance of adequate diatom feeding and its biochemical composition, for growth of post-larvae and early juveniles of *H. tuberculata coccinea,* by the significant divergences in growth rates among abalone fed the different feeding regimes appearing in post-larvae of 0.8-1mm SL and onwards. No effect of the feeding regime was detected before week 4 (0.8-1 mm SL) since during this period post-larval nutrition is greatly derived from the biofilm components such as extra cellular mucus of diatoms and associated bacteria (Daume et al., 2000; Kawamura & Takami, 1995). Besides at this stage post-larvae still rely on residual nutritional reserves from the yolk and present

incomplete development of their digestive system or of their radula, only taking place as the larvae grow (Kawamura et al., 1998b; Roberts et al., 1999). The marked increase in post-larvae's growth rates occurring during week six to eight on *N. incerta* fed abalone illustrates the evolution of these morphological developments corroborating that the dietary benefits of diatoms are size dependant for *H. tuberculata coccinea* post-larvae as observed in other abalone species (Daume et al., 2000; Kawamura et al., 1998a; Kawamura et al., 1995; Kawamura & Takami, 1995; Roberts et al., 1999). Eight weeks after settlement, around 2 mm SL, size at which the first respiratory pore is formed (Hahn, 1989), *H. tuberculata coccinea* post-larvae exhibited increased growth rates subsequently further increasing, until reaching rates of 94 μm day^{-1}, as found for *Haliotis discus hannai* feeding on juvenile sporophytes of *Laminaria japonica* (Takami et al., 2003) and for *Haliotis rubra* feeding on diatoms and *U. lens* (Daume et al., 2004). These increased growth rates in all treatments, suggest a shift in feeding towards green algae germlings when juveniles average 2mm SL, denoting the use of *U. lens* and *U. rigida* as a complement to the diatom diet, as observed by Daume et al., (2004). Nevertheless, *U. lens* alone has been reported insufficient to sustain initial growth of *H. discus hannai* and *H. rubra* post-larvae (Daume et al., 2000; Kawamura, 1996).

Survival rates at 10 weeks post settlement were quite high, similar among treatments and in the same order as the ones recorded by Roberts et al., (1999). This high survival could be related to an adequate post-settlement density (0.5 larvae cm^{-2}) that would have spared the post-larvae from suffering a high competition for food and space. Competition for food and space were the cause of critical density-dependent mortality in previous studies (Daume et al., 2004). In addition, *U. lens* and *U. rigida*, acting as a feed supplement, from the substrates helped assuring constant food supply even when grazers pressures are high.

The benefits of diatoms as *Amphora sp*. or *N. incerta* on abalone growth in the present study were at least partly due to the higher protein contents of these diatoms and a positive significant correlation was found between growth and diatoms protein content. Nevertheless, all the diatom species tested, except *Proschkinia* sp., were high enough in proteins and lipids contents to meet the theoretical nutritional needs of post-larvae stated in previous studies (Daume et al., 2003; Gordon et al., 2006; Mai et al., 1995a, 1994; Viana et al., 2007) and were in agreement to the ones described in a previous study (Courtois de Viçose et al., Submitted-a). In other abalone species, the influence of the diatoms protein (Daume et al., 2003) or lipid (Viana et al., 2007) contents for early growth has been also demonstrated. On the contrary, *Proschkinia* sp. had the lowest dietary value for *H. tuberculata coccinea* post-larvae what could be related with the highest ash content of this diatom. Increase in dietary ash not only

reduces the energy content of the feed, but also can markedly reduce digestibility of other dietary nutrients. Moreover, the high ash content could also indicate a thicker mineral capsule in this diatom, implying an increase in the specific dynamic action employed by the abalone to ingest its feed, what together with the lower energy content of *Proschkinia sp.* would reduce the recovered energy available for growth. In agreement, abalone fed *Proschkinia sp.* not only grew less but it also had the lowest lipid content. Moreover, abalone fed *Nitzschia sp.*, whose ash content was similar to that of *Proschkinia sp.*, also had lower growth and lipid contents.

H. tuberculata coccinea larvae has a lipid content of 25% before the beginning of exogenous feeding, highlighting the significant role of lipids in larval development (Mai et al., 1995b), while juveniles at the end of the experiment presented lipid levels close to 3%, reflecting the ones of the diatoms they had been feeding on as observed by Mai et al., (1995a).

The fatty acid profiles of the diatoms tested were characteristic of most diatoms, presenting high proportions of 16:0 and 16:1n-7, together with variable but usually high, proportions of 14:0 and 20:5n-3. EPA was the predominant n-3 PUFA (Brown et al., 1996; Brown et al., 1997) while DHA proportion was low (Gordon et al., 2006; Renaud et al., 1999; Dunstan et al., 1994) being the lowest in *N. incerta*. Regardless of the diatoms used, the substrates of the four feeding regimes, presented an increased *U. lens* and *U. rigida* cover, with an average of 84%, at the end of the experiment in agreement with the competition for the space between diatoms and *U. lens* on the settlement plates described by Daume et al., (2004). This high final cover of *U. lens* and *U. rigida* suggest that it is mainly *U. lens* and *U. rigida* proximate biochemical and fatty acid composition that is reflected in the results of substrates analysis. This would explain the similar proximate biochemical and fatty acid composition of the substrates in all treatments. This is illustrated by the low levels of C_{20} and C_{22} PUFAs, as well as the high C_{18} PUFAs, observed in all substrates, which are characteristics of green algae (Brown et al., 1997; Mai et al., 1996).

The higher 18:2n-6 and 18:3n-3 contents found in abalone at the end of the feeding trial reflected the grazing on *U. lens* and *U. rigida* since the substrates were found to be rich in these two fatty acids in the present experiment. Moreover, their contents in abalone were directly related with the substrate contents indicating that they are essencials for this abalone species since they are dietary dependant and cannot be synthesized. Despite the contents in these fatty acids in the feed were not correlated with growth, these fatty acids are major growth factors for other *Haliotis* species (Mai et al., 1996), what could indicate that their contents in the substrate cover the essential fatty acid requirements for this species. In the present study, the high abalone contents in ARA and 22:5n-3, products derived from 18:2n-6 and 18:3n-3, even

several times over their levels in the feed, indicating their importance and the presence in *H.t. coccinea* of the respective elongases and desaturases (Δ4 and Δ5 desaturases) that synthetise them. This conversion of C_{18}, PUFA into the C_{20} and C_{22} PUFAs is present in many marine invertebrates including other *Haliotis* species (Uki et al., 1986; Floreto et al., 1996).) The content in 22:5n-3 was higher than the one in 22:6n-3 as observed for *H. laevigata* and *H. rubra* (Dunstan et al., 1996) what could be related to the ability of abalone species to retroconvert 22:6n-3 or to a low Δ5-desaturase activity and could also result from an adaptation to low lipid diets. On the contrary, the contents in DHA were very low denoting the lack of Δ6 desaturase activity in this species. The contents in 18:2n-6, 18:3n-3 and 20:5n-3 found in the juveniles reflect the contribution of both diatoms and substrate to juvenile's nutrition; this coupled with the similar trend in lipids contents observed between juveniles and diatoms could indicate an accumulative effect of the initial nutrition on diatoms and therefore reinforce the importance of early diatom feeding on general growth.

In summary, this study denotes the importance of diatom diet and their proximate biochemical composition, particularly *Amphora* sp., for early growth of post-larval *H. t. coccinea*, especially between the weeks 4 and 8 post-settlement. It also corroborates the good nutritional value of *U. lens* and *U. rigida* germlings employed as settlement cues for older *H. t. coccinea* post-larvae (after week 8) as a continuation feed, after the diatoms, during a period of increasing food demand. Improved understanding and control of substrates nutritional quality as well as implication in term of food value would improve abalone post-larval growth and consequently the efficiency and consistency of juvenile production.

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EARLY LIFE OF THE ABALONE *HALIOTIS TUBERCULATA COCCINEA*: DEVELOPMENT, SETTLEMENT AND GROWTH

9. CONCLUSIONS

9. CONCLUSIONS

Study I: Embryonic and larval development of *Haliotis tuberculata coccinea* Reeve: an indexed micro-photographic sequence.

1. Embryonic and larval development of *H. tuberculata coccinea* took place in thirty-nine distinct, consecutive, stages, presenting recognizable external features, from fertilization until the apparition of the third tubule on the cephalic tentacles, differing in order of appearance to that of other abalone species.
2. Eggs of *H. tuberculata coccinea* presented dark violet pigmentation and an average diameter of 205 ± 8 µm after fertilization. Egg pigmentation was reflected in the larvae presenting violet-coloured foot, velum and cephalic tentacles and orange-yellowish-coloured visceral mass. Larval size was constant with a length of 216.6 ± 5.3 µm and a width of 172 ± 8.8 µm.
3. The occurrence rates of embryonic and larval development stages of *H. tuberculata coccinea* were closer to the ones of a tropical abalone species like *H. asinina* than to the ones of a temperate species.
4. Knowledge gained on embryonic and larval development rates has enabled to adapt culture techniques of other abalone species to the particular characteristics of *H. tuberculata coccinea*.

Study II: Larval settlement of *Haliotis tuberculata coccinea* in response to different inductive cues and the effect of larval density on settlement, early growth and survival.

5. Crustose coralline algae were found to be the best settlement inducing substrate for *H. tuberculata coccinea* whereas settlement induction with 1 µM GABA and on conspecific mucus, in the absence of diatom films, led to low settlement rates.

6. None of the diatom species tested were successful in the induction of *H. tuberculata coccinea* larval settlement, a fact possibly linked to species specificity as well as age, growth phase or culture conditions of the diatoms.
7. *U. lens*, was found to be an efficient and consistent settlement substrate for *H. tuberculata coccinea* larvae, while the colonization of *U. lens* by the diatom *Navicula incerta* reduced its settlement induction potential.
8. Higher larval density had a negative effect on settlement rates and post-larval survival of *H. tuberculata coccinea*.

Study III: Larval settlement, early growth and survival of *Haliotis tuberculata coccinea* using several algal cues.

9. Algal developmental stages and proximate composition, specifically protein content, markedly affected *H. tuberculata coccinea* settlement, which was higher on CCA, on a combination of mature *U. lens* and *U. rigida* and on mature *U. rigida* alone.
10. Algal substrates were differentiated by their fatty acids compositions and fatty acids correlated to settlement were different from the ones linked to survival rates; only EPA levels were correlated with both.
11. Germlings of the green macroalgae *U. rigida* were found potent and suitable for settlement, growth and survival of *H. tuberculata coccinea*. The highest growth and survival rates were observed on a combination of old *U. lens* and *U. rigida*, also yielding high settlement rates, and denoting that green macroalgae play an important role in the early life of abalone and that *Ulvaceae* species, being ubiquitous and easily cultured, present great potential to satisfy abalone´s larval requirements.

Study IV: Potential value of *Navicula incerta, Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp. as feed for *Haliotis tuberculata coccinea* post-larvae: Effect of density on algal growth rates.

12. The four diatom species investigated showed adequate size range, to be potentially used as feed for *H. tuberculata coccinea* post-larvae throughout the nursery stage of production.

13. Higher growth rates were found for all species at lower inoculums of 0.10 x 10^6 cells mL^{-1} densities and suggest a nutrient limitation and/or the attenuation of average irradiance in the cultures due to the increase in cell density.
14. The fatty acid profiles of the diatoms tested were characteristic of most diatoms: presenting high proportions of 16:1n-7 and 16:0, with variable, but usually high, proportions of 14:0 and 20:5n-3, EPA being the predominant n-3PUFA in the diatoms studied while DHA was low in all diatoms tested, being the lowest for *N. incerta*.
15. The diatoms from the present study that would best fit *H. tuberculata coccinea* post-larvae nutritional requirements would be *Amphora* sp. in log-phase of growth, considering its good energy contents; its highest cell attachment capacity; its high protein and lipid contents, and carbohydrate content within the range needed for juvenile abalone.

Study V: Improving nursery performances of *Haliotis tuberculata coccinea:* Nutritional value of four species of benthic diatoms

16. The high lipid content (25%) of *H. tuberculata coccinea* larvae before the beginning of exogenous feeding denotes the significant role of lipids in larval development of this species.
17. Diatoms, particularly *Amphora sp*., are determinant for early growth of post-larval *H. tuberculata coccinea*, especially between the weeks 4 and 8 post-settlement. Their nutritional value for abalone growth markedly depends, among other factors, on their total protein, lipid, energy and ash contents.
18. *U. lens* and *U. rigida* germlings, employed as settlement cues, were found of good nutritional value to be employed as complement feed to the diatom diets and cover the, growth phase dependant, nutritional requirements of *H. tuberculata coccinea* post-larvae, especially after week 8, a period of increasing food demand

1. Both linoleic and linolenic acid were identified as essential for *H. tuberculata coccinea*, whereas ARA, EPA and 22:5n-3, despite of being important, were synthetised by this abalone species, denoting the presence of the respective elongases Δ4 and Δ5 desaturases. However, the low content in DHA denoted a low Δ5 activity in those species.

As a general conclusion, the improved knowledge gained, in this thesis, on *H. tuberculata coccinea:* embryo and larval development; factors affecting settlement process and early post-larvae and juvenile nutrition, has led to the development of improved early rearing techniques for this species; the experimental mass production of seeds and improved abalone post-larval growth.

EARLY LIFE OF THE ABALONE *HALIOTIS TUBERCULATA COCCINEA*: DEVELOPMENT, SETTLEMENT AND GROWTH

10. REFERENCES

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EARLY LIFE OF THE ABALONE *HALIOTIS TUBERCULATA COCCINEA*: DEVELOPMENT, SETTLEMENT AND GROWTH

11. ANNEX

11. ANNEX

ESTADIOS TEMPRANOS DE LA OREJA DE MAR *HALIOTIS TUBERCULATA COCCÍNEA*: DESARROLLO, FIJACIÓN Y CRECIMIENTO

11.1 RESUMEN

En vista del interés por la diversificación de la producción de especies de oreja de mar a nivel mundial, es necesaria la identificación de los procesos vitales en las etapas iniciales del desarrollo para la puesta a punto de técnicas de producción de semillas adaptadas a *Haliotis tuberculata coccinea*. Por lo tanto el objetivo principal de este estudio fue "*Adquirir conocimientos sobre la historia de vida inicial de la oreja de mar Haliotis tuberculata coccinea y contribuir al desarrollo de sus técnicas de producción a través de la mejora de la fijación larval, del crecimiento y de las tasas de supervivencia de los juveniles*". En concreto, el desarrollo inicial, los procesos de fijación así como el crecimiento y la supervivencia de las post-larvas y juveniles, así como diversos factores que afectan a estos procesos, se abordaron en este estudio a través del desarrollo de cinco objetivos específicos.

La primera descripción completa del desarrollo embrionario y larvario de *Haliotis tuberculata coccinea,* Reeve se puso de manifiesto en una secuencia de micro-fotografías correspondientes a en treinta y nueve etapas consecutivas y distintas. Los tiempos de ocurrencia de las diferentes etapas, observadas a 23 ± 0.5 ºC, difieren en orden de aparición a las de otras especies, asemejándose más a las de una especie de abalón tropical como *Haliotis asinina* que a las de un abalón de clima templado, adquiriendo la competencia de fijación (aparición del túbulo tercero en los tentáculos cefálicos) a las 62h después de la fecundación.

La eficacia de diferentes sustratos de inducción al asentamiento, así como efecto de sus edades, niveles de enriquecimiento y combinaciones, se determinaron en funcción de las tasas de fijación y metamorfosis de las larvas de *H. tuberculata coccinea* y la supervivencia de las post-larvas. En todos los experimentos, la mayor fijación larval se obtuvo sobre las algas coralinas incrustantes (CCA), lo que confirma que dichas algas se encuentran entre los mejores sustratos de inducción al asentamiento. Las larvas también exhibieron una alta tasa de fijación sobre *Ulvella lens* lo que sugiere su uso como sustrato de fijación eficaz para las larvas de *H. tuberculata coccínea.* Sin embargo la eficiencia de inducción al asentamiento de

dicho sustrato se vió reducida cuando éste fue colonizado por diatomeas. En relación a los esporofitos del alga verde, *Ulva rigida*, el uso de este sustrato mejoró en gran medida la fijación de las larvas, revelando un fuerte potencial para la producción futura de semillas de abalón. Por otra parte, las diferencias significativas encontradas en las tasas de fijación y supervivencia de las larvas, se correlacionaron con la composición bioquímica de los diferentes tipos de sustratos algales, a su vez, influenciada por las diferentes especies algales, así como las etapas de desarrollo y las condiciones de cultivo de los sustratos. Esto puso de relieve la capacidad de las larvas para distinguir las diferentes etapas de desarrollo y la composición proximal de las algas del sustrato antes de la fijación y metamorfosis.

Por el contrario, la inducción al asentamiento con sustratos, libre de películas de diatomeas y tratados con 1 μM GABA o con el mucus proveniente de la misma especie, dio lugar a bajas tasas de fijación y ninguna de las especies de diatomeas probadas indujeron con éxito la fijación de larvas de *H. tuberculata coccinea*, lo que sugiere especificidad de especie así como los efectos de la edad, fase de crecimiento o de las condiciones de cultivo de las diatomeas.

Una alta densidad de larvas tuvo un efecto negativo sobre las tasas de fijación y la supervivencia de las post-larvas de *H. tuberculata coccinea* mientras que sus tasas de crecimiento no se vieron influenciadas por este factor.

Además se ha encontrado que las características de los sustratos de fijación influenciaban las tasas de crecimiento, independientemente de la fuente de alimento empleado, lo que sugiere una competencia por espacio entre las algas del sustrato y las diatomeas, específicamente con las CCA, sus altas actividades exfoliativas podrían haber contribuido a la reducción del número de células de diatomeas a ser ingeridas por las post-larvas. Los resultados de crecimiento obtenidos en el presente estudio también apoyan la idea de que los patrones de asentamiento se ven reflejados y se manifiestan en el crecimiento y la supervivencia de las larvas de *Haliotis* considerando que las tasas crecimiento y de supervivencia más altas se observaron en el sustrato que también produjo altas tasas de fijación.

Teniendo en cuenta que las diatomeas bentónicas son la principal fuente de alimento de las post-larvas de abalón se realizó un estudio para adaptar las condiciones de cultivo de las diatomeas a los requisitos de las post-larvas.

Las cuatro especies de diatomeas investigadas mostraron tamaños adecuados, para ser potencialmente utilizadas para la alimentación de las post-larvas de *H. tuberculata coccinea* a lo largo de la etapa de semillero de la producción. Los perfiles de ácidos grasos fueron

característicos de la mayoría de las diatomeas, con una alta proporción de 14:0, 16:0, 16:1n-7 y 20:5n-3, siendo el ácido eicosapentaenóico (EPA), el n-3PUFA predominante mientras que el ácido docosahexaenóico (DHA) fue representado en bajas proporciones, siendo *Navicula incerta* la diatomea con un menor nivel de este ácido graso. Los resultados de este estudio revelaron que una baja densidad de inóculo era más adecuada para mantener las tasas de crecimiento de las diatomeas, así como su producción celular y su adhesión y que los cultivos cosechados en fase exponencial de crecimiento presentan valores nutricionales superiores siendo *Amphora* sp. y *Proschkinia* sp., en fase exponencial de crecimiento, las que mejor se ajustan a los requerimientos nutricionales de las post-larvas de *H. tuberculata coccínea*.

Por último, se evaluó los efectos de las diatomeas sobre las tasas de crecimiento de las post-larvas de *H. tuberculata coccínea*, mostrando divergencias en tasas de crecimiento en función de las dietas ingeridas cuando llegaron a un tamaño medio de concha de 0.8-1 mm y presentando mayores tasas de crecimiento alrededor de 2 mm de longitud de concha.

Estas diferencias en tasas de crecimiento indican, en primer lugar, un acceso al contenido de las células de diatomeas seguido de un posible cambio en la alimentación hacia esporofitos de algas verdes, como complemento de la dieta de diatomeas, cuando los juveniles alcanzan un tamaño promedio de 2 mm de longitud de concha. Las post-larvas alimentadas con la diatomea de mayor contenido en proteína y lípidos (*Amphora* sp.), presentaron las mayores tasas de crecimiento, relacionándose el contenido en proteína con un mayor crecimiento y con la longitud final de la concha.

El perfil de ácidos grasos de las post-larvas sugiere que 18:2n-6 y 18:3n-3 son esenciales para *H. tuberculata coccinea* y que esta especie es capaz de absorber una selección de ácidos grasos provenientes de la fuente de alimentación o tiene capacidad de sintetizar ácidos grasos a partir de acidos grasos n-3 precursores presentes en la dieta.

Los resultados obtenidos proporcionan un conocimiento técnico crucial y necesario para la adaptación de las técnicas de cultivo a las exigencias de *H. tuberculata coccínea*. En particular: especies de *Ulvaceas* presentan un gran potencial para mejorar la fijación, el crecimiento y las tasas de supervivencia. Ademas, el control de las diatomeas así como la calidad nutricional de los sustratos han demostrado que permiten mejorar el crecimiento de las post-larvas de abalón y su supervivencia, influyendo por lo tanto en la eficiencia y la fiabilidad de la producción de juveniles necesarias para el desarrollo y estandarización de los métodos de producción.

11.2 INTRODUCCIÓN

11.2.1. Ciclo de vida del abalón

La oreja de mar o abalón es un molusco gasterópodo marino herbívoro, univalvo (sólo una concha), que pertenece a la familia Haliotidae. Se clasifican en 56 especies existentes perteneciente todas al género *Haliotis* (Geiger, 2000). Están presentes en todos los océanos. A pesar de que el estudio de la biología del abalón comenzó hace mucho tiempo (Stephenson, 1924; Bonnot, 1930) su investigación se ha sido intensificando durante los últimos 30 años, debido al creciente interés en su cultivo. Los abalones son reproductores gonocóricos, con machos y hembras que liberan sus gametos en la columna de agua, de forma sincrónica, para la reproducción (Stephenson, 1924; Crofts, 1930). Los machos adultos y las hembras pueden ser fácilmente reconocidos por las diferencias de color de las gónadas (Bardach et al., 1972).

Su historia de vida se puede describir en cinco etapas: embriones, larvas, post-larvas, juveniles y adultos (Fig. 1).

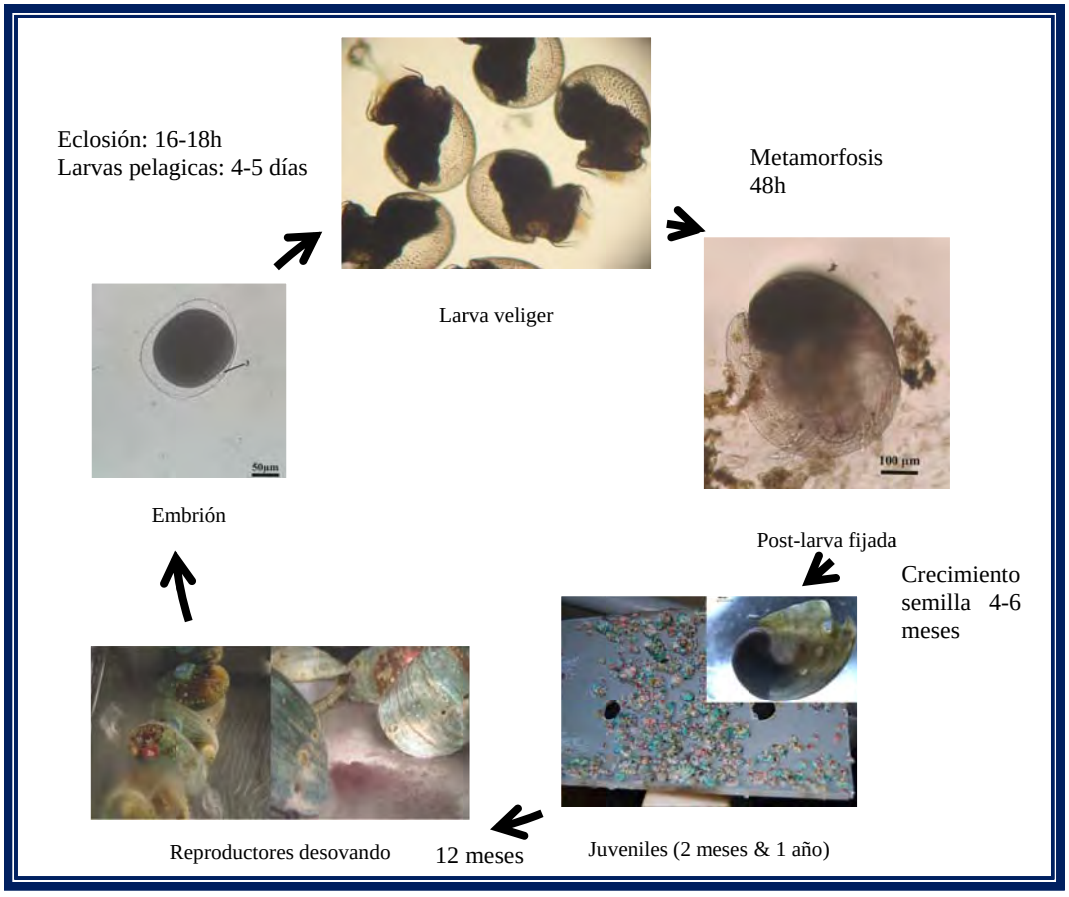

Fig. 1: Ciclo de vida del abalón

Los huevos fertilizados pasan primero por la etapa embrionaria, que sólo dura unas pocas horas y, a continuación eclosionan en una larva trocófora ciliada. Las larvas resultantes son pelágicas y lecitotróficas, nadando en la columna de agua durante días o semanas, dependiendo de la especie y la temperatura del agua, antes de estar preparadas para fijarse (Leighton, 1972; Sawatpeera et al., 2001). Las larvas lecitotróficas dependen de sus reservas energéticas y de la absorción de materia orgánica disuelta en el agua hasta la fijación (Jaeckle & Manahan, 1992; Shilling et al., 1996; Kawamura et al., 1998b). Las larvas competentes se fijan una vez encontrados estímulos adecuados de fijación. La fijación es una fase crítica que consiste en la transición de la vida pelágica a una bentónica e implica la fijación y la metamorfosis (Roberts, 2001). Durante la fijación, las larvas abandonan su actividad natatoria y se asientan sobre el sustrato conservando su velo, mientras que la metamorfosis implica cambios irreversibles constriñendo el abalón a la vida bentónica. Desde este momento se les califica de post-larvas, empiezan a alimentarse de la microflora bentónica, principalmente *biofilms* de diatomeas y bacterias que crecen en la superficie de los sustratos de fijación (Pastor & Daume, 1996; Kawamura et al., 1998b; Daume et al., 1999a).

La etapa de post-larva se termina con la aparición de los poros respiratorios después de la cual los abalones se consideran juveniles hasta llegar a la madurez sexual. El tamaño y la edad a la que se alcanza la madurez sexual varían entre las especies y dependen de la temperatura y de la disponibilidad de alimentos. De juveniles a adultos, los abalones cambian su dieta de microalgas a una de macro algas y experimentan un crecimiento exponencial en las etapas de post-larvas y juveniles que se ve disminuyendo cuando la madurez sexual se alcanza. Las especies de oreja de mar subtropicales y tropicales crecen más rápido, maduran antes y presentar menor tamaño que las especies de aguas templadas.

11.2.2 Acuicultura y producción mundial de oreja de mar

A nivel mundial, la producción total de abalón (incluidas la pesca y la acuicultura) ha mostrado un aumento significativo durante las últimas décadas, pasando de 20370mt en 1970 a 48405mt en 2008 (Fig. 2). Los productos se venden vivos, frescos, congelados, enlatados o de forma seca, siendo los principales mercados los de China, Japón, Hong Kong, el Sureste Asiático, EE.UU., México, Corea y Europa (Oakes &Ponte, 1996).

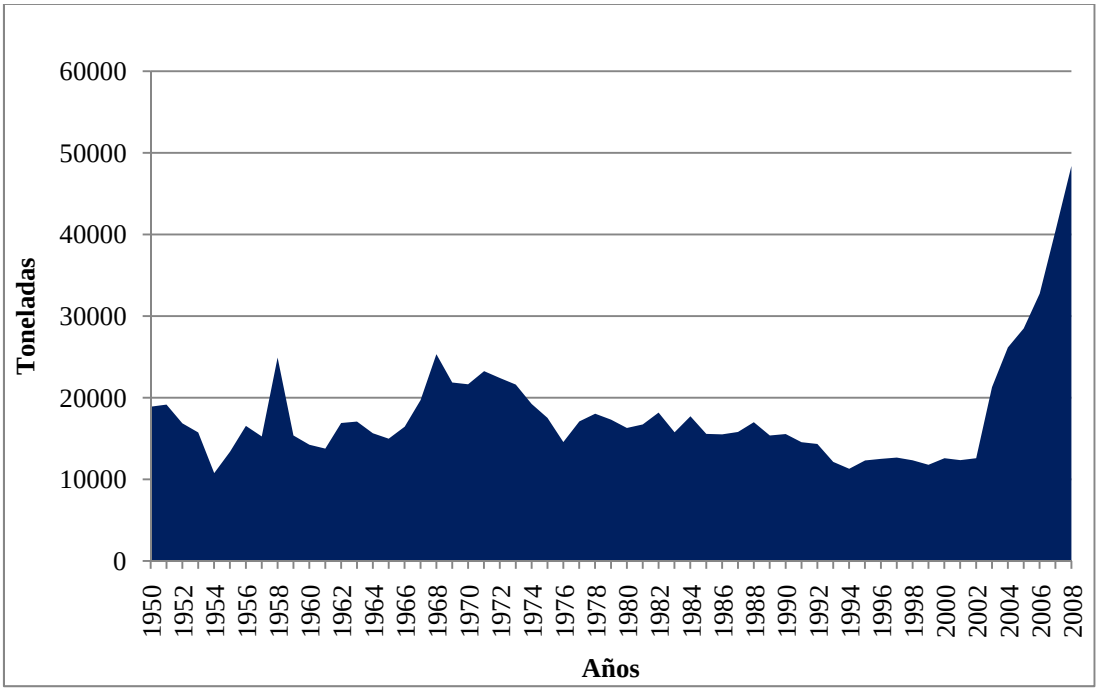

Fig. 2: Producción mundial de abalón incluyendo pesca y acuicultura (FAO, 2009).

Este aumento se debe a la contribución exponencial del abalón proveniente de la acuicultura a la producción mundial estando la pesca de abalón en continuo descenso durante las últimas cuatro décadas (Fig. 3). Son varias las especies de abalón (*Haliotis* spp) de interés para la producción acuícola debido a su precio de mercado y la sobreexplotación de las poblaciones silvestres. Se estima que en los próximos años más del 70% de las poblaciones silvestres se incluirán en las listas de especies en peligro de extinción. Como consecuencia, de la sobreexplotación, la acuicultura será la única vía posible para satisfacer la creciente demanda del mercado. Los productos provenientes de la acuicultura se ajustan a un nicho de mercado diferente a los de los productos de la pesca comercial, ya que pueden ser cosechados en diferentes tamaños y de menor dimensión. Así mismo, tienen también la capacidad de

proporcionar productos totalmente trazables y certificados que se pueden identificar y diferenciar de las poblaciones silvestres, ademas ofrecen posibilidades de mejora genética que puede permitir la optimización de la producción.

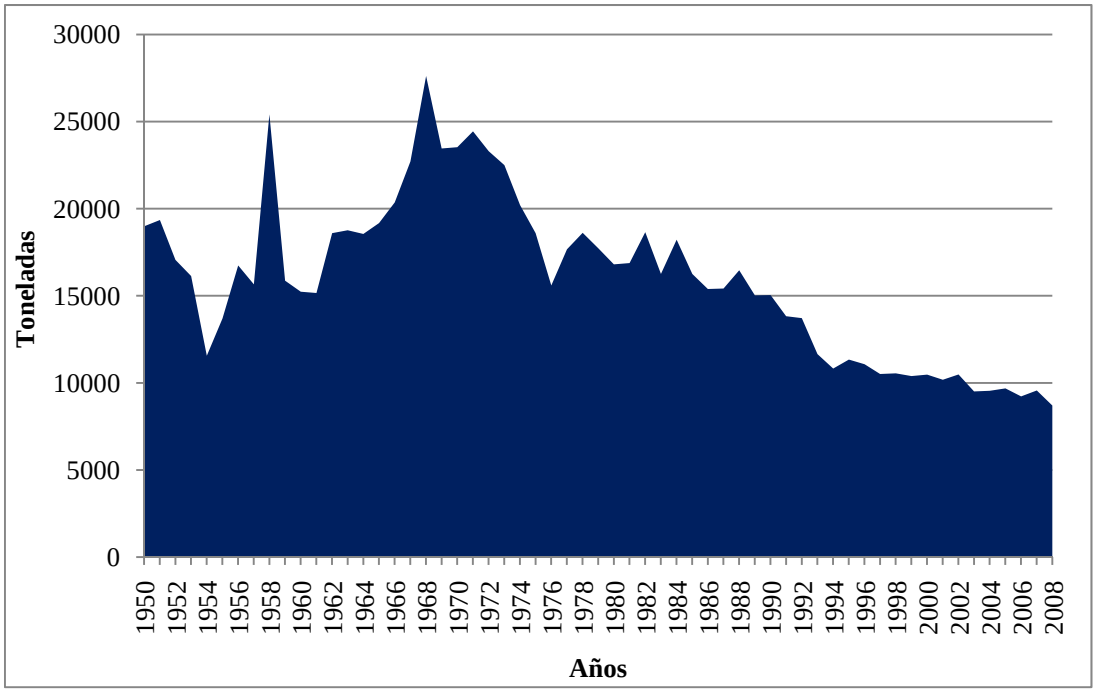

Fig. 3: Capturas mundiales de abalón (FAO, 2009).

La pesca excesiva, las enfermedades, la pérdida de hábitat, y la mala gestión gubernamental de las capturas ilegales, han contribuido a la disminución de la pesca de abalón en las últimas cuatro décadas (Gordon & Cook, 2004). En 1970, las capturas mundiales de abalón llegaron a 23.532 toneladas, mientras que en 2008 este valor había bajado a 8701mt.

La situación con respecto al cultivo de abalón es contraria, con un aumento de más del 600% en los últimos diez años. Este aumento ha sido posible gracias al desarrollo de recientes prácticas de producción, especialmente para las etapas juveniles, que promovieron la rápida expansión de la industria (Daume et al., 2004; Roberts et al., 2004.). El crecimiento exponencial de la oreja de mar proveniente de cultivo se evidencia al comparar las 40.486 toneladas producidas en el año 2008 con sólo 689 toneladas 20 años antes (Fig. 4).

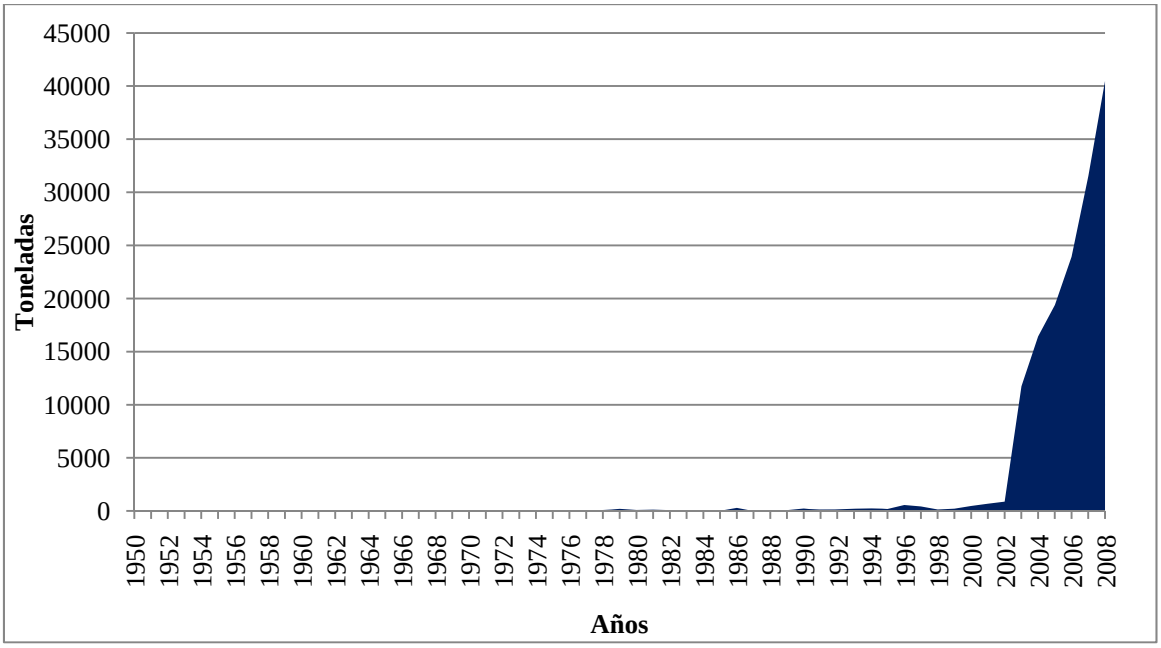

Fig. 4: Producción mundial de abalón (FAO, 2009)

El aumento de la producción de abalón cultivado ha sido posible gracias al desarrollo de la investigación sobre su cultivo, que comenzó en Japón con *Haliotis discus* (Ino, 1952). En la década de los 60, se desarrollaron los sistemas de criadero de oreja de mar (Leighton, 2000), con los aportes más significativos a principios de los 70, incluyendo los estudios sobre la reproducción controlada por Nagahisa Uki & Shōgo Kikuchi de *Haliotis discus*, *Haliotis discus hannai* y *Haliotis gigantea* y abarcando aspectos tales como los efectos de la temperatura y la nutrición sobre el acondicionamiento de reproductores, radiación ultravioleta (UV) para la inducción al desove y la densidad óptima de espermatozoides para la fecundación. Estos resultados mejoraron considerablemente las producciones de criaderos y semilleros (Uki & Kikuchi, 1984).

Estas técnicas desarrolladas para criadero, junto al descubrimiento del uso del peróxido de hidrógeno (H_2O_2) para la inducción al desove del abalón (Morse et al., 1977) se adaptaron pronto a las necesidades de otras especies en otras partes del mundo, permitiendo el desarrollo de la acuicultura de abalón en varios países como EE.UU., Corea del Sur, China, Taiwán, México, Chile, Sudáfrica, Nueva Zelanda, Australia y Europa. Actualmente, más de 15 especies de abalón son cultivadas comercialmente a nivel mundial en unidades de producción de diferentes magnitudes que van desde menos de una tonelada a 200 toneladas (Gordon & Cook, 2004). Los principales productores son China, Australia y Corea, liderando China la producción con 33.010 toneladas en 2008. Australia no ha dejado de desarrollar su producción de abalón alcanzando 5320 toneladas en 2008, mientras que Corea

ha experimentado un aumento exponencial de su producción en los últimos diez años con un total de 5318 toneladas en 2008 (Fig. 5).

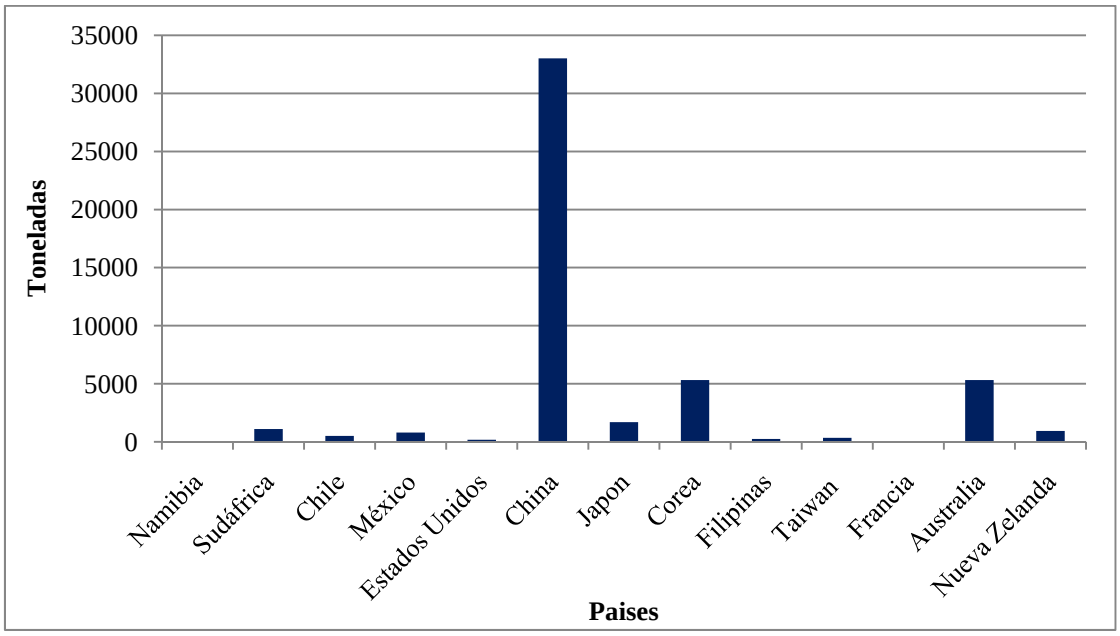

Fig. 5: Productores mundial de abalón (FAO, 2009).

11.2.2.1 Importancia del control de los estados iniciales de vida para el cultivo de abalón

A pesar de que las tasas de supervivencia, durante el cultivo larvario, pueden llegar a un 90-95%, la supervivencia durante la etapa de fijación sólo alcanza una media del 10% (Hahn, 1989). Por lo tanto, la inducción a la fijación y a la metamorfosis se considera la etapa más crítica en la producción de semillas de abalón (Ebert & Houk, 1984). Los estudios sobre la fijación de las larvas de abalón, revisados por Roberts en 2001, demostraron que las larvas preparadas para la metamorfosis necesitan encontrar y ponerse en contacto con estímulos de inducción a la fijación, adecuados, para pasar con éxito de su vida pelágica a la vida bentónica.

Tradicionalmente, a nivel mundial, se han utilizado *biofilms* y películas de diatomeas en los criaderos de abalón, para inducir la fijación de las larvas y como fuente de alimentación inicial de las post-larvas (Seki, 1980; Seki & Kan-No, 1981b; Hahn, 1989; Kawamura & Kikuchi, 1992; Takami et al., 1997a; Daume et al., 1999a; Daume et al., 2000). Sin embargo estudios sobre, el crecimiento y la supervivencia de las post-larvas, revelaron que las claves, necesarias para la fijación larval y el crecimiento de las post-larvas, no están

siempre proporcionadas de manera eficiente por los sustratos, tradicionalmente utilizados, colonizados por los *biofilms* y las diatomeas, tradicionalmente utilizados (Kawamura & Takami, de 1995; Buchal et al., 1998; Bernal et al., 2000; Daume et al., 2000). Por otra parte los *biofilms* son difíciles de mantener a largo plazo, ya que es complicado ejercer el control sobre la composición de sus especies, su densidad y, por lo tanto, su calidad (Hahn, 1989). La calidad y la composición de los *biofilms* y de las películas de diatomeas influencian profundamente la fijación de las larvas, así como la supervivencia y el crecimiento de las post-larvas (Searcy-Bernal et al., 2001; Daume et al., 2003; Searcy-Bernal et al., 2003; Daume & Ryan, 2004; Watson et al., 2004; Gordon et al., 2006; Uriarte et al., 2006; Roberts et al., 2007; Viana et al., 2007a).

En paralelo con los estudios anteriores, se probaron diversos estímulos de inducción a la fijación para diferentes especies de abalón que incluyen, las algas coralinas incrustantes (CCA) (Morse et al., 1980c; Morse & Morse, 1984; Roberts & Nicholson, 1997; Takami et al., 1997b; Daume et al., 1999a; Daume et al., 2000); el aminoácido gamma butírico (GABA) (Morse et al., 1980a; Searcy-Bernal et al., 1992; Slattery, 1992, Bryan & Qian, 1998) o el mucus de abalón (Seki & Kan-No, 1981a; Slattery, 1992; Seki, 1997; Bryan & Qian, 1998). En Japón, la macroalga verde, *Ulvella lens*, se emplea en criaderos para mejorar el asentamiento de las larvas y el crecimiento de las post-larvas de *H. discus hannai* (Takahashi & Koganezawa, 1988), extendiéndose posteriormente a los criaderos australianos (Daume et al., 2004). El abalón es un molusco de crecimiento lento que a su vez presenta tasas de fijación generalmente inestables en los criaderos. Por tanto, cualquier mejora en el éxito de fijación o en la tasa de crecimiento, que permita mejorar la fiabilidad así como reducir el tiempo de producción será de gran beneficio para la producción, permitira aumentar el coste-beneficio de la producción. Las mejoras en los procesos que tienen lugar en las etapas iniciales vitales son esenciales para la producción de abalón, considerando que el suministro regular de larvas y de producción de semillas son factores clave para el cultivo de este molusco.

11.2.2.2 Haliotis tuberculata coccinea

Sólo una especie de abalón está presente en Europa, *Haliotis tuberculata* (Mgaya et al., 1995). Considerando las características morfológicas, se han descrito tres sub-especies: *Haliotis tuberculata tuberculata* Linnaeus, 1758 en el Atlántico; *Haliotis tuberculata lamellosa* Lamarck, 1822 en el Mar Mediterráneo, y *Haliotis tuberculata coccinea* Reeve,

1846 en el archipiélago de la Macaronésia (Mgaya et al., 1995; Geiger, 2000). En Canarias, *Haliotis tuberculata coccínea,* se distribuye desde la zona intermareal hasta 15 m de profundidad en zonas semi-expuestas y expuestas. *Haliotis tuberculata coccinea* crece hasta un tamaño máximo de unos 80 mm de longitud y ha sido explotado comercialmente durante décadas para el mercado local, dando lugar a una sobreexplotación de sus poblaciones, que se encuentran próximas a la extinción. Los estudios preliminares, sobre la producción de *H. tuberculata coccinea*, que se han centrado sobre técnicas de reproducción (Peña, 1986) y nutrición (Toledo et al., 2000; Viera et al., 2005); sugieren la posibilidad de reproducción artificial de esta especie y la identifican como especie potencial para la diversificación de la producción acuícola. Sin embargo, no existen estudios previos sobre sus procesos de fijación larval, así como los estímulos de fijación adecuados, la densidad larvaria, y su efecto sobre el crecimiento post-larval y la supervivencia.

Con el fin de desarrollar técnicas de producción de semillas adaptadas a esta especie, es necesario investigar temas de producción larvaria, fijación larvaria y de nutrición de las post-larvas. Mejoras en el ámbito de la producción larvaria requieren del control de la reproducción y de la comprensión de las diferentes técnicas de inducción, así como del estudio del ciclo embrionario y larvario en condiciones naturales. En el caso de las post-larvas, los efectos de varios estímulos de fijación sobre la fijación y la metamorfosis y el instante en el que tienen que ser proporcionados deben ser estudiados con el fin de mejorar esa etapa del cultivo. Por último, en relación a la nutrición de las post-larvas, es importante controlar la calidad y la densidad de las diatomeas bentónicas, suministradas como alimento para las post-larvas, y estudiar su efecto sobre el crecimiento y la supervivencia de las mismas. La comprensión y el control de estas diferentes fases del cultivo son cruciales para la mejora, el desarrollo y la estandarización de la producción de juveniles de *H. tuberculata coccínea*.

11.2.3 Larvas de abalón

11.2.3.1 Inducción a la puesta

Una mayor comprensión de los procesos de maduración gonadal y de los métodos de inducción al desove de los reproductores se ha desarrollado a lo largo de los años.

La influencia del acondicionamiento de los reproductores sobre la calidad de los gametos se ha estudiado para diversas especies de abalón, prestando especial atención a la

influencia de factores externos, tales como las dietas o la temperatura, sobre el desarrollo gonadal y la maduración (Uki & Kikuchi, 1984), así como la calidad de los gametos y el éxito del desove (Ebert & Houk, 1984; Moss, 1998; Teruel-Bautista et al., 2001; Grubert et al., 2004; Grubert & Ritar, 2005).

Gametos de alta calidad, provenientes de reproductores mantenidos en sistemas de acondicionamiento óptimos, son fácilmente obtenidos a través de la inducción controlada al desove. Esto es generalmente el primer paso en el cultivo de nuevas especies y su éxito es determinante para la producción de larvas, a su vez esenciales para el desarrollo de criaderos de nuevas especies. Las técnicas de inducción al desove han sido desarrolladas para varias especies de abalón y se pueden realizar por estímulo único o con una combinación de varios tales como: shock térmico; agua de mar tratada con rayos ultravioletas (UV); ozono o peróxido de hidrógeno; el manejo o la exposición al aire. Los métodos de inducción al desove más comúnmente empleados y eficientes son el tratamiento de agua de mar con UV o el método del peróxido de hidrógeno (Moss et al., 1995). Morse et al., (1977) descubrieron que bajas concentraciones de peróxido de hidrógeno (H_2O_2) inducen al abalón al desove, sugiriendo que los productos de la descomposición de H_2O_2, como por ejemplo, el hidroperoxi radical libre, HOO^-, o el peróxido dirradical, OO^-, actúan sobre el sistema enzimático que produce prostaglandinas, que a su vez permiten iniciar el desove. Uki & Kikuchi, (1974), propusieron el uso de agua de mar irradiada por UV para inducir el desove de abalón. Se cree que la radiación UV del agua del mar provoca la descomposición energética de la molécula de agua, produciendo radicales libres similares a los del método del peróxido de hidrógeno, siendo la molécula de ozono (O_3) la donante en vez de H_2O_2. El éxito de la fertilización está vinculado a la calidad de los gametos, influenciados por el proceso de fertilización empleado, especialmente en términos de ratio óvulos – espermatozoides y el tiempo de fertilización aplicado. La densidad óptima de espermatozoides y el tiempo de contacto de los gametos para la fertilización han sido documentados para varias especies de abalones (Kikuchi & Uki, 1974, Leighton & Lewis, 1982; Clavier, 1992; Encena et al., 1998; Babcock & Keesing, 1999, Baker & Tyler, 2001).

11.2.3.2 Desarrollo embrionario y larvario

Los huevos fertilizados tienen una flotabilidad negativa y la mantienen durante la totalidad del desarrollo embrionario. Una vez eclosionados, los abalones inician su etapa pelágica que incluye dos etapas larvarias, planctónicas y lecitotróficas, la larva trocófora y la

velíger. La duración de la fase trocófora es breve (unas horas), mientras que la de la velíger puede durar días o semanas, dependiendo de la especie y de las condiciones ambientales. Siendo lecitotróficas, las larvas dependen de sus reservas de energía endógena, que consiste principalmente en lípidos (Moran & Manahan, 2003). Sin embargo, estas reservas endógenas podrían obtenerse a través del aumento de la captación de materia orgánica disuelta (DOM) presente en el agua (Jaeckle & Manahan, 1989). De hecho se ha reportado para varias especies que la energía proveniente de la DOM contribuye al desarrollo lecitotrófico (Crisp et al., 1985; Gallager et al., 1986; Dawirs, 1987; Angers et al., 1989; Nates & McKenney, 2000; Marsh et al., 2001).

Diversos estudios han documentado el desarrollo inicial de diversas especies de abalón tales como, *H. discus hannai, H discus, H. fulgens, H. sorenseni, H. tuberculata* y *H. asinina* (Ino, 1952; Leighton, 1972; Seki & Kan-no, 1977; Peña, 1986; Sawatpeera et al., 2001), sobre una base morfológica. Estos estudios han reconocido que el desarrollo larvario es un proceso gradual que no se produce en etapas concretas, sino paso a paso en varias y distintas etapas larvarias con reconocibles rasgos externos. Por lo tanto, la etapa larvaria se inicia con la fecundación y se completa con la formación del cuarto túbulo sobre los tentáculos cefálicos, aunque las larvas se consideran competentes para la fijación cuando el tercer túbulo aparece y las larvas empiezan a explorar la superficie de fijación (Hahn, 1989). La comparación entre diversos estudios detallados sobre las secuencias del desarrollo larvario revelan que éstas son similares entre las especies, aunque si bien pueden tener lugar en diferentes orden o momento de aparición (Hahn, 1989). Por lo tanto, la ocurrencia de las etapas de desarrollo depende de la especie y es función de la temperatura del agua y del tiempo, ya que el abalón no se alimenta antes de la fijación (Hahn, 1989).

El desarrollo larvario del abalón está bloqueado por debajo de un umbral de temperatura determinado, conocido como " punto cero biológico " (BZP). El BZP del abalón fue descrito descrito por primera vez por Seki & Kan-no., (1977), para *H. discus hannai*, y se sitúa a temperaturas más bajas para especies de abalón de clima templado (Seki & Kan-no, 1977) que para las especies tropicales (Sawatpeera et al., 2001). A temperaturas por encima de la BZP, la aparición de cada etapa de desarrollo es función de la diferencia acumulada entre la temperatura del cultivo y la BZP, factor conocido como temperatura acumulativa eficaz (EAT, expresado en ºC-h). Las temperaturas más altas inducen un desarrollo más rápido y reducen el intervalo de tiempo entre la eclosión y el logro de la competencia metamórfica (la formación del tercer túbulo en los tentáculos cefálicos (Hahn, 1989)), mientras que lo contrario ocurre a temperaturas más bajas. Sin embargo, la tasa de desarrollo

de las larvas de abalón no es dos veces más rápida al doble de la temperatura del agua (Hahn, 1989). Por lo tanto, el conocimiento sobre los patrones de la embriogénesis y sobre la morfología de las larvas y sus tasas de aparición es crucial para el desarrollo del cultivo de una nueva especie de abalón, ya que permitirá predecir la aparición de cada etapa del desarrollo a una temperatura determinada y un tiempo de fertilización identificado. La capacidad de predecir la duración del desarrollo larvario es de especial relevancia para pronosticar el tiempo necesario para la adquisición de la competencia metamórfica, considerando que la edad larvaria y la etapa del desarrollo afectan la respuesta de fijación (Barlow, 1990), y la planificación de la transferencia de las larvas a los tanques de fijación. El control de la aparición de estas etapas es de especial relevancia para las actividades de criadero, ya que evita los retrasos en la inducción de la metamorfosis en presencia de estímulos de inducción a la fijación. Este retraso se tiene que diferenciar de los descensos de supervivencia larvaria y de crecimiento, observados en ausencia de adecuados estímulos de inducción a la fijación, causados por el agotamiento de las reservas endógenas, y provocados por la falta prolongada de contacto con una señal ambiental adecuada. (Roberts & Lapworth, 2001; Takami et al., 2002).

El conocimiento de la vida inicial de *H. tuberculata* ha recibido poca atención (Crofts, 1938; Koike, 1978). Por otra parte, el estudio del desarrollo embrionario y larvario de *H. tuberculata coccinea* se limita al estudio de Peña (1986), en el cual se describen brevemente las etapas de desarrollo para un rango de temperatura que no refleja el de su hábitat natural. Aunque varios estudios se han realizado para mejorar el conocimiento de la fisiología de esta especie y su comportamiento bajo condiciones de cautiverio (Peña, 1986; Toledo et al., 2000; Viera et al., 2003; Viera et al., 2005) ninguno de ellos se ha centrado en el desarrollo temprano de *H. tuberculata coccinea* en su área de distribución térmica natural (18-24 ºC). Por lo tanto, el objetivo del estudio del desarrollo inicial de *H. tuberculata coccinea* es de determinar sus etapas de desarrollo, su orden de aparición y el tiempo requerido a una temperatura determinada. Tales conocimientos son esenciales para desarrollar y mejorar las técnicas de cría larvaria, evitar retrasos en la inducción a la metamorfosis y mejorar la producción de semillas, fase del cultivo que sigue siendo un importante cuello de botella para la producción de esta especie.

11.2.4 Fijación y metamorfosis del abalón

Una vez competentes para la fijación, después de la aparición del cuarto túbulo sobre los tentáculos cefálicos, las larvas se transforman en organismos bentónicos. La fijación del abalón implica primero la etapa de asentamiento de las larvas seguida de la de la metamorfosis. Durante la etapa de asentamiento las larvas se unen al sustrato por su pie y todavía conservan su velum para poder reanudar su actividad natatoria en caso de que durante la exploración se denotara un sustrato inadecuado. Por el contrario, irreversibles cambios físicos tienen lugar durante la metamorfosis. Estos cambios incluyen la pérdida del velum, el desarrollo de las branquias y del pie, la apertura de la boca y el principio de la alimentación, así como la deposición de la concha peristomial. La etapa de la metamorfosis de las larvas de abalón ha recibido una atención especial, ya que es una etapa crucial de desarrollo que implica la reorganización del tejido existente y la producción de uno nuevo, aumentando el riesgo de padecer elevados índices de mortalidad. Como consecuencia, se considera como la etapa la más crítica del cultivo de abalón.

Los procesos involucrados en la metamorfosis se llevan a cabo después de la fijación de las larvas competentes sobre el bentos en respuesta a estímulos ambientales (Hadfield, 1986). Estos estímulos proporcionan información química o nutricional relacionada con el hábitat que las post-larvas y los juveniles posteriormente habitarán. El conocimiento de estos estímulos y su poder de inducción a la fijación es fundamental en el cultivo de la oreja de mar en el que se requiere una fijación completa, rápida y previsible pero difícil de lograr (Roberts, 2001). La ausencia de tales estímulos conduce a bajas tasas de fijación y de supervivencia de las post-larvas. (Searcy-Bernal et al., 1992; Slattery, 1992; Daume et al., 1999a; Roberts et al., 1999a; Takami et al., 2002).

En las últimas décadas, se han estudiado, para diversas especies de abalón, una amplia gama de estímulos y sus efectos sobre el asentamiento de las larvas. Estos incluyen algas coralinas incrustantes (CCA) y su extracto químico (el ácido γ aminobutírico, GABA), los *biofilms*, incluyendo las diatomeas; las películas de bacterias; productos químicos puros o mucus de abalón y macroalgas.

La literatura publicada sobre la capacidad de inducción a la fijación y a la metamorfosis de estos sustratos relata resultados contradictorios y una amplia variación en las tasas de fijación según las especies de abalón y la metodología experimental empleada en los diferentes estudios. Estos resultados se detallan a continuación para cada conjunto de estímulos.

11.2.4.1 Algas coralinas incrustantes (CCA) y ácido γ aminobutírico (GABA)

Las algas coralinas costrosas (CCA) son algas rojas calcáreas que se encuentran abundantemente sobre sustratos duros en comunidades submareales de latitudes tropicales a polares. La investigación inicial, sobre las propiedades de inducción, de las CCA, a la fijación del abalón, se basó sobre observaciones de campo e identifico que los abalones juveniles están estrechamente asociados con ellas en la naturaleza (Shepherd & Turner, 1985; McShane et al., 1988; Daume & Shepherd, 1996; Day & Branch, 2000). La actividad, importante y rápida, de inducción a la fijación de las CCA fue confirmada por experimentos de laboratorio que demostraron que están entre los mejores inductores de fijación para la oreja de mar, independientemente de las especies de abalón probadas (Morse et al., 1980c; Morse & Morse, 1984; Moss & Tong, 1992b; Roberts & Nicholson, 1997; Takami et al., 1997b; Daume et al., 1999b, a; Moss, 1999; Roberts et al., 2004; Roberts et al., 2010). Sin embargo, el uso de CCA en los criaderos no es práctico, ya que son algas de crecimiento lento que no se propagan fácilmente a gran escala (Daume, 2006).

Morse et al., (1979) encontraron que el ácido γ-aminobutírico (GABA), un aminoácido y neurotransmisor en los animales superiores, puede ser extraído de las algas rojas incrustantes e inducir a la fijación de las larvas de abalón. Este descubrimiento permitió entender mejor los mecanismos de fijación de las larvas de oreja de mar, las vías bioquímicas implicadas y los receptores implicados en la inducción a la fijación (Morse, 1985; Trapido-Rosenthal & Morse, 1986; Barlow, 1990). Además se encontró que el asentamiento y la metamorfosis podrían ser provocados artificialmente mediante el uso de diversas sustancias químicas que afectan a las vías de transducción de señales, tales como el transporte de iones a través de la membrana (Jensen et al., 1990; Morse, 1992). La eficacia del GABA para la inducción a la fijación ha sido un caso de controversia, en la literatura publicada. Podría estar relacionado con la susceptibilidad del GABA a la degradación por los microbios marinos, lo que probablemente, explicaría los diferentes resultados obtenidos entre los distintos estudios realizados (Morse, 1992; Slattery, 1992; Kaspar & Mountfort, 1995). La otra cuestión sobre el uso del GABA es la concentración a la que tiene que ser empleado, así como el tiempo de exposición al cual tienen que estar sometidas las larvas. Morse, (1979) reportó una inducción eficaz a la fijación del GABA a una concentración de 10^{-6} M para larvas de *H. rufescens*, mientras que concentraciones más altas se han encontrado tóxicas e inhibitorias del crecimiento de la concha o de la metamorfosis (Morse et al., 1980b). Searcy-Bernal & Anguiano-Beltrán, 1998). Por el contrario, Searcy-Bernal & Anguiano Beltrán (1998),

demostraron que las concentraciones de GABA se podían duplicar o triplicar sin comprometer la metamorfosis o la supervivencia de las post-larvas y el crecimiento de *H. rufescens*. Los resultados de inducción a la fijación con GABA también varían en función de las especies de abalón probadas (Roberts & Nicholson, 1997), lo que sugiere que las estímulos químicas de inducción a la fijación podrían variar según las especies.

11.2.4.2 Otros sustancias químicas puras

Las investigaciones sobre la inducción a la fijación con GABA han permitido una mejor comprensión, a nivel molecular, de la forma en que los inductores provocan una respuesta en el abalón y revelaron que incumbía la unión de una sustancia a un receptor específico de las larvas. Como consecuencia, otras sustancias químicas puras, sospechosas de tener un posible efecto sobre la vía de transducción de señales, se probaron como sustancias de inducción a la fijación del abalón. Los productos químicos puros, testados para inducir la fijación del abalón fueron el dibromometano y la manipulación de la concentración del cloruro de potasio (KCl) en agua de mar lo que afecta a la concentración de iones de potasio (K^+). El efecto de la adición de KCl al agua de mar indujo el asentamiento, pero no la metamorfosis en *Haliotis iris* y *Haliotis virginea* (Roberts & Nicholson, 1997) y sus efectos sobre la inducción de la metamorfosis de *Haliotis rufescens*, *Haliotis diversicolor* y *Haliotis discus hannai* se describen como poco eficientes y función de la concentración de la exposición y del tiempo empleado (Baloun & Morse, 1984; Bryan & Qian, 1998; Kang et al., 2004). El dibromometano, una sustancia química volátil naturalmente liberada por las algas coralinas y otras algas, ha sido citado como inductor de metamorfosis en larvas de erizo (Taniguchi et al., 1994). Así mismo, ha sido implicado en la inducción de la metamorfosis de *H. discus hannai* (Kang et al., 2004) mientras que sus efectos fueron insignificantes para la inducción de *H. iris* (Roberts & Nicholson, 1997). Sin embargo, su combinación con mucus de abalón provocó una fijación rápida y completa de larvas de *H. discus hannai* y de *H. iris*, lo que sugiere efectos sinérgicos entre estos estímulos (Roberts & Nicholson, 1997; Seki, 1997).

11.2.4.3 Biofilms de diatomeas bentónicas

Los *biofilms* de diatomeas han sido utilizados, a nivel mundial, para inducir la fijación de larvas de abalón en los criaderos. La composición en especies de diatomeas de los *biofilms*

es amplia y las diatomeas están asociadas a bacterias; esta combinación hace difícil la descripción precisa de las características del *biofilm* y la identificación de las características específicas responsables de la inducción a la fijación larval. En los primeros estudios de inducción a la fijación, no se controlaba la composición en diatomeas del *biofilm*, y las diatomeas filamentosas demostraron ser inadecuadas para la inducción a la fijación de la oreja de mar (Seki & Kan-No, 1981b). Esta observación conllevó la realización de diversos estudios sobre el efecto de: la edad del *biofilm*; la composición en especies de diatomeas; la forma de crecimiento de las diatomeas o su densidad de colonización del sustrato, sobre la fijación de las larvas de abalón con el fin de entender los mecanismos implicados en la inducción a la fijación por *biofilms* de diatomeas. La respuesta de las larvas de abalón a diferentes densidades y cepas de diatomeas ha puesto de manifiesto diferencias significativas en el porcentaje de metamorfosis según las cepas o la densidad de diatomeas (Kawamura & Kikuchi, 1992). Además, las características de las diatomeas también influyen sobre el tiempo requerido para completar la fijación (Kawamura & Kikuchi, 1992; Kawamura, 1996).

Ocho modos de crecimiento de las diatomeas han sido identificados (Fig. 6), las cepas que forman comunidades planas indujeron un alto porcentaje de fijación, mientras que las que tienen formas de crecimiento en tres dimensiones enredan las larvas y les impide cumplir su metamorfosis (Kawamura, 1996). Además del efecto de las formas de crecimiento de las diatomeas sobre la inducción a la fijación, la abundancia y la edad del *biofilm* de diatomeas se consideran también factores influyentes. A menudo, los *biofilms* más antiguos y más densos inducen un mayor asentamiento de las larvas de abalón (Kawamura & Kikuchi, 1992; Moss & Tong, 1992a; Daume et al., 1999a; Roberts et al., 2007). Como consecuencia, la selección de las formas idóneas de diatomeas que crecen sobre las placas de fijación en cultivo se lleva a cabo por el pre-pastoreado de las placas por juveniles (Seki & Kan-No, 1981a; Searcy-Bernal et al., 1992; Bryan & Qian, 1998) o, en el caso de *biofilms* sin pastoreo, mediante la filtración del agua o el cultivo de especies específicas de diatomeas (Hahn, 1989; Gallardo & Buen, 2003, Gordon et al., 2004). A pesar del uso generalizado de las diatomeas en los criaderos de abalón, las tasas de metamorfosis sobre películas de diatomeas son a menudo bajas (Roberts, 2001; Daume, 2006, Roberts & Watts, 2010) y la investigación hacia la identificación de las diatomeas potencialmente idóneas para una inducción a una fijación eficiente, ha revelado diferencias significativas entre las cepas y especies de abalón. Por ejemplo, *Cocconeis* spp. fue descrita como diatomea apropiada, siendo a menudo dominante sobre las placas pre-pastoreadas que inducen a fijación en los criaderos del abalón japonés (Seki, 1997, Kawamura et al., 1998b; Roberts, 2001). Sin embargo, algunas cepas de

Cocconeis han inducido bajas tasas de fijación en otras especies, lo que implica diferencias en los requisitos de fijación entre especies de abalón (Daume et al., 1999a; Daume et al., 2000). Otras diatomeas de relevancia en los criaderos de abalón son *Cylindrotheca* spp., *Nitzschia* spp. y *Navicula* spp, todas ellas dando lugar a diferentes tasas de inducción a la fijación en función de las cepas de diatomeas o de las especies de abalón probadas (Ohgai et al., 1991; Kawamura & Kikuchi, 1992; Kawamura & Takami, de 1995, Roberts & Nicholson, 1997; Daume et al., 1999a). Las películas de diatomeas ofrecen poca consistencia en la inducción a la fijación lo que podría estar relacionado con variaciones físicas o químicas. Efectivamente, otros factores que a menudo covarian con la abundancia de diatomeas en el *biofilm* son la fase de crecimiento de la película, la movilidad y fuerza de adherencia de las diatomeas y la densidad de bacterias presente en la película. La eficiencia de inducción a la fijación de las cepas de diatomeas también ha sido probado en base a sus propiedades físico-químicas en un intento de entender la base química de la respuesta sensorial para la fijación de las larvas de abalón (Gordon et al., 2004).

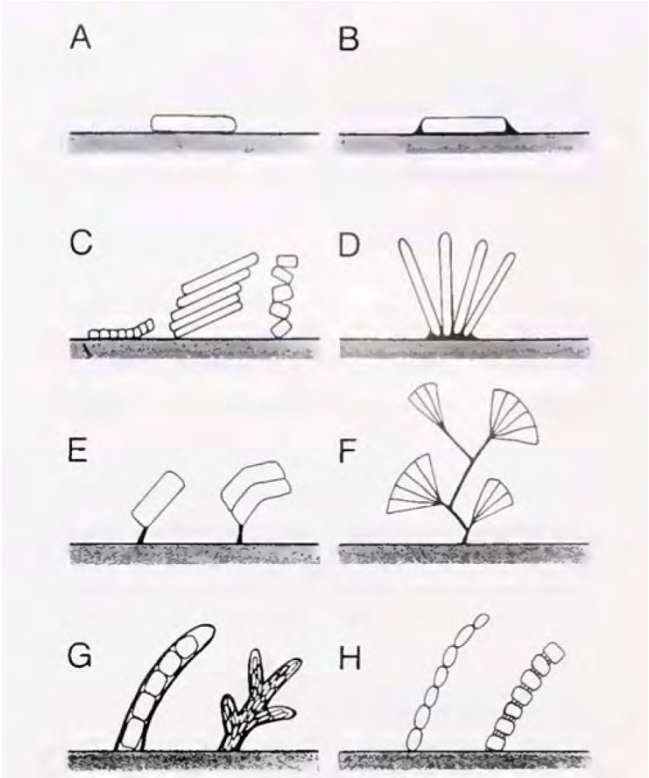

Fig. 6: Esquema de los ochos modos de crecimiento de las diatomeas (Kawamura, 1994).

11.2.4.4 Bacterias

Las películas bacterianas han sido reseñadas como inductoras de fijación para varias especies de moluscos (Fitt et al., 1990). Se ha descrito que las bacterias, junto con las diatomeas, también desempeñan un papel en la inducción a la fijación de la oreja de mar, ya que las películas de diatomeas son colonizadas por una amplia gama de bacterias en los tanques de cultivo. La fijación de las larvas de *Haliotis diversicolor* ha sido inducida en bajas proporciones por tres especies de bacterias (Bryan & Qian, 1998) y algunas cepas bacterianas han inducido un 50% de metamorfosis en larvas de *H. iris* (Roberts et al., 2010). Larvas de *Haliotis virginea* han sido inducidas a la fijación por bacterias, con una lente respuesta, requiriendo una semana para alcanzar el 50% de metamorfosis (Roberts, 2001), lo que demostró que la actividad bacteriana influye en gran medida en el efecto de la película de diatomeas mediante la activación de la fijación (Roberts et al., 2007). Sin embargo, han sido pocos los estudios realizados sobre la capacidad de inducción a la fijación larvaria de abalón de las bacterias no quedando claro si las sustancias de inducción a la fijación son sintetizadas por las bacterias de forma independiente o si estas últimas son sintetizadas a partir de compuestos derivados de las diatomeas.

11.2.4.5 Mucus de abalón

Biofilms de diatomeas pastoreados por abalones, juveniles o adultos, se utilizan extensamente como inductores eficaces de fijación de larvas de abalón (Roberts, 2001). Se considera que la acción del mucus requiere que haya contacto y es específica del género como lo ilustran las larvas de *H. discus hannai* que son inducidas por las mucosidades de cuatro especies de abalón japonés, pero no por la mucosidad de otros gasterópodos (Seki & Taniguchi, 1996). Para *H. australis* el efecto de pastoreo parece ser especifico de la especie, puesto que el pre-pastoreo de *Haliotis iris* no tiene ningún efecto sobre la inducción a la fijación de las larvas de *H. australis* (Roberts & Watts, 2010). Sin embargo, la eficiencia de inducción a la fijación del mucus es muy variable según las especies de abalón (Slattery, 1992; Seki, 1997; Bryan & Qian, 1998). Esta variabilidad podría explicarse por el hecho de que las larvas son capaces de distinguir entre diferentes tipos de mucosidades segregadas por el pie de los gasterópodos (Hahn, 1989). Cuatro tipos diferentes de mucosidades ("de pastoreo", "de arrastre", "de adhesión" y "de frotar") se describen y han sido investigadas. La fijación de larvas de *H. discus hannai* sólo se ha observado en mucosidades de tipo "de

arrastre" y, en particular, las de tipo "de pastoreo" (Seki & Taniguchi, 1996). Estas diferencias pueden deberse a variaciones en las propiedades físicas o químicas de los distintos tipos de mucus (Seki & Kan-No, 1981a) o por bacterias asociadas a los pies del abalón (Bryan & Qian, 1998).

11.2.4.6 Macroalgas

Escasos estudios describen el uso de macroalgas como inductores de fijación de las larvas de abalón, investigando su potencial para la inducción a la fijación y como fuente de nutrición para las post-larvas. Las larvas de *H. rubra* presentan una elevada tasa de fijación inducida por las algas verde *Ulva australis* y *Ulva compressa* (Huggett et al., 2005). Seki, (1997) también relató que las macroalgas pardas, rojas y verdes inducen a la metamorfosis de *H. discus hannai* y (Strain et al., 2006) sugieren que las esporas de *Ulva* sp. podrían proporcionar una fuente de alimentación adecuada para abalones juveniles. Además, diversos estudios han demostrado que la oreja de mar se puede fijar con éxito sobre las algas verde incrustantes tales como, *Ulvella lens*, que crecen como rosetas sobre el sustrato (Takahashi & Koganezawa, 1988; Daume et al., 2000; Daume et al., 2004; Daume & Ryan, 2004). *U. lens* es adecuada para mejorar la fijación de las larvas de *Haliotis laevigata* a escala comercial y en la actualidad esta cultivada para ser utilizada como sustrato de inducción a la fijación larvaria en la mayoría de las granjas comercial de orejas de mar en Australia (Daume, 2006). Daume, (2006) también señala la influencia de la edad y de las características bioquímicas de *U. lens* sobre la fijación de las larvas de abalón, basándose en el hecho de que las larvas de *Haliotis rubra* y *Haliotis laevigata* demuestran tener una clara preferencia por *U. lens* de edad avanzada en lugar *U. lens* más joven. Sin embargo, ningún estudio ha investigado la utilización de las esporas de macroalgas como inductores de fijación de la oreja de mar, a excepción de la investigación realizada con *U. lens* (Takahashi & Koganezawa, 1988; Daume et al., 2000; Daume et al., 2004; Daume & Ryan, 2004). Este tipo de investigación sería útil para determinar nuevos potenciales estímulos de inducción a la fijación que podrían ser fácilmente reproducidos y manipulados a escala comercial y que también podrían servir como fuente potencial de alimento para juveniles de abalón.

11.2.4.7 Técnicas artificiales de cultivo en criadero

Como se expuso anteriormente, la calidad del *biofilm* de algas es muy variable en función de su densidad, edad, y de las cepas de diatomeas. En la búsqueda de sustratos de inducción fiable y estable, unos sistemas alternativos, como sustitución de las algas vivas, se han propuesto recientemente en Japón para *H. discus discus* y *H. diversicolor*, como soluciones para la fijación larvaria y el crecimiento de las post-larvas. El sistema propuesto consiste en pulverizar una solución de agar mezclado con polvo de algas secas sobre placas acondicionadas con mucus proveniente de abalón juveniles (Stott et al., 2004b). No hubo diferencias significativas entre las tasas de fijación de *H. discus discus* observadas sobre los tratamientos de microalgas en polvo y los *biofilms* naturales y las tasas de fijación fueron significativamente más altas que las del control negativo (Stott et al., 2004a). Es un sistema interesante que puede servir el doble propósito de inducción a la fijación y de suministro de alimentación para las post-larvas, aunque medios mecanizados y rentables de pulverización de las placas tendrían que ser desarrollados antes de que esa solución sea comercialmente viable.

11.2.5 Nutrición post-larval y crecimiento del abalón

11.2.5.1 Factores de influencia sobre el crecimiento post-larval y la supervivencia

Las diatomeas bentónicas son la principal fuente de alimento para las post-larvas de abalón una vez cumplida la metamorfosis (Seki & Kan-No, 1981b; Hahn, 1989; Kawamura, 1996; Daume, 2006). Una nutrición adecuada es fundamental para el crecimiento y la supervivencia de las post-larvas y juveniles de abalón (Hahn, 1989, Kawamura et al., 1998b). Las tasas de crecimiento y supervivencia durante las primeras etapas de las post-larvas han sido descritas en la literatura como muy variables y generalmente bajas (Kawamura & Takami, 1995; Kawamura, 1996; Kawamura et al., 1998a; Kawamura et al., 1998b; Daume et al., 1999a, Roberts et al., 1999a; Daume et al., 2000; Searcy-Bernal et al., 2001). Estas variaciones se han atribuido a diferencias en el *biofilm* del que se alimentan las post-larvas, como pueden ser, las especies de diatomeas y las cepas; su densidad de células; las condiciones de cultivo; la etapa de desarrollo del cultivo y las especies de abalón (Kawamura & Takami, 1995, Kawamura et al., 1998b; Roberts et al., 1999a; Daume et al., 2000; Searcy Bernal et al., 2001; Searcy Bernal et al., 2003). Por lo tanto, el mantenimiento de una película

de diatomeas adecuada es un factor crítico en el éxito de criaderos de oreja de mar a nivel mundial (Hahn, 1989).

La cantidad de alimento disponible para las post-larvas es un factor importante que determina su crecimiento. El pastoreo y las tasas de crecimiento de las post-larvas de *H. fulgens* aumentan cuando la densidad de la diatomea *Navicula incerta* aumenta de 500 a 4.000 células/mm^2 (Searcy-Bernal et al., 2001). Sin embargo, una alta densidad de diatomeas puede tener un impacto negativo sobre el crecimiento post-larval siendo las diversas razones posibles para explicar este fenómeno: la asfixia (Tong & Moss, 1992); una movilidad reducida; así como la acumulación de depredadores (Ebert & Houk, 1984) y una mala calidad del agua (Searcy-Bernal, 1996).

Sin embargo, la ingestión de células de diatomeas no implica que se utilicen como alimento (Kawamura et al., 1995). La proporción de células de diatomeas que se rompen durante el pastoreo parece ser un factor crítico que controla el valor nutricional de las diatomeas para las post-larvas de abalón, puesto que esta sugerido que las post-larvas son incapaces de utilizar el contenido de las diatomeas para su nutrición celular a menos que las diatomeas hayan sido rotas durante el pastoreo (Kawamura et al., 1998a). La diferencia en la eficiencia de digestión entre las diatomeas está descrita como un factor importante que determina su valor nutricional para las post-larvas de abalón. La eficiencia de digestión de las diatomeas provoca variaciones en las tasas de crecimiento; se ha observado que las cepas digestibles producen un crecimiento significativamente más rápido que las diatomeas digeridas de manera ineficaz, y estas diferencias se han vinculado a las características físicas, como son el tamaño y la fuerza de adhesión, de las células de diatomeas (Seki & Kan-No, 1981b; Kawamura et al., 1995; Matthews & Cook, 1995, Kawamura et al., 1998b). Post-larvas de 0,8-2 mm de longitud de la concha crecen aproximadamente unas 40-60 μm day^{-1} con diatomeas digeribles, mientras que crecen sólo aproximadamente de 15 a 30 μm day^{-1} con diatomeas no digeribles. (Kawamura et al., 1998a).

Otros factores pueden también influir sobre el valor nutricional de las diatomeas. Estos incluyen la naturaleza y la cantidad de sus polisacáridos extracelulares; la flora microbiana asociada (Kawamura, 1996; Roberts et al., 1999a; Searcy-Bernal et al., 2001; Daume, 2006) y la composición bioquímica y en ácidos grasos del contenido de las células de diatomeas (Dunstan et al., 1994), factores que varían entre especies de diatomeas. Además, se sugiere que la influencia de las diferencias bioquímicas sólo tendrá un efecto sobre el crecimiento a partir del momento en que las post-larvas habrán adquirido la capacidad de digerir y beneficiarse de los contenidos de las células de diatomeas (Kawamura et al., 1998b)

Otra característica importante que influye sobre el crecimiento post-larval y la supervivencia es la etapa de desarrollo de las post-larvas, ya que afecta el consumo selectivo de especies de diatomeas, y la capacidad de digestión de la oreja de mar (Kawamura et al., 1998a; Roberts et al., 1999a). Tres grandes transiciones se han descrito en la alimentación de las post-larvas y juveniles: la primera es la transición del estado lecitotrofíco a la alimentación a base de partículas; la segunda corresponde a los principios de una ingestión y digestión eficaz de las células de diatomeas y aparece a una longitud de la concha media de 600-800µm; por último la tercera, es el cambio nutricional de las diatomeas a las macroalgas (Kawamura et al., 1998b). Estas transiciones en la alimentación, en conjunto con la capacidad de las post-larvas para consumir diatomeas, están vinculadas al desarrollo del sistema digestivo, así como al estado del desarrollo morfológico de la rádula, correspondiente a una boca circular albergando un órgano similar a una lengua que presenta hileras de pequeños dientes que permiten al abalón raspar las partículas de algas (Chitramvong et al., 1998; Takami et al., 1998; Roberts et al., 1999b; Takami et al., 2000; Onitsuka et al, 2004; Johnston et al, 2005).

Las diferencias en las tasas de crecimiento en respuesta a diferentes algas se han observado sólo desde la segunda transición nutricional y en adelante, dado que durante la primera transición las post-larvas se siguen beneficiando de los efectos residuales de sus reservas vitelinas. (Kawamura et al., 1998b; Roberts et al., 1999a; Daume et al., 2000).

Dos días después del inicio de la metamorfosis se observa la apertura de la boca así como el principio de la alimentación (Seki & Kan-No, 1981b; Norman-Boudreau et al., 1986; Roberts et al., 1999a) a base de bacterias y secreciones extracelulares (Garland et al., 1985; Kawamura, 1996) y diatomeas adecuadas (Martínez-Ponce & Searcy-Bernal, 1998, Roberts et al., 1999a). Una nutrición adecuada durante esta etapa es un elemento clave para la supervivencia, ya que la reorganización de los tejidos durante la metamorfosis es energéticamente costosa y puede resultar en el agotamiento de las reservas endógenas. En el caso de no disponibilidad de diatomeas adecuadas o de aumento drástico del consumo de alimento, dando lugar a una escasez de alimento teniendo en cuenta que los criaderos luchan para satisfacer la demanda de alimentos cada vez mayores de las post-larvas (Ebert & Houk, 1984, Hahn, 1989), se pueden observar períodos de hambruna (Roberts, 2001; Onitsuka et al., 2010) que pueden dar lugar a altas tasas de mortalidad (Roberts et al., 1999a). La escasez de alimentos en esta etapa se puede reducir mediante el uso de *U. lens* para alimentar a juveniles con una longitud de concha de 2-3 mm (Daume & Ryan, 2004). Las bajas tasas de crecimiento observadas con *U. lens* en post-larvas que no habían alcanzado los 2-3 mm de

longitud de concha (Kawamura et al., 1998b; Daume et al., 2000) sugieren su imposibilidad de utilizar esta fuente de alimento, probablemente debido a la inmadurez del sistema digestivo y de la rádula antes de alcanzar este tamaño (Roberts et al., 1999b).

11.2.5.2 Calidad del alga

La composición bioquímica de las algas varía según las especies y está influenciada por las condiciones de cultivo y la etapa de desarrollo del mismo (Thompson et al., 1993; Brown et al., 1996; Liang et al., 2001; Simental-Trinidad et al., 2001; Liang et al., 2002; Mercado et al., 2004). La composición bioquímica de las algas está directamente relacionada con sus valores nutricionales (Brown et al., 1997; Renaud et al., 1999), y por lo tanto tiene un efecto directo sobre el crecimiento de los animales y su supervivencia. El efecto de la composición bioquímica de las algas se ha estudiado para abalones juveniles y adultos (Mai et al., 1994, 1995b, a; Dunstan et al., 1996; Mai et al., 1996; Shpigel et al., 1999; Boarder y Shpigel, 2001). Sin embargo, son pocos los estudios que han investigado los efectos de la variación de la composición proximal y en ácidos grasos de la dieta, sobre el crecimiento y la supervivencia de las post-larvas y de los juveniles (Daume et al., 2003; Daume & Ryan, 2004; Gordon et al., 2006; Uriarte et al., 2006; Viana et al., 2007b). Las post-larvas solo dependen de las películas de diatomeas, así como de su flora microbiana, como fuente de alimentos, hasta llegar a 2-3mm cuando pueden ser destetadas con esporas de macroalgas.

Se ha visto que las condiciones de cultivo afectan al valor nutricional de las diatomeas y en consecuencia pueden afectar fuertemente el crecimiento y la supervivencia da las post-larvas y juveniles de abalón (Daume, 2006). Un aumento en la cantidad de nitrato del medio de cultivo incrementó el contenido en proteína de *Navicula* sp., lo que llevo a un aumento de las tasas de crecimiento de juveniles de *H. rubra* de 1-3 mm de longitud de concha (Daume et al., 2003). La composición bioquímica de *Navicula jeffreyi* se vio modificada según las intensidades de luz y las concentraciones de nitrato del cultivo, presentando un menor contenido en proteínas en condiciones de luz alta, y juveniles *H. laevigata* alimentados con *N. jeffreyi* de diferentes composiciones bioquímicas se observaron pastoreando sobre un mayor número de diatomeas de contenido bajo en proteína, posiblemente para compensar los niveles inferiores de proteína (Watson et al., 2005). Además, se ha observado que los niveles óptimos de proteínas en la dieta de abalones juveniles y adultos varían de un 20% a un 35% según la especie y la edad (Mai et al., 1995a; Britz & Hecht, 1997).

Los carbohidratos son una fuente adecuada de energía alimentaria para la oreja de mar considerando que el metabolismo de la oreja de mar tiene como base este nutriente y que sus enzimas digestivas son capaces de hidrolizar carbohidratos complejos. De hecho, su dieta natural consiste en un 40-50% de carbohidratos (Fleming et al., 1996).

Los lípidos son también importantes componentes de la dieta no sólo por su alto valor energético y como fuente de ácidos grasos esenciales, sino también porque son los vehículos de las vitaminas liposolubles (Fleming et al., 1996). Se ha visto que los requerimientos en lípidos de la oreja de mar son muy bajos como corresponde a animales herbívoros (Mai et al., 1995b) y que un aumento de los niveles de lípidos en la alimentación puede disminuir sus tasas de crecimiento, ya que se cree que reduce la digestibilidad de otros nutrientes (Britz & Hecht, 1997). Los niveles de lípidos de un 4% a 5% han demostrado ser óptimos para maximizar las tasas de crecimiento y la salud de la oreja de mar y, como para las proteínas, sus requerimientos son específicos de la especie (Brito & Hecht, 1997).

Los ácidos grasos (AG) son quizás los lípidos más importantes, ya que son fuentes de energía y componentes celulares de las membranas. Como todos los animales, los abalones no pueden sintetizar todos los ácidos grasos requeridos para su función celular y su crecimiento (Uki et al., 1986), y se basan en fuentes alimenticias de estos ácidos grasos esenciales (AGE) para cumplir con sus necesidades. La restricción de la ingesta, ya sea a través de las raciones de alimento reducidas o el suministro de alimentos bajos en AGE, resultan en un crecimiento sub-óptimo del abulón (Uki et al., 1986; Floreto et al., 1996; Mai et al., 1996). El tipo de algas consumidas afecta de forma significativa las tasas de crecimiento del abalón (Uki et al., 1986) y ofrecen en diferentes proporciones los ácidos grasos poliinsaturados (PUFA) importantes a nivel nutricional. Una característica que distingue el abalón del resto de los animales marinos son sus necesidades dietéticas en ácido docosapentaenoico (22:5 n-3, DPA) en lugar del ácido docosahexaenoico (22:6 n-3, DHA) (Dunstan et al., 1996). Esto se ha observado para el abalón alimentado con diferentes dietas por Dunstan et al., (1996) llegando a la conclusión de que esta distribución de C_{22} PUFA, podría ser el resultado de una adaptación a una dieta de macroalgas, lo que implica una dieta baja en lípidos, así como una baja tasa de conversión. PUFA de ambos familias, n-3 y n-6, se han descritos como esenciales para el crecimiento de juveniles de *H.discus hannai* (Mai et al., 1996). Entre los ácidos grasos altamente insaturados (HUFA), el ácido eicosapentaenoico (20:5 n-3, EPA) promueve un crecimiento rápido de juveniles de *H. discus hannai* (Dunstan et al., 1996; Mai et al., 1996). Se sugiere que EPA también también tiene una especial relevancia durante la alimentación de las post-larvas (Viana et al., 2007b), ya que parece que

este ácido graso podría fácilmente ser absorbido por las diatomeas (Gordon et al., 2006) conocidas por ofrecer altos niveles de lípidos y PUFA, sobre todo de EPA (Dunstan et al., 1994; Brown et al., 1997). Así mismo, se ha sugerido la importancia del ácido araquidónico (20:4 n-6, ARA) teniendo en cuenta su alta proporción en los tejidos de abalón (Fleming et al., 1996) y su aumento en los tejidos de las post-larvas una vez que empiezan a pastar sobre las diatomeas (Viana et al., 2007b). Por el contrario, el DHA se encuentra en baja proporción en los tejidos de abalón indicando una menor importancia de este ácido graso en la alimentación del abalón (Dunstan et al., 1996; Mai et al., 1996). Ademas es sabido que las diatomeas contienen bajas cantidades de DHA (Dunstan et al., 1994). Por lo tanto, el desarrollo de técnicas optimas de cultivo que proporcionen y mantengan una densidad adecuada de algas, de buena calidad nutricional, durante las primeras etapas del ciclo de vida es un factor clave para el éxito de los criaderos de abalón.

11.3 OBJETIVOS

En vista del interés por la diversificación de la producción de especies de abalón y la identificación de los procesos de vida inicial como principales limitaciones para el desarrollo futuro de esta producción, el objetivo principal de este estudio fue "***Adquirir conocimientos sobre la historia de vida inicial de la oreja de mar Haliotis tuberculata coccinea y contribuir al desarrollo de las técnicas de su producción a través de la mejora de la fijación larval, así como del crecimiento y de las tasas de supervivencia de los juveniles***". En concreto, el desarrollo inicial, los procesos de fijación, el crecimiento y la supervivencia de las post-larvas y juveniles, así como diversos factores que afectan a estos procesos, se abordaron en este estudio. Por lo tanto, para alcanzar el objetivo general cinco objetivos específicos fueron seleccionados:

Objetivo I: Estudiar el desarrollo embrionario y larvario de *Haliotis tuberculata coccinea*, en comparación con otras especies, y adaptar las técnicas de cultivo a las fases del desarrollo, factor crítico para el éxito de la producción.

Objetivo II: Estudiar la fijación de larvas de *Haliotis tuberculata coccinea* en respuesta a diferentes estímulos de inducción.

Objetivo III: Mejorar la fijación de *Haliotis tuberculata coccinea* con estímulos de inducción innovadores que promueven de forma conjunta el crecimiento y la supervivencia.

Objetivo IV: Mejorar las técnicas de cultivo de diatomeas bentónicas y determinar el potencial valor nutricional de las especies de diatomeas para las post-larvas de *Haliotis tuberculata coccínea*.

Objetivo V: Estudiar la nutrición inicial de *Haliotis tuberculata coccínea*, y de manera especifica, el efecto de las diatomeas bentónicas sobre el crecimiento y la supervivencia de las post-larvas y de los juveniles.

Estos objetivos fueron abordados con los siguientes estudios:

Estudio I: Desarrollo embrionario y larvario de *Haliotis tuberculata coccinea* Reeve: secuencia de de micro-fotografías indexadas

Con el fin de lograr el objetivo I de este estudio se describe el desarrollo completo de embriones y larvas de *Haliotis tuberculata coccinea* en condiciones naturales de temperatura, se ilustran las características morfológicas propias de cada etapa y el tiempo necesario para su aparición. También se abordan aspectos del desarrollo morfológico inicial con el objetivo de evaluar el período de tiempo necesario a las larvas para adquirir su competencia de fijación y permitir la transferencia de las larvas competentes a sustratos de fijación en el momento adecuado para garantizar mayores posibilidades de éxito de fijación.

Estudio II: Fijación de larvas de *Haliotis tuberculata coccinea* en respuesta a diferentes estímulos inductivos y el efecto de la densidad larvaria sobre la fijación, el crecimiento inicial y la supervivencia

Con el fin de estudiar la fijación de las larvas de *Haliotis tuberculata coccinea* propuesta en el objetivo II se llevaron a cabo cuatro experimentos. Por un lado, se estudió la eficiencia de la inducción a la fijación de diferentes sustratos utilizados como inductores de fijación de otras especies de abalón, incluyendo diferentes especies de diatomeas. Por otro lado, se evaluó el efecto de la densidad de larvas sobre el rendimiento de las post-larvas. Este estudio permitió identificar sustratos adecuados de fijación para esta especie de abalón y determinar la influencia de la densidad de larvas sobre las tasas de fijación y el rendimiento de las post-larvas en términos de tasas de crecimiento y de supervivencia.

Estudio III: Efecto de diferentes estímulos de inducción procedentes de algas sobre la fijación larvaria, el crecimiento inicial y la supervivencia de *Haliotis tuberculata coccínea*.

Para abordar el objetivo III de mejora de la fijación de *Haliotis tuberculata coccinea* utilizando estímulos de inducción innovadores, se probó por primera vez el uso de esporas de algas, que no sean *U. lens*, como inductores de fijación de abalón. El estudio también investigó los efectos de la edad y la composición proximal y en ácidos grasos de esporofitos de *U. lens* y *U. rigida*, además de las algas coralinas incrustantes, sobre la fijación larvaria, la supervivencia y el crecimiento de las post-larvas de *H. tuberculata coccinea*. Este estudio permite una mejor comprensión de la fijación de *H. tuberculata coccinea* inducida por macroalgas para mejorar aún más el reclutamiento y el crecimiento de las post-larvas con el fin de producción de semillas.

Estudio IV: Valor potencial de *Navicula incerta, Proschkinia* sp., *Nitzschia* sp. y *Amphora* sp. para la alimentación de post-larvas de *Haliotis tuberculata coccinea*: Efecto de la densidad sobre las tasas de crecimiento de las algas.

Este estudio examinó las características de cuatro especies de diatomeas bentónicas y el efecto de la densidad sobre su crecimiento y su calidad nutricional para la oreja de mar, en términos de composición proximal y en ácidos grasos. Este estudio se abordó con el objetivo de mejorar las técnicas de cultivo de diatomeas bentónicas para ofrecer a la especie de abalón estudiada una fuente nutritiva de alimento, tanto en calidad como en cantidad, durante las primeras etapas de su ciclo de vida para sostener mayores tasas de crecimiento y supervivencia.

Estudio V: Mejora de la fase de criadero de *Haliotis tuberculata coccínea*: valor nutricional de cuatro especies de diatomeas bentónicas

El último estudio tuvo como objetivo determinar el efecto de diferentes diatomeas bentónicas, y su composición proximal y en ácidos grasos, sobre el crecimiento y la supervivencia de las post-larvas, siendo subministradas a *H. tuberculata coccinea* durante todo el período post-larvario hasta la etapa juvenil. El objetivo de este estudio fue obtener una mejor comprensión de la nutrición y su relación con el crecimiento inicial de post-larvas de *H. tuberculata coccínea*, a partir del inicio de su alimentación exógena, con el fin de mejorar el crecimiento y la supervivencia para el desarrollo de protocolos estándar de cría post-larvaria para esta especie de abalón.

11.4 MATERIAL Y MÉTODOS

11.4.1 Localización e instalaciones generales

Los estudios han sido realizados en las instalaciones de cultivo del Grupo de Investigación en Acuicultura (GIA) en el Instituto Canario de Ciencias Marinas (ICCM). Este Instituto se encuentra ubicado en la localidad de Melenara, Municipio de Telde, provincia de Las Palmas con una situación geográfica de latitud 27º59'31''N y longitud 15º22'31''. Las instalaciones de cultivo de abalón consisten en áreas de: acondicionamiento de reproductores, inducción al desove y cría larvaria (Fig. 7); criadero, para la producción de post-larvas (Fig. 8); ensayos de engorde y alimentación (Fig. 9); producción de microalgas (Fig. 10) y unidades de biofiltración y de reciclaje de efluentes de tanques de peces para la producción de macroalgas (Fig. 11).

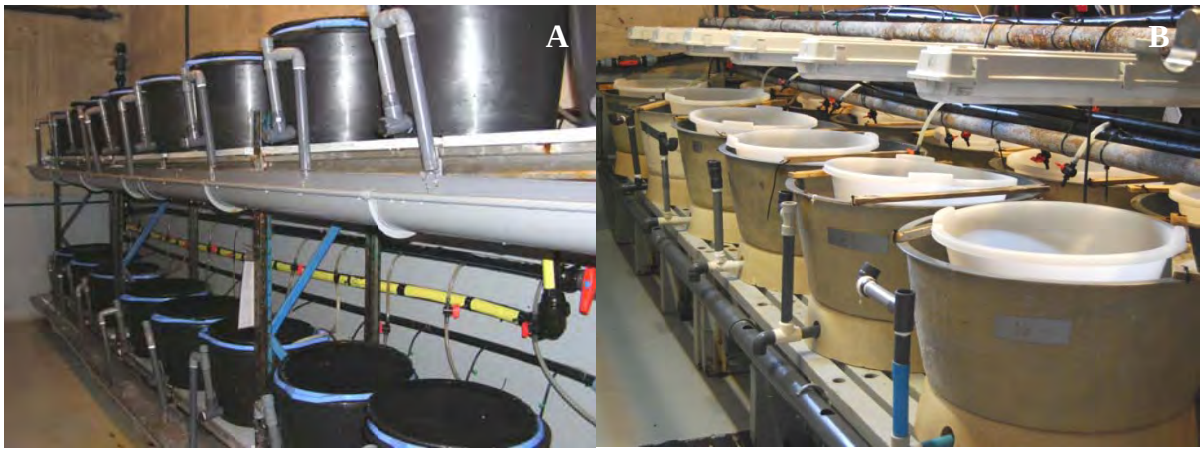

Fig. 7: Acondicionamiento de reproductores (A) y instalaciones de producción larvaria (B).

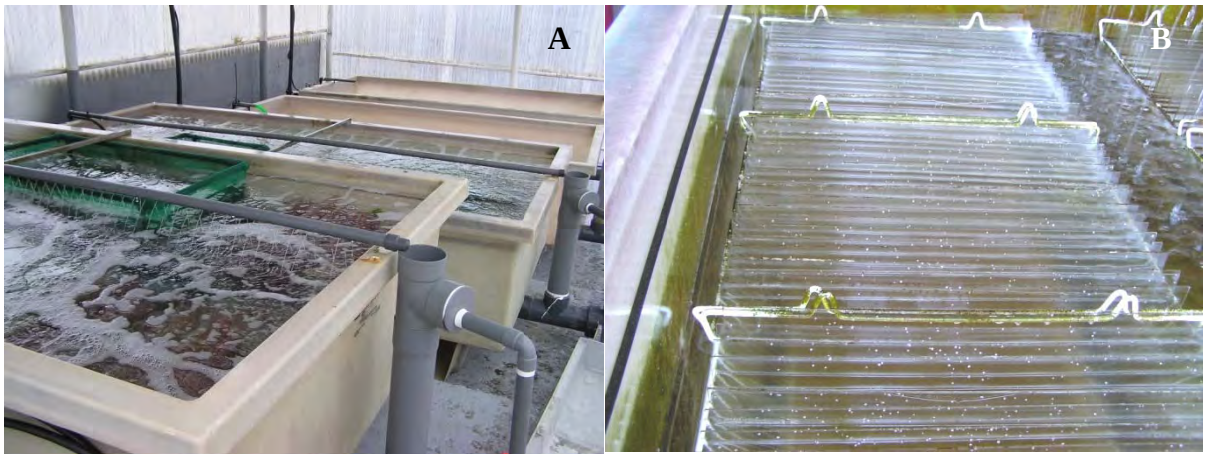

Fig. 8: Vista general del criadero experimental en el ICCM (A). Detalle de las placas de fijación (B).

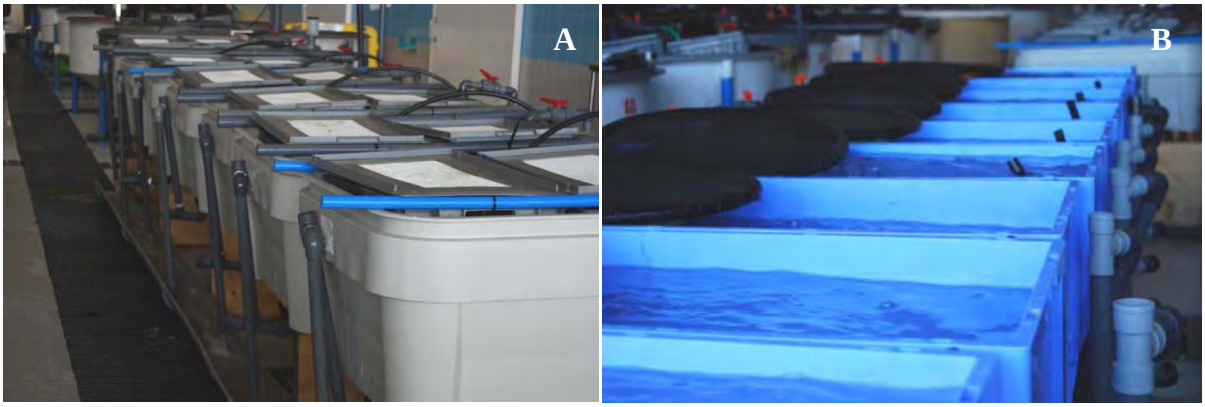

Fig. 9: Area de engorde (A) y alimentación experimental de abalón (B).

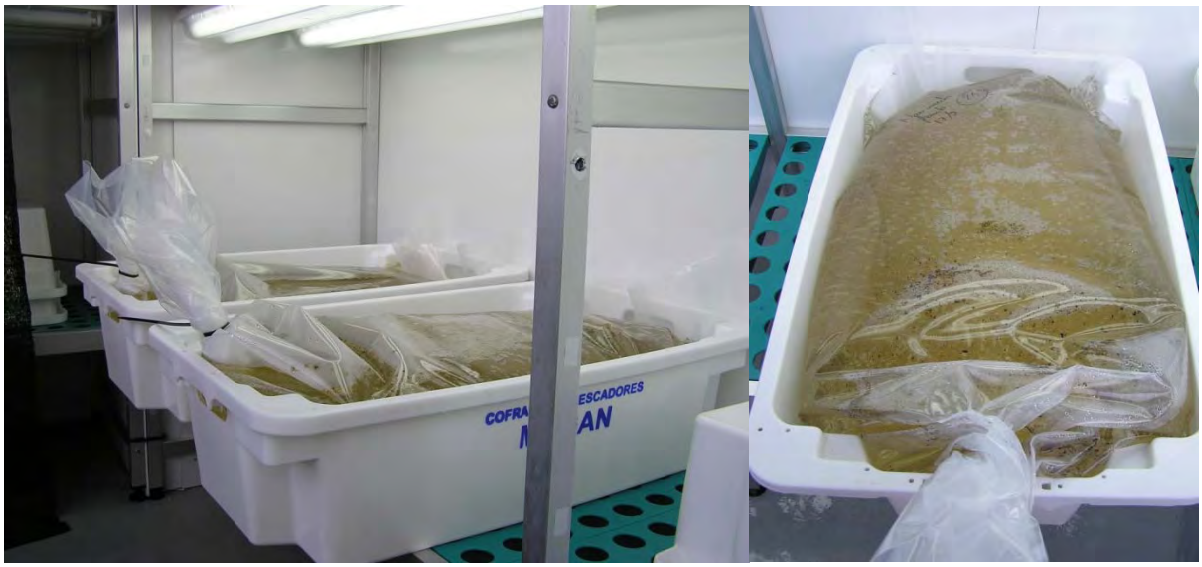

Fig. 10: Producción de diatomeas bentónicas.

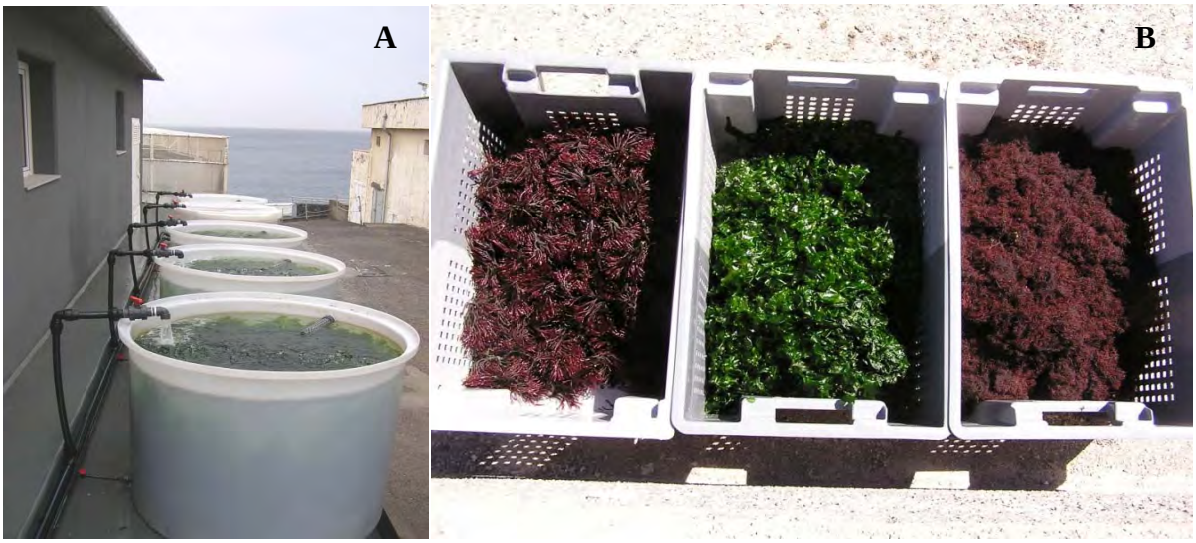

Fig. 11: Unidades de biofiltración (A) y producción de macroalgas resultante (B).

11.4.2 Acondicionamiento de reproductores y selección

Los reproductores de *Haliotis tuberculata coccinea* se mantuvieron en cautividad en condiciones de fotoperiodo natural y de temperatura de agua ambiente, en la oscuridad, en tanques de 60 l, en sistemas de acondicionamiento de reproductores, en flujo abierto (fig. 7, fig. 12). Dentro de cada uno de los 24 tanques de acondicionamiento de reproductores, 10-15 animales (dependiendo de su tamaño) se mantuvieron bajo tejas de PVC que les proporcionaban refugio, y fueron separados de los desechos, en la parte inferior del tanque, a través de una pared perforada. Machos y hembras, diferenciados por el color de las gónadas (de color blanco cremoso para los machos y gris oscuro a violeta para las hembras) (Fig. 13), se mantuvieron por separado en tanques de acondicionamiento. Los reproductores se alimentaron dos veces por semana con una dieta mixta de *Ulva rigida*, *Gracilaria cornea* e *Hypnea Spinella* producidas en las unidades de biofiltración. Los abalones seleccionados para ser inducidos al desove fueron los que presentaban gónadas maduras en la etapa 3 o entre la etapa 2 y 3 (Fig. 13).

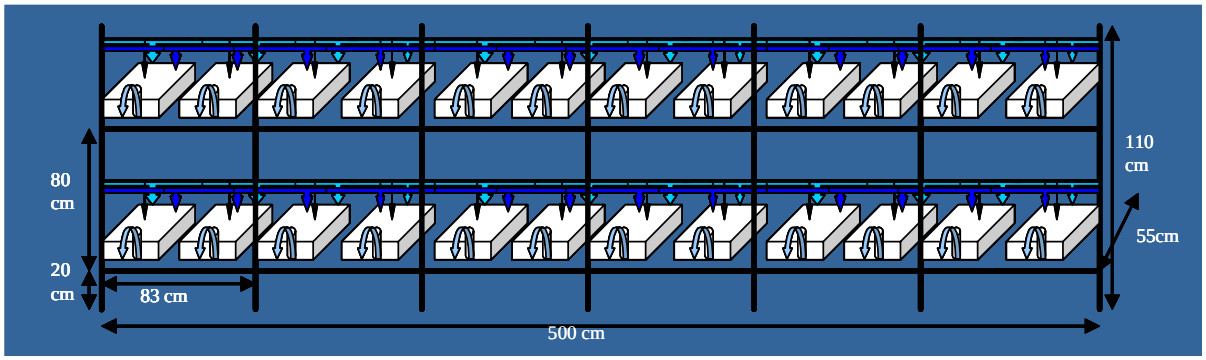

Fig. 12: Diagrama del sistema de acondicionamiento de reproductores.

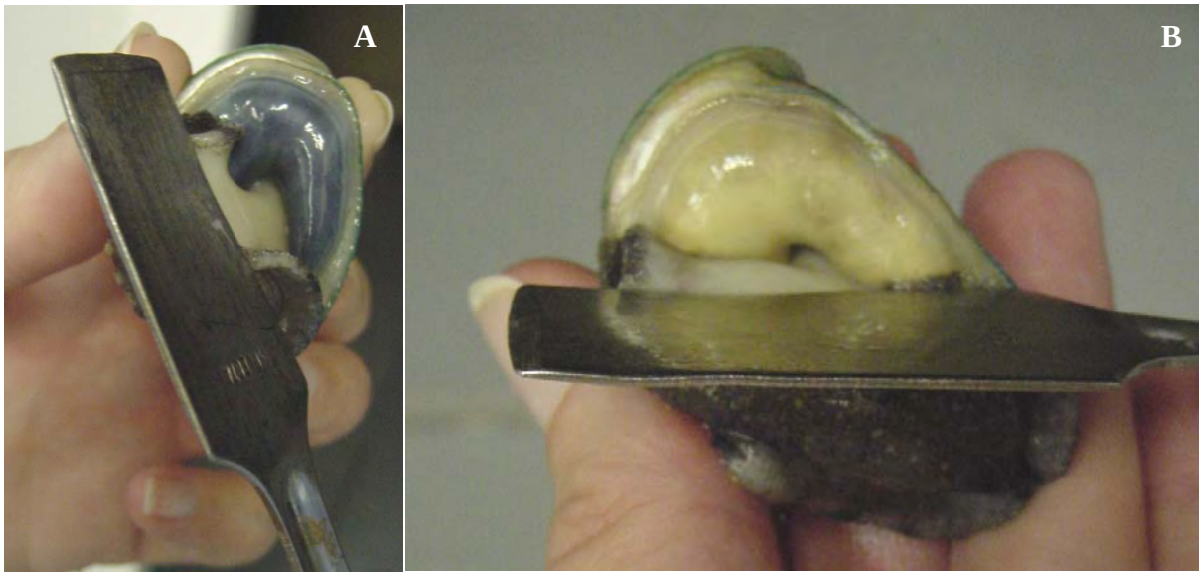

Fig. 13: Hembra (A) y macho (B) de *H. tuberculata coccinea* en etapa 3 del índice gonadal.

Las etapas de desarrollo gonadal del abalón han sido identificadas por Ebert & Houk, (1984), que la diferenciaron de acuerdo con el índice gonadal siguiente:

0 – Inmaduros; sexo indeterminado, la glándula digestiva es fácilmente visible como una masa gris-marrón.

1 – Desarrollo de gametos iniciado; los gametos aparecen en un patrón irregular, de color diferenciado, en la superficie de la glándula digestiva. La determinación del sexo es fácil para los machos en esta etapa debido al color cremoso, sin embargo, la determinación del sexo femenino es difícil.

2 – Los gametos envuelven completamente el apéndice cónical, el sexo es fácil de determinar, pero las gónadas no son voluminosas.

3 – Similar a la condición para la etapa de índice 2, salvo que la gónada es muy voluminosa. Este volumen se extiende hasta la punta de las gónadas.

11.4.3 Inducción a la puesta

Los machos y hembras adultos, con un ratio macho/hembra de 1:2, fueron inducidos a desovar, separados por sexo, en recipientes de desove rellenados con agua de mar filtrada a 1-µm a través de filtros de cartuchos y esterilizada por UV. La esterilización UV del agua se realizó a 254 nm de longitud de onda con los equipos industriales (Mod REX 1x36HO15-PE;

Wedeco, AG, Herford, Alemania). Los gametos de distinto sexo se obtuvieron por separado con el fin de controlar el ratio de gametos empleados durante la fecundación y tener un mejor control sobre el proceso de fertilización. Durante la inducción al desove los recipientes se quedaron en la oscuridad. Dos métodos de inducción al desove fueron utilizados para llevar a cabo estos estudios, el método del peróxido de hidrógeno (Morse et al., 1977) y el método del ultravioleta (Kikuchi & Uki, 1974).

11.4.3.1 Método de inducción al desove del peróxido de hidrógeno

Se ha descrito que este método de inducción esta incitado por el radical libre HOO^- o el peróxido di-radical $^-OO^-$ que se pueden producir cuando el peróxido de hidrógeno (H_2O_2), se añade al agua. Por otra parte un pH alto permite aumentar la descomposición del peróxido de hidrógeno, añadido, en estos radicales libres oxidantes altamente reactivos y de actuación corta.

Los machos y hembras de *H. tuberculata coccinea*, a inducir al desove, se colocaron en depósitos de 10L conteniendo agua de mar filtrada a 1µm a través de filtros de cartuchos y esterilizada con UV y aireación. El pH del agua se incrementó primero a 9,1 mediante la adición de 6,6 ml de 2 M de Tris - (hidroxi-metilamino) metano por litro de agua de mar en los depósitos de desove. La solución de 2M de Tris se realizó mediante la adición de 24.2g de Tris a 75 ml de agua destilada y rellenando este hasta 100 ml una vez totalmente disuelto. Quince minutos después de agregar el Tris, 3 ml de solución, recién preparada, de peróxido de hidrógeno al 6% se añadió por cada litro de agua en el recipiente. La solución de peróxido de hidrógeno al 6% se preparó por dilución de una solución de peróxido de hidrógeno al 30%, añadiendo 20 ml de peróxido de hidrógeno al 30% a 80 ml de agua destilada, para obtener un volumen final de 100 ml de peróxido de hidrógeno al 6%. El agua en el recipiente se agitó vigorosamente para mezclar la solución. Los abalones permanecieron en esta mezcla durante 1,5 a 2 horas después de las cuales se vaciaron los depósitos y las orejas de mar se enjuagaron con agua de mar isotérmica para eliminar trazas de los productos químicos. Este paso es esencial, ya que los productos químicos empleados destruyen los gametos liberados. A continuación, los recipientes fueron rellenados con agua de mar, isotérmica y después de alrededor de 30 minutos o una hora y media más tarde, los abalones se han observados desprendiéndose de sus gametos (Fig. 14).

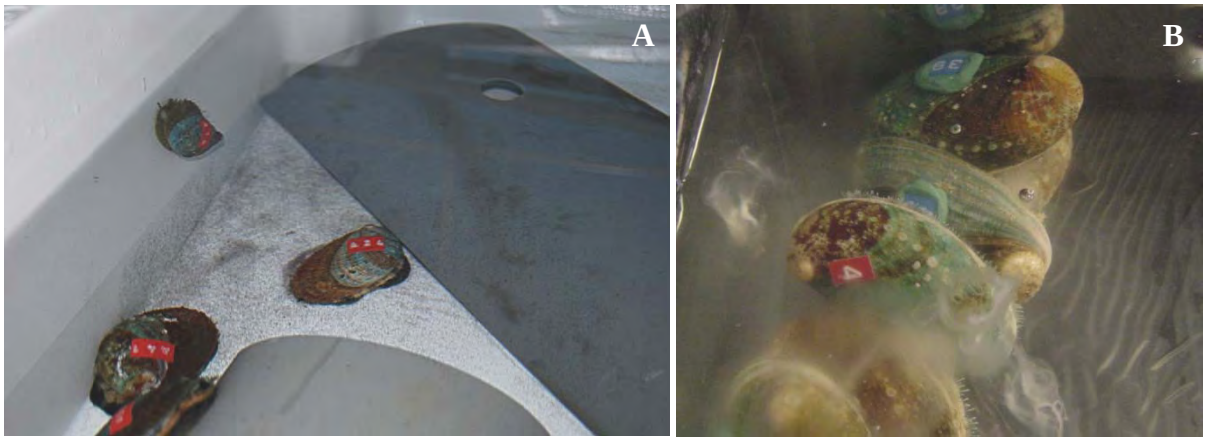

Fig. 14: Expulsión de gametos de hembras (A) y machos (B).

11.4.3.2 Método de inducción al desove del Ultra Violeta

Este método de inducción al desove fue originalmente descrito por Kikuchi & Uki (1974) quienes afirmaron que la radiación ultravioleta causa la descomposición de la molécula de agua en radicales libres hidroperoxi HOO^- o en peróxido dirradical $^-OO^-$, responsables de la inducción al desove (Hahn, 1989). Este método es generalmente considerado como el mejor y el más fiable para la inducción al desove en la mayoría de las instalaciones de cultivo de abalón a nivel mundial.

Los machos y hembras de *H. tuberculata coccinea*, a inducir al desove, se colocaron en depósitos de desove, conteniendo agua de mar filtrada a 1µm a través de filtros de cartuchos y esterilizada con UV y aireación. Los reproductores permanecieron en la oscuridad hasta el inicio del desove. La radiación ultravioleta del agua se realizó a 254 nm de longitud de onda con los equipos industriales (Mod REX 1PE10; Wedeco, AG, Herford, Alemania). El tiempo entre el inicio de la estimulación UV y el desove es proporcional a la cantidad de radiación UV, por lo que se recomienda que el potencial ideal del UV sea de 800 milivatios hora por litro. Utilizando este método, se observó que los reproductores de *H. tuberculata coccinea* empezaron a desovar entre 2 y 3 horas después del inicio de la estimulación UV (Fig. 14).

Una de las ventajas de la utilización de rayos UV para la inducción al desove es que su uso es totalmente inofensivo para los gametos. Como consecuencia no se requieren cambios de agua durante el proceso de reproducción.

11.4.4 Método de fertilización y su estimación

Una vez obtenidos los gametos, los ovocitos liberados, que presentan una flotabilidad negativa, fueron sifonados de los depósitos de desove y tamizados por una malla de 300 micras con el fin de retener las heces y otros residuos (Fig. 15). Los ovocitos fueron colectados en recipientes de 10L y fertilizados con una concentración de espermatozoides final de 10^5/ml durante 30 minutos. La concentración de esperma fue controlada con el fin de garantizar la máxima tasa de fertilización sin riesgo de occurir polispermia, observado en caso de exceso de densidad de espermatozoides (Hahn, 1989). Con el fin de determinar la concentración de esperma, una gota de solución de lugol se añadió a las muestras de esperma y la cantidad de espermatozoides se contabilizó con un hemocitómetro (Mod. Neubauer, Alemania) (Fig. 15). Otro parámetro a considerar durante la fecundación es la viabilidad de los gametos, por lo que con el fin de maximizar el éxito, la fecundación se realizó con gametos de tras máximo una hora después de su expulsión. El tiempo para la fecundación fue de 30 minutos, después de los cuales, los huevos se lavaron con agua de mar para eliminar el exceso de esperma y las tasas de fertilización se determinaron mediante el registro de la proporción de huevos que presentaban células en división 1 h después de la fecundación. La estimación de la tasa de fertilización se realizó tomando tres muestras de 3 ml de los depósitos de fertilización y registrando el número de huevos fertilizados observados en cámaras de recuento bajo una lupa (Mod. SL 260004, Optech, Alemania). Los huevos fertilizados, del mismo lote y obtenidos a partir de gametos fertilizados procedentes de varios machos y hembras, se colocaron en bandejas (bandejas de incubación) de fondo plano hasta formar una monocapa de huevos permaneciendo en el interior hasta llegar a la etapa de larva trocófora. Las bandejas de incubación son rectangulares y de 60 L provistas de una toma de agua en un extremo y de una salida de agua en el extremo opuesto evacuando el flujo hacia tanques de cría larvaria (Fig. 16). Se dispuso un flujo suave de agua en las bandejas de incubación una vez observado que la totalidad de los huevos se había distribuido uniformemente sobre el fondo de las bandejas.

Tras la eclosión, las larvas trocóforas, que nadan hacia la superficie fueron separadas de los huevos no eclosionados y de los desechos de la eclosión por desbordamiento, y fueron entonces transferidas por medio del flujo hacia los tanques de cría larvaria.

Fig. 15: Recogida de huevos (A) y eliminación de los desechos (B). (C) Estimación de la concentración de espermatozoides.

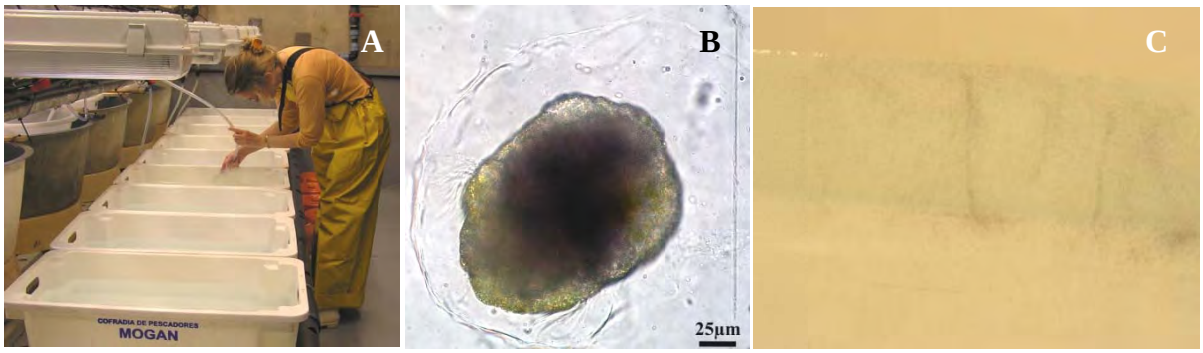

Fig. 16: Bandejas de incubación (A) y larva trocófora tras la eclosión, presentado cilios (B) que permiten la actividad natatoria (C).

11.4.5 Cultivo larvario

Durante la fase de cultivo larvario las larvas de abalón empiezan en fase trocófora hasta ser caracterizadas como competentes para la fijación y la metamorfosis.

Dos métodos se pueden utilizar para la cría larvaria: cultivo estático o en flujo abierto. En el método estático, las larvas se crían en grandes tanques (5-20 m^3) sin flujo continuo de agua, cambiándose el agua en cada tanque periódicamente, cada uno o dos días. En el presente estudio las larvas de *H. tuberculata coccinea* se criaron, bajo fotoperiodo natural y a temperatura ambiente del agua, en sistema de cultivo larvario en flujo abierto que consiste en doce tanques larvarios de 100 L, que se describen a continuación (Fig. 17).

Durante esta fase del cultivo, se debe prestar una atención especial a la calidad del agua y, por lo tanto, la instalación de cultivo larvario fue equipada con un equipo de filtración y esterilización. El agua suministrada a los tanques larvarios se filtró a través de filtros de cartucho de 1µm y se esterilizó por irradiación UV a 254 nm de longitud de onda con un equipo industrial (Mod REX 1x36HO15-PE; Wedeco, AG, Herford, Alemania) (Fig. 17).

Cada tanque de cultivo larvario, de 100 L, fue equipado con malla de 63µm en la salida para evitar la pérdida de las larvas, realizándose el cultivo en flujo abierto. Las mallas fueron sumergidas en el agua del cultivo para asegurar una superficie máxima de contacto entre las larvas y la malla para evitar la obstrucción. Una aireación suave se proporcionó a través de piedras de aeración y la renovación del agua se reguló para generar una tasa aproximada de renovación por hora del 20% (Fig. 18).

Las larvas nadando en superficie de las bandejas de incubación fueron transferidas directamente por desbordamiento a los tanques de cultivo larvario a una densidad de larvas de 10-20 ml^{-1} (Fig. 18). La densidad en los tanques larvarios fue controlada por el registro del número de huevos fecundados introducidos en cada bandeja de incubación y por una distribución uniforme entre las bandejas de incubación.

Las larvas oligotróficas permanecieron sin comer en los mismos tanques durante la totalidad del periodo de cultivo larvario, siendo trasladadas para su fijación después de la aparición del tercer túbulo en los tentáculos cefálicos, una indicación de competencia para la fijación de las larvas. La competencia para la fijación se observó entre 62 y 72 horas después de la fertilización en función de la temperatura durante el cultivo larvario.

Las larvas utilizadas en cada experimento pertenecían al mismo lote y fueron obtenidas a partir de gametos fertilizados de diferentes machos y hembras.

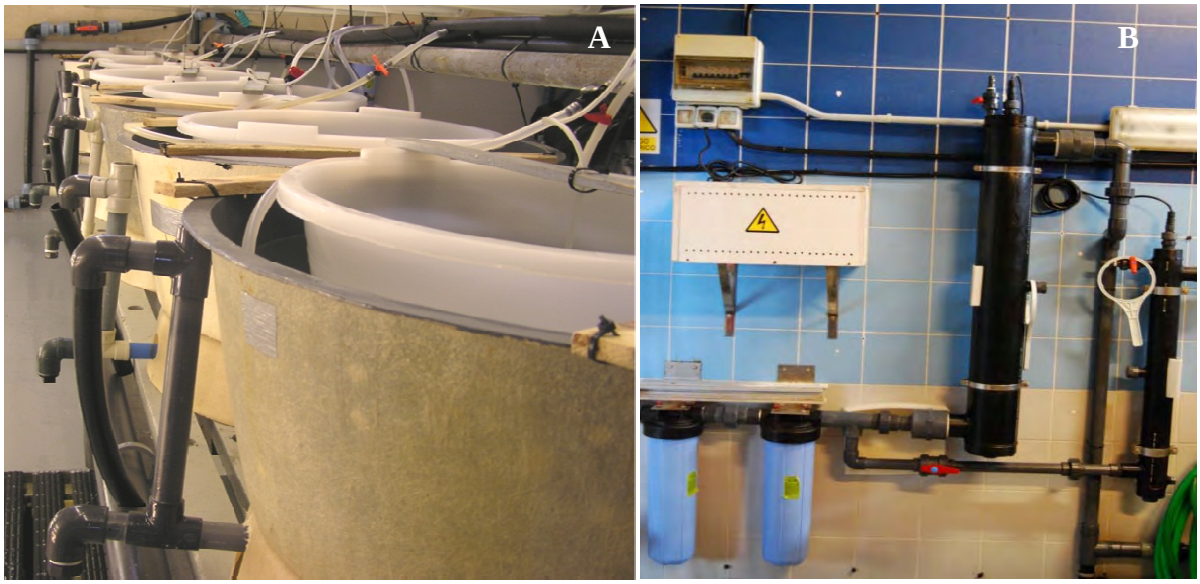

Fig. 17: Instalación de cultivo larvario (A) y equipamiento de filtración y esterilización (B).

Fig. 18: Tanque de cultivo larvario (A) y bandeja de incubación vinculada al tanque de cultivo larvario en sistema de flujo abierto (B).

11.4.5.1 Condiciones experimentales

El estudio sobre el desarrollo embrionario y larvario de *H. tuberculata coccinea* se inició inmediatamente después de la fertilización y se llevó a cabo de manera continua hasta la aparición del tercer túbulo en los tentáculos cefálicos. Todas las observaciones se realizaron con muestras de larvas mantenidas en depósitos de 5L. Las muestras de larvas provenían del mismo período de fertilización y del mismo lote y se tomaron muestras de las bandejas de incubación y de los tanques de cultivo larvario a intervalos regulares. Todas las observaciones se llevaron a cabo continuamente durante la totalidad del desarrollo estudiado y se realizaron bajo luz transmitida con un microscopio Leitz DMRBE (Leica, Wetzler, Alemania) a un aumento de x400 (Fig. 19). Se registró el tiempo requerido por los huevos y larvas para alcanzar cada etapa. Imágenes de cada etapa fueron tomadas por una cámara digital (Evolt E-300, Olympus) acoplada al microscopio. El diámetro de los huevos así como la longitud y anchura de las larvas se midieron a partir de fotografías (Fig. 19).

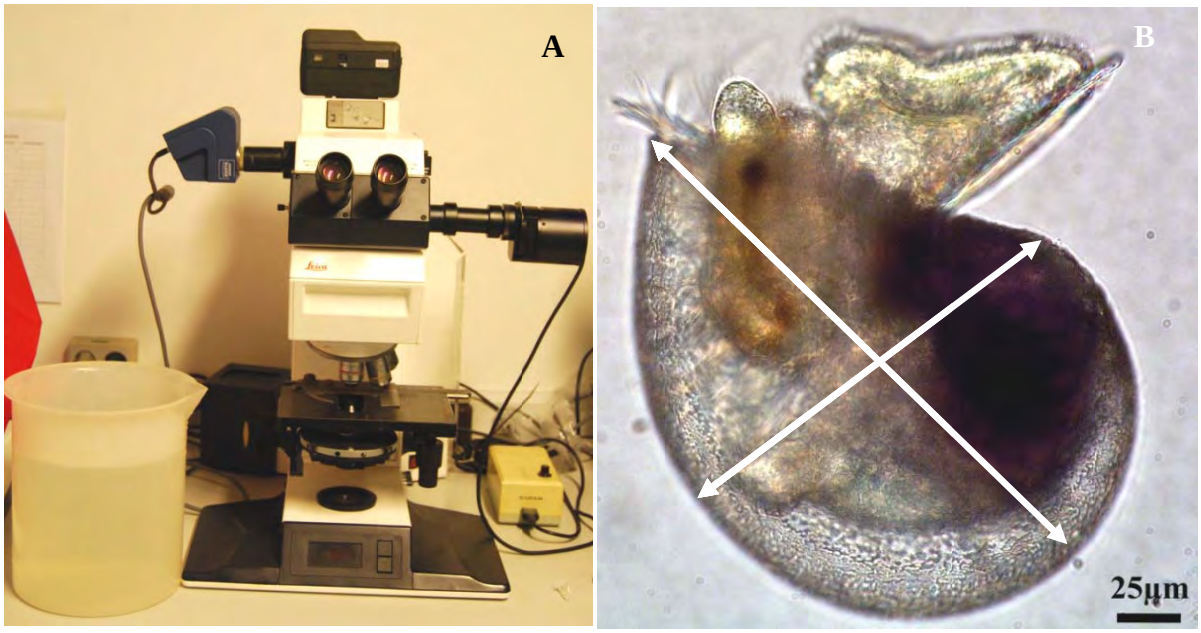

Fig. 19: Microscopio Leitz (A) y medidas de la longitud y anchura de las larvas (B).

11.4.6 Fijación larvaria y crecimiento post-larvario

La inducción a la fijación de *H. tuberculata coccinea*, en el criadero, se realizó en placas verticales de fijación agrupadas en cestas en el interior de los tanques de fijación. Cada tanque de fijación tenía una capacidad de 2500 litros y contenía 12 cestas con 20 placas de fijación de 60x30 cm cada una. La aireación se proporcionó a través de cuatro líneas de aeración en la parte inferior del tanque, localizadas a lo largo de los lados de las cestas, y perpendicularmente a las placas (Fig. 20). Cada tanque de fijación constaba de flujo abierto y de agua de mar filtrada. El agua de mar se filtró mecánicamente a través de un filtro de arena (Mod. 00689, piscina Astra, Barcelona, España), seguido de una filtración por cartucho situada a la entrada de cada tanque de fijación (Fig. 21). Una vez fijadas, las post-larvas se alimentaron de una mezcla de diatomeas bentónicas, cultivadas y proporcionadas a las post-larvas una vez en semana. Las especies de diatomeas y las condiciones de cultivo se describen con más detalle a continuación.

Para llevar a cabo experimentos de fijación, crecimiento y supervivencia a pequeña escala, en condiciones de cultivo que sean representativas de las condiciones de criaderos viveros, todos los experimentos de fijación se realizaron según la metodología descrita en las siguientes secciones.

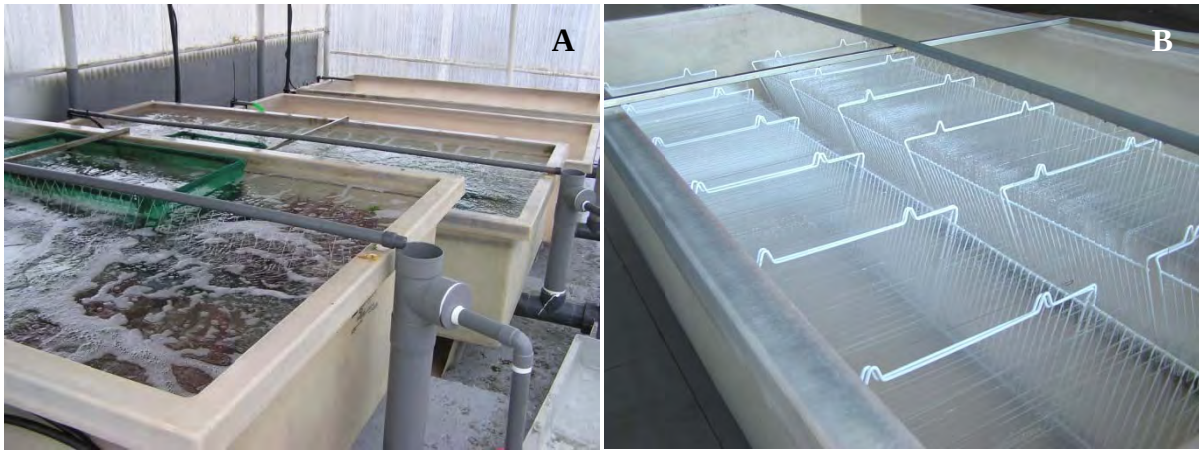

Fig. 20: Á rea de criadero de *H. tuberculata coccinea* (A) y detalle de un tanque de fijación (B).

Fig. 21: Unidades de filtración de agua en área de criadero.

11.4.6.1 Condiciones experimentales

En la primera serie de estudios de fijación, las placas experimentales de fijación consistieron en cuadrados de 50 cm^2 de plástico colonizados por los estímulos de fijación a estudiar, a diferencia de las placas de control negativo que no fueron colonizadas. Para cada tratamiento, cada replica consistió en cuatro placas experimentales de fijación colocadas verticalmente en depósitos de 12L rellenos de de agua de mar filtrada a 1µm por filtro de cartucho y suministrados con aireación suave (Fig. 22). La densidad de larvas introducidas en los depósitos se estimó mediante el recuento de tres sub-muestras de 3 ml procedentes del lote global de larvas. Las larvas, en todos los experimentos, provenían del mismo lote y las densidades estudiadas fueron 2.000 y 200 larvas por contenedor de 12L, correspondientes a

10 y 1 larvas por cm^2 de sustrato, respectivamente. Veinticuatro horas después de la introducción de las larvas, se dispuso flujo en los contenedores experimentales a una tasa de renovación del 1% por hora, incrementándose hasta una renovación del 20% por hora tras 72 horas de cultivo. La salida de agua se equipó con una malla de 125 micras para evitar la pérdida de larvas en condiciones de flujo abierto (Fig. 22). Los experimentos de fijación se realizaron a temperatura de agua ambiente (19-21 º C) y bajo un fotoperiodo artificial de 12:12 horas L: D con una intensidad de luz de 2000 lux que se midió utilizando un medidor de luz digital (HT170N, HT ITALIA, Italia).

Una vez fijadas y tras haber sufrido la metamorfosis, las post-larvas se alimentaron semanalmente con 200 ml de una mezcla de diatomeas (10^6 células ml^{-1}). Las especies de diatomeas, las condiciones de cultivo y el recuento celular se describen con más detalle a continuación. El crecimiento post-larval y la supervivencia fueron seguidos durante 4 semanas después de la fijación y la metodología empleada se describe más ampliamente a continuación.

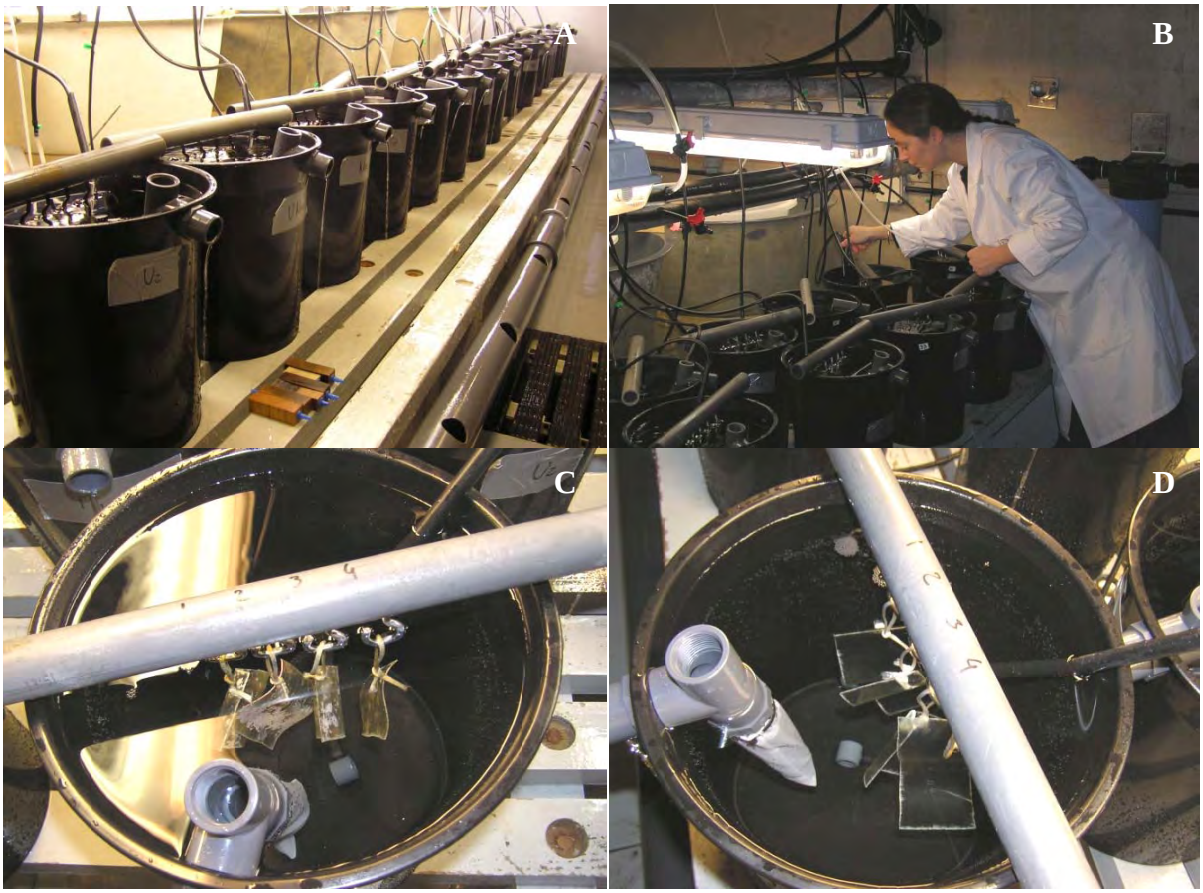

Fig. 22: Vista general de la instalación experimental de inducción a la fijación (A & B). Detalle de las placas de fijación en los diferentes tratamientos, así como la aeración y las salidas de los depósitos (C & D).

En el otro conjunto de estudios de fijación, las larvas competentes fueron inducidas a la fijación sobre placas colonizadas con una mezcla de esporofitos de *U. lens* y *U. rigida* cultivados durante 45 días. Para cada tratamiento, cada replica consistió en un tanque de 100 litros conteniendo cuatro placas de de fijación de 60 x 30 cm, colonizadas por los estímulos, mas cuatro plaquetas de 50 cm^2. Las placas y plaquetas de fijación fueron colocadas verticalmente en los tanques de fijación, llenos de agua de mar filtrada a 1μm por filtro de cartucho, y suministrados con aireación suave. Larvas competentes, del mismo lote, se introdujeron a una densidad de 7.200 larvas por tanque de 100 L, lo que representa 0,5 larva por cm^2 del sustrato de fijación. Veinticuatro horas después de la introducción de las larvas, el flujo se inició en los tanques de experimentación, a razón de una renovación del 1% por hora y se incrementó hasta una renovación del 20% por hora después de 72 horas. La salida de agua fue equipada con una malla de 125 micras para evitar la pérdida de larvas cultivadas en condiciones de flujo abierto. Tres días después de la fijación soluciones de diatomeas procedentes de cultivo se añadieron a los tanques de 100 litros como suministro de alimentos. Cuatro diferentes tratamientos de diatomeas, *Amphora* sp., *Navicula incerta, Nitzschia* sp. y *Proschkinia* sp., fueron probados, por triplicado, como fuente de alimento para las post-larvas de *H. tuberculata coccinea*. En cada replica, las post-larvas se alimentaron semanalmente con 2 litros de diatomeas respectivas (10^5 a 10^6 células ml^{-1}) cuyas condiciones de cultivo y estimaciones del recuento celular se describen con más detalle a continuación. La temperatura del agua en los depósitos de cultivo fue de 21 ± 1 º C y el experimento se realizó bajo un fotoperiodo artificial 12:12 horas L: D siempre a una intensidad de luz de 2000 lux que se midió utilizando un medidor de luz digital (HT170N, HT ITALIA, Italia). El crecimiento post-larval y la supervivencia fueron seguidos durante 10 semanas tras la fijación y la metodología empleada se describe con más detalle a continuación. La composición proximal y el análisis de los ácidos grasos de las fuentes de alimentación y de las muestras de juveniles se llevaron a cabo una vez finalizado el período experimental.

Fig. 23: Tanques experimentales de 100-L.

11.4.6.2 Medidas

El número de post-larvas fijadas y su supervivencia se estimó mediante el registro de la cantidad de post-larvas vivas en las placas de fijación en cada replica bajo una lupa (Mod. SL 260004, Optech, Alemania) (Fig. 24). La tasa de fijación se estimó 48 horas después de la introducción de las larvas a los depósitos de fijación, mientras que la supervivencia fue objeto de un seguimiento semanal. Las larvas se consideraron fijadas una vez adheridas permanentemente al sustrato después de perder el velum para completar su metamorfosis. El crecimiento post-larval fue revisado semanalmente o cada quince días muestreando la longitud de la concha de las post-larvas seleccionadas al azar para cada tratamiento. Las medidas se tomaron con un proyector de perfil (Mod. PJ-H3000, MITUTOYO, Japón) (Fig. 24 y 25). Las placas se mantuvieron inmersas en todo momento durante la observación y fueron reemplazadas inmediatamente, en los depósitos experimentales, después de la observación y medición (Fig. 25). En base a los datos obtenidos, la tasa de crecimiento diario (DGR) se calculó según la fórmula:

$$\frac{Lf - Li}{t}$$

Lf siendo la longitud final de concha en micrómetros y *Li* la longitud inicial de concha en micrómetros. *t* representa el tiempo en días.

Fig. 24: Lupa (A) y proyector de perfil (B).

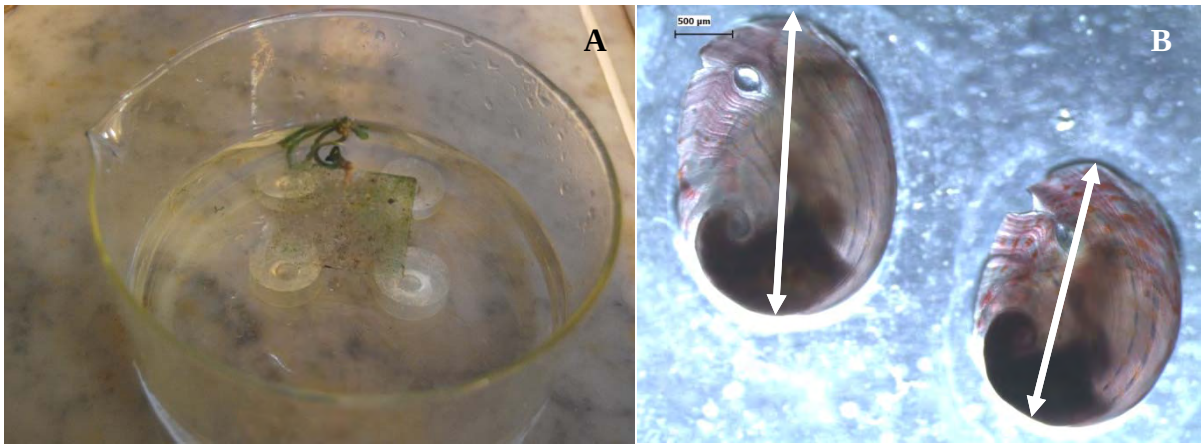

Fig. 25: Placa de fijación en inmersión (A) y medidas de conchas (B).

11.4.6.3 Estimación de la densidad celular y del porcentaje de cobertura del alga

Con el fin de inocular y controlar la tasa de crecimiento, se registró el número de células de diatomeas. Las células se contaron con un hemotocitómetro (Mod. Neubauer, Alemania). Para evitar posibles agregaciones de células, las muestras fueron sometidas a ultrasonidos (Mod. 3510 E-MT, Branson, EE.UU.) (1 o 3 minutos) antes de la evaluación de

su concentración. Las tasas medias de crecimiento fueron estimadas utilizando la fórmula siguiente:

$$\mu=Ln\ (N_1/N_0)/\ t_1-t_0$$

N_1 = densidad celular a tiempo t_1 y N_0 = densidad celular a tiempo t_0, (Guillard, 1973).

El porcentaje de cobertura de los sustratos de fijación estudiados, así como el número de células de diatomeas adheridas por cm^2 fueron estimados al azar eligiendo diez campos de vista de las placas de fijación, para cada tratamiento, y fotografiándolos a un aumento de 400 x en el momento de la fijación. El número de células y el porcentaje de cobertura se calculó mediante el procesamiento de las imágenes con el Software Image J (National Institutes of Health, USA, Image J 1.42q) (Fig. 26).

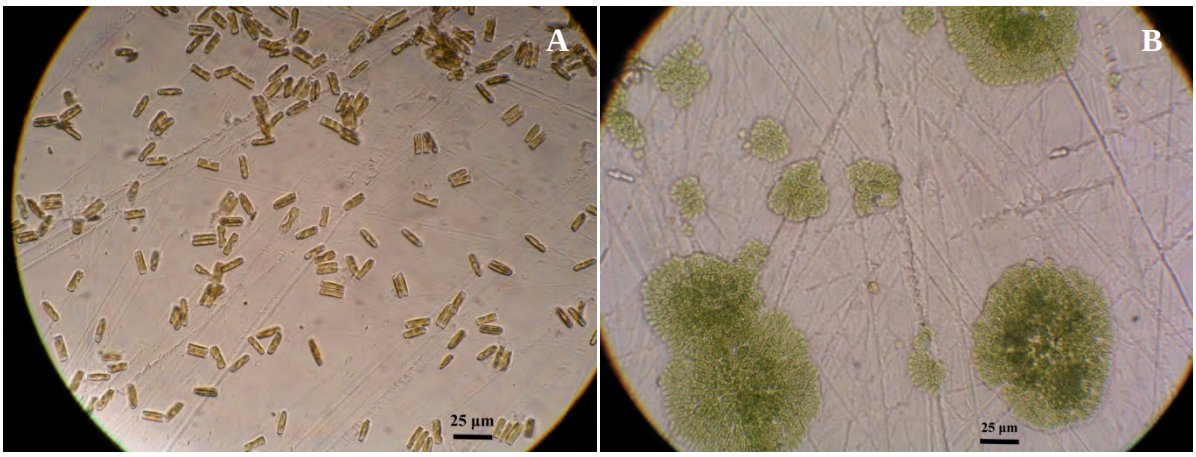

Fig. 26: Campos de vista, a aumento x400, de places de fijación colonizadas por diatomeas (A) y *U. Lens* (B).

11.4.7 Cultivo de algas

11.4.7.1 Algas coralinas incrustantes

Con el fin de colonizar las placas de fijación con algas coralinas incrustantes, las placas de fijación se dejaron ser colonizadas en los tanques de criadero hasta alcanzar una media del 50% de cobertura (Fig. 27).

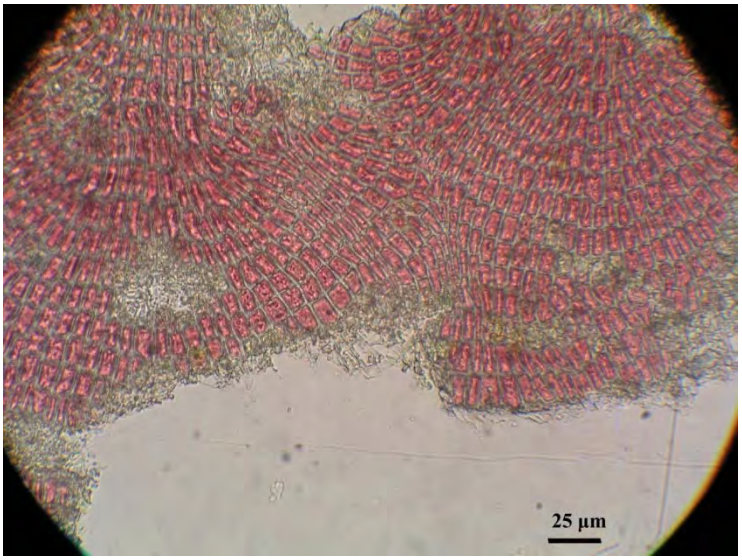

Fig. 27: Campo de vista de CCA colonizando una placa de fijación experimental

11.4.7.2 Macroalgas verdes

Las esporas de algas verdes utilizadas como sustratos de inducción a la fijación fueron *U. lens* y *U. rigida*. Esporofitos de ambas especies se obtuvieron a partir de talos maduros (produciendo esporas) de *U. lens* y *U. rigida*. *U. lens* se mantuvo en cultivo bajo fotoperiodo natural y con la adición cada dos semanas de una mezcla f / 2 (Guillard, 1975) (Fig. 28). *U. rigida* se cultivó bajo fotoperiodo natural en unidades de biofiltración localizadas en el exterior y que reciben efluentes de agua procedentes de tanques de peces (Fig. 28).

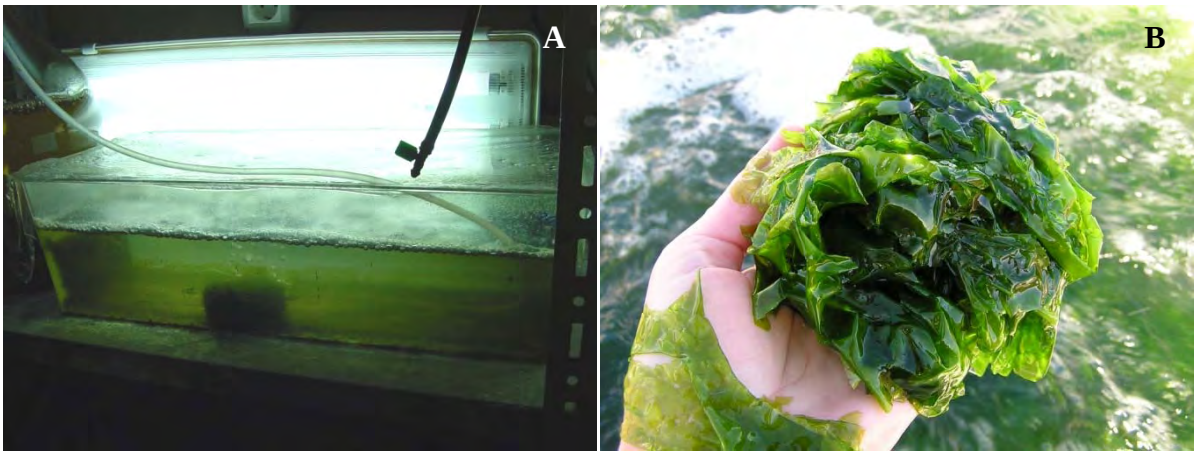

Fig. 28: Cultivo madre de (A) *U. lens* y (B) *U. rigida.*

Placas que presentaban grandes manchas verde oscuras, de *U. lens* fueron libradas de la película de diatomeas y se dejaron en la oscuridad durante 2 semanas. Los talos de *U.*

rigida fueron sometidos a 7 días de tratamiento en frío (4 ºC) antes de iniciar el acondicionamiento de las placas de experimentación. Con el fin de inducir la liberación de esporas, ejemplares de cada especie fueron colocados en los tanques, que contenían las placas experimentales, mantenidos sin flujo de agua, con aireación suave y a los cuales se aportó una mezcla de f / 2 completa. La mayor liberación de esporas se observó 4-5 días después de la introducción de las macroalgas maduras en los tanques conteniendo las placas de experimentación. Los métodos para la producción de esporofitos y su cultivo han sido adaptados de Daume et al., (2004) y la Strain et al., (2006). Los esporofitos de las placas experimentales de fijación se mantuvieron en cultivo durante períodos diferentes para evaluar el efecto de la edad del sustrato y su calidad sobre la inducción a la fijación. Durante este período, los esporofitos se mantuvieron con aeración, bajo fotoperiodo natural y con una renovación semanal del medio de cultivo f / 2 (Fig. 29).

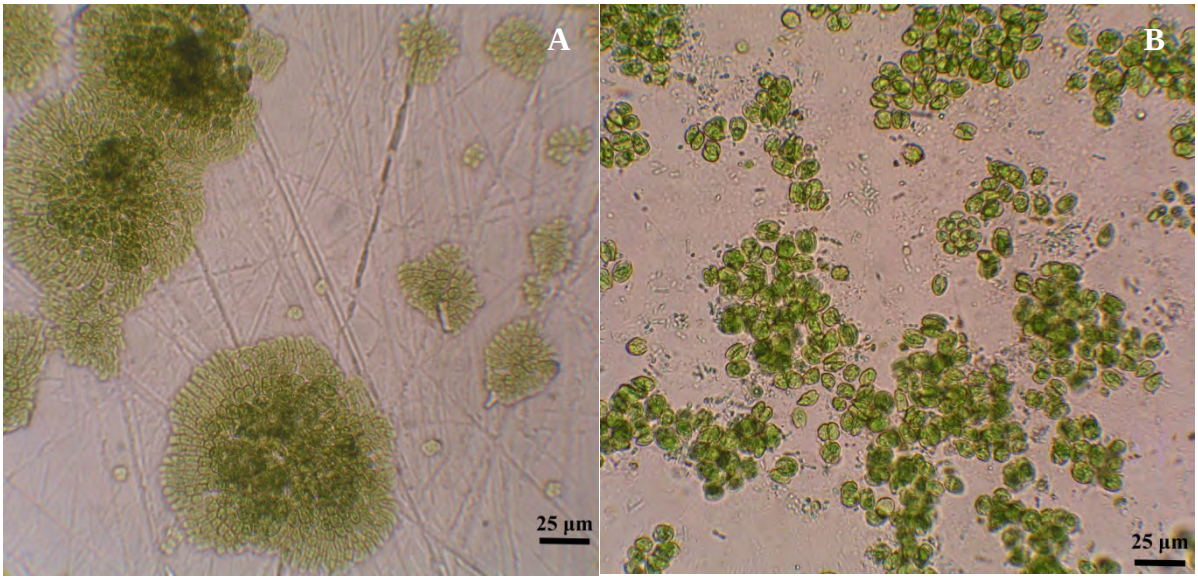

Fig. 29: Esporofitos de *U. lens* mayor (A) y *U. rigida* joven (B).

11.4.7.3 Diatomeas bentónicas

El cultivo a gran escala de las diatomeas bentónicas se realizó en forma horizontal en bolsas de polietileno de 40 L. Cuatro especies de diatomeas *N. incerta*, *Proschkinia* sp., *Nitzschia* sp. y *Amphora* sp. se cultivaron de forma individual y se escalonaron hasta llegar a un cultivo a gran escala. Se mantuvieron, estimándose su crecimiento en tubos de ensayo de 10 ml, los cultivos se escalonaron a frascos Erlenmeyer de 4L y, finalmente, se cultivaron en bolsas de 40 L (Fig. 30). Las diatomeas fueron cultivadas en medio f/2, más silicato ($1mgL^{-1}$)

(Guillard 1975), a temperatura ambiente y bajo luz continua de 62 ± 8 mmol fotones m^{-2} s^{-1}. En el protocolo de cultivo tipo "Batch", todas las diatomeas fueron cultivadas con un inoculo inicial de 10^5 células ml^{-1} y se cosecharon después de 5 días de cultivo, correspondiente a la fase de crecimiento exponencial. Los cultivos fueron unialgales, pero no axénicos, y se mantuvieron sin aeración ni agitación. La densidad celular de diatomeas se evaluó con un hemocitómetro (Mod. Neubauer, Alemania). Se aplicaron ultrasonidos (Mod. 3510 E-MT, Branson, EE.UU.) (1 o 3 minutos) a las muestras de diatomeas, antes del recuento, para evitar posibles agregaciones de células. La densidad de flujo de fotones (radiación) se midió con un medidor de luz digital (HT170N, HT ITALIA, Italia). El agua empleada para el cultivo de diatomeas bentónicas fue filtrada mecánicamente a través de un filtro de cartucho y se esterilizo mediante radiación UV como se describió anteriormente.

Las diatomeas cultivadas fueron empleadas como fuente de nutrición para las post-larvas, y en el caso de ser utilizadas como sustrato de inducción a la fijación, las diatomeas fueron cosechadas después de 5 días de cultivo y transferidas a depósitos que contenían placas experimentales de fijación a colonizar por diatomeas hasta llegar a un densidad aproximada de 10^5 células por cm^2.

Fig. 30: Cultivo tipo "Batch" de diatomeas bentónicas.

11.4.8 Análisis bioquímico y de ácidos grasos

Durante el curso de los diferentes estudios, muestras de diatomeas, de algas del sustrato, de larvas y post-larvas, se recolectaron por triplicado para el análisis de su composición nutricional. Concretamente se analizaron los contenidos en materia seca, cenizas, proteínas y lípidos, así como los ácidos grasos. Los métodos de recolección de las muestras difieren en función de su naturaleza. Las muestras de diatomeas se recogieron por la filtración de las bolsas de algas y se enjuagaron con agua dulce destilada para eliminar las partículas de sal antes de ser almacenadas. Las algas de los sustratos se recolectaron mediante

el raspado de la superficie de las placas de fijación al inicio y final de los experimentos. Las larvas fueron sifonadas de los tanques de larvas, recolectadas en una malla de 63µm y enjuagadas con agua dulce destilada. Por último, las post-larvas se obtuvieron de las placas de fijación. Todas las muestras fueron almacenadas a -80 º C antes de ser analizadas. Antes del análisis, todas las muestras se homogeneizaron con mortero antes de ser pesadas para su análisis posterior. Todos los análisis se realizaron en el laboratorio del Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA, ULPGC).

11.4.8.1 Determinación de la humedad

Se determinó secando una cantidad conocida de muestra fresca (0,5-10g) (*Pi*) en una estufa a 105 º C hasta obtener un peso constante (*Pf*). Antes de ser ponderadas las muestras fueron sometidas a la desecación durante 30 minutos hasta llegar a temperatura ambiente. El porcentaje de humedad de la muestra se calculó según la siguiente fórmula:

$$\% H = \frac{(Pi - Pf) \times 100}{Pi}$$

11.4.8.2 Determinación de las cenizas

El contenido en cenizas se determinó gravimétricamente después de la incineración de una cantidad conocida de muestra (1-2g) (*Pi*) en un horno mufla a una temperatura de 600 º C durante 24 horas. La cantidad resultante de cenizas fue registrada y pesada hasta alcanzar un peso constante (*Pf*) de acuerdo a las recomendaciones establecidas de la AOAC (2005). El contenido en cenizas final de las muestras se calculó según la siguiente fórmula:

$$\% Cenizas = \frac{Pi \times 100}{pf}$$

11.4.8.3 Determinación de las proteínas

El contenido proteico se calculó a partir del contenido de nitrógeno total de las muestras según el método de Kjeldahl de acuerdo con la AOAC, (2005). La técnica consiste en digerir por ebullición, a 400 º C durante una hora, las muestras (de 0.2-0.4 g) en ácido sulfúrico concentrado (H_2SO_4), en presencia de un catalizador de cobre. Este proceso permite obtener una solución de sulfato de amonio ($(NH_4)_2SO_4$). Un exceso de base (NaOH) se añade

al producto de la digestión para convertir NH_4 a NH_3 recuperado por destilación del producto de la reacción. En la unidad de destilación (Mod. Foss Tecator, 1002, Höganäs, Suecia) una valoración directa de la solución se realiza con ácido bórico (H_3BO_3), para cuantificar la cantidad de amoníaco en la solución receptora. La titulación se realiza con HCl a 0,1 M. El contenido en proteína de las muestras se calculó de acuerdo a la fórmula siguiente:

$$\%\ Proteina = \frac{(Vs - Vb) \times N \times 14.007}{W} \times F$$

Siendo:

Vb = volumen de ácido consumido durante la valoración del blanco

Vs = Volumen en ml de ácido consumido durante la valoración de la muestra

N = Normalidad del ácido titulante (HCl)

14.007 = Peso molecular del nitrógeno

W = Peso de la muestra en mg

F = Factor de conversión empírico para convertir el porcentaje de nitrógeno de la muestra en porcentaje de proteína, con un valor de 6.25.

11.4.8.4 Determinación de los lípidos totales

La extracción de lípidos se realizó según la metodología descrita por (Folch et al., 1957). De 50 a 200 mg de muestra se mezclaron con 5 ml de una solución de Cloroformo: Metanol (2:1) con 0,01% de antioxidantes butilhidroxitolueno (BHT); se homogeneizaron en un Ultra Turrax (IKA-Werke, BASIC T25, Staufen, Alemania) durante 5 min. El homogeneizado obtenido se enjuagó con 5 ml de Cloroformo: Metanol por filtración añadiendo KCl al 0,88% para aumentar la polaridad de fase acuosa. La mezcla se centrifugó a baja velocidad (2000 rpm) durante 5 minutos para separar la fase acuosa de la orgánica. La fase acuosa en la parte superior fue descartada, mientras que la inferior, que contiene los lípidos, se filtró y se evaporó con vapor de nitrógeno hasta la obtención de la sequedad total. El contenido seco en lípidos en seco se determinó posteriormente por gravimetría.

11.4.8.5 Determinación de ácidos grasos

Los ácidos grasos de los extractos de lípidos se transesterificaron a esteres metílicos (FAMES) con 1% de ácido sulfúrico: metanol (Christie, 1982) y se conservaron en atmósfera de N_2. La mezcla se dejó incubando a 50 ºC durante 16 horas, y se enfrió. Agua destilada, junta con hexano 1:1 ester dietílico y BHT al 0,01% se añadieron a continuación. Los FAMES purificados se evaporaron hasta sequedad con N_2 y se pesaron. Por último, los FAMES fueron extraídos en hexano y se almacenaron a -80 ºC. Los FAMES se analizaron con un cromatógrafo de gas de Thermo Finnigan GC-gas (Mod. Shimadzu GC-14A; Analytical instrument division Kyoto, Japón) equipado con un detector de ionización de llama (260 ºC). Los FAMES fueron separados con columna capilar (28m x 0.32mm Supercowax x 0,25 id) utilizando helio como gas portador bajo la presión de gas siguiente: He 1 kg cm^{-2}, H_2 0.5 kg cm^{-2}, N_2 1 kg cm^{-2}, aire 0,5 kg cm^{-2}. Las condiciones fueron las siguientes: temperatura del inyector 260 º C, temperatura de la columna 180 º C durante 10 minutos, aumentando a 215 ºC a una velocidad de 2.5 ºC min^{-1} y se mantuvo a 215 ºC durante 15 minutos. Los ácidos grasos fueron identificados por comparación con el estándar EPA 28.

11.4.9 Análisis estadístico

El análisis estadístico se realizó mediante el programa Statgraphics Plus 5.1. Análisis de varianza (ANOVA de una vía) se llevaron a cabo para comparar la composición bioquímica proximal, así como el número de células, la fijación, la supervivencia y la tasa de crecimiento diario (DGR) entre tratamientos. Los datos que mostraron diferencias significativas ($P < 0.05$) se analizaron mediante comparaciones pareadas con la prueba de Tukey HSD. La normalidad y homogeneidad de varianza fueron evaluados con Skewness y Kurtosis estandarizados y la prueba de Bartlett. Cuando las varianzas fueron heterogéneas y/ o en el caso de la no distribución normal de los datos se transformaron en su logaritmos o con la función arco seno.

Los análisis de regresión múltiple se llevaron a cabo para explicar la variación observada de fijación, de crecimiento y de supervivencia entre los tratamientos. La composición bioquímica próxima de las algas de los sustratos y de las diatomeas, sus contenidos en proteínas, lípidos, cenizas y carbohidratos, fueron los factores seleccionados para el análisis.

Los análisis en componentes principales se llevaron a cabo para datos de composición en ácidos grasos de las algas de sustratos y de las diatomeas y los componentes principales se identificaron.

Una correlación de rango de Kendall se realizó a, continuación, entre los principales componentes principales y los datos de fijación, de crecimiento y supervivencia con el fin de identificar los efectos determinantes de la composición en ácidos grasos entre los tratamientos.

El análisis en clúster se realizó sobre datos de composición en ácidos grasos de las larvas, post-larvas, diatomeas y sustratos con el fin de establecer grupos en función de su porcentaje de similitud.

11.5 CONCLUSIONES

Estudio I: Desarrollo embrionario y larvario de *Haliotis tuberculata coccinea* Reeve: secuencia de de micro-fotografías indexadas

1. El desarrollo embrionario y larvario de *H. tuberculata coccinea* se llevó a cabo en treinta y nueve distintas, etapas consecutivas, con características externas identificables, desde la fecundación hasta la aparición del tercer túbulo en los tentáculos cefálicos, que se differenciaron en order de apparencia en comparación con otras especies de oreja de mar.
2. Los huevos de *H. tuberculata coccinea* presentaron una pigmentación de color violeta oscura y un diámetro de 205 ± 8 micras después de la fecundación. La pigmentación del huevo se vio reflejada en las larvas que presentaron pies, velum y tentáculos cefálicos de color violeta, y una masa visceral de color naranja-amarillento. El tamaño de las larvas se mantuvo constante con una longitud de 216,6 ± 5,3 micras y un ancho de 172 ± 8,8 micras.
3. Los tiempos de ocurrencia de las etapas del desarrollo embrionario y larvario de *H. tuberculata coccinea* fueron más cercanas a las de una especie de abalón tropical, tal como *H. asinina,* que a las de abalones de climas templados.
4. Los conocimientos adquiridos sobre las tasas de desarrollo embrionario y larvario han permitido adaptar las técnicas de cultivo de otras especies a las exigencias de *H. tuberculata coccinea*.

Estudio II: Fijación de larvas de *Haliotis tuberculata coccinea* en respuesta a diferentes estímulos inductivos y el efecto de la densidad larvaria sobre la fijación, el crecimiento inicial y la supervivencia

5. Las algas coralinas incrustantes se encuentran entre los mejores sustratos de inducción a la fijación para *H. tuberculata coccínea* mientras que las inducciones a la fijación realizadas con 1 μM GABA y con el mucus de la misma especie, sin presencia de películas de diatomeas, dieron lugar a bajas tasas de fijación.
6. Ninguna de las especies de diatomeas probadas han inducido con éxito la fijación de las larvas de *H. tuberculata coccinea,* un hecho posiblemente vinculado a la

especificidad de las especies, así como a las condiciones de edad, fase de crecimiento o de cultivo de las diatomeas.

7. *U. lens*, se identifico como sustrato eficaz y consistente para inducir a la fijación las larvas de *H. tuberculata coccínea* mientras la colonización de *U. lens* por la diatomea *Navicula incerta* ha reducido su potencial de inducción a la fijación.
8. Una densidad elevada de larvas tuvo un efecto negativo sobre las tasas de fijación y la supervivencia de las post-larvas de *H. tuberculata coccinea.*

Estudio III: Efecto de diferentes estímulos de inducción, procedentes de algas, sobre la fijación larvaria, el crecimiento inicial y la supervivencia de *Haliotis tuberculata coccínea*.

9. El estado de desarrollo algal y su composición bioquímica proximal, especialmente el contenido en proteínas, influenciaron notablemente la fijación de *H. tuberculata coccínea* que fue superior sobre las CCA, en una combinación de *U. lens* y *U. rigida* maduras y en *U. rigida* madura.
10. Los sustratos de algas se diferenciaron por su composición en ácidos grasos y los ácidos grasos correlacionados con la fijación fueron diferentes de los vinculados a la supervivencia, sólo los niveles de EPA se correlacionaron con los dos.
11. Los esporofitos del alga verde *U. rigida* fueron potentes y adecuados para la fijación, el crecimiento y la supervivencia de *H. tuberculata coccinea*. Las mayores tasas de crecimiento y supervivencia se observaron en una combinación de *U. rigida* y *U. lens* maduras, induciendo así mismo altas tasas de fijación y denotando que macroalgas verdes juegan un papel importante en la vida inicial de la oreja de mar y que las especies de *Ulvaceas*, siendo presentes a nivel mundial y cultivadas con facilidad, presentan un gran potencial para satisfacer los requisitos de las larvas de abalón.

Estudio IV: Valor potencial de *Navicula incerta, Proschkinia* sp., *Nitzschia* sp. y *Amphora* sp. para la alimentación de post-larvas de *Haliotis tuberculata coccinea*: Efecto de la densidad sobre las tasas de crecimiento de las algas.

12. Las cuatro especies de diatomeas investigadas mostraron tamaños adecuados, para ser potencialmente utilizadas para la alimentación de las post-larvas de *H. tuberculata coccinea* a lo largo de la etapa de criadero de la producción.

13. Altas tasas de crecimiento fueron observadas, para todas las especies, a bajas densidades de inoculo de 0.10 x 10^6 cells mL^{-1}, y sugieren una limitación de nutrientes y/o la atenuación de la radiación media en los cultivos debido al aumento de la densidad celular.
14. Los perfiles de ácidos grasos de las diatomeas estudiadas fueron característicos de la mayoría de las diatomeas: presentando una alta proporción de 16:1n-7 y 16:0, y cantidades de 14:0 y 20:5n-3 variables, pero por lo general altas, EPA fue el n-3 PUFA predominante en las diatomeas estudiadas mientras DHA fue baja en todas las diatomeas estudiadas, siendo la más baja la de *N. incerta*..
15. La diatomea del presente estudio, que mejor se ajusta a los requisitos nutricionales de las post-larvas de *H. tuberculata coccinea* es *Amphora* sp. en fase exponencial de crecimiento, ya que presentan un buen contenido en energía, la mayor capacidad de fijación celular, el más alto contenido en proteínas y lípidos, y unos contenidos en carbohidrato dentro del rango necesario para juveniles de abalón.

Estudio V: Mejora de la fase de criadero de *Haliotis tuberculata coccínea*: valor nutricional de cuatro especies de diatomeas bentónicas

16. El alto contenido lipidico (25%) de las larvas de *H. tuberculata coccínea* antes del inicio de la alimentación exógena, destaca el importante papel de los lípidos en el desarrollo larvario de esta especie.
17. Las diatomeas, en particular *Amphora* sp., son determinantes para el crecimiento inicial de post-larvas de *H. tuberculata coccínea*, especialmente entre las semanas 4 y 8 posteriores a la fijación. Sus valores nutritivos para el crecimiento del abalón dependen notablemente, entre otros factores, de la cantidad total de proteína; de lípidos; de energía y de cenizas.
18. Esporofitos de *U. lens* y *U. rigida*, utilizados como inductores de fijación larval, presentarón un valor nutricional adecuado para ser empleados como complemento alimenticio a las dietas de diatomeas, cubriendo los requerimientos nutricionales de las post-larvas de *H. tuberculata coccínea,* que dependen de las fases de crecimiento, especialmente después de la semana 8, correspondiendo a un periodo de incremento en la demanda en alimento.
19. Tanto el ácido linoleico como el linolénico se consideran esenciales para *H. tuberculata coccínea*, mientras que ARA, EPA y 22:5 n-3, a pesar de ser

importantes, fueron sintetizados por esta especie de abalón, lo que denota la presencia de las respectivas elongasas Δ4 y desaturasas Δ5. Sin embargo, la baja cantidad de DHA denota una actividad de Δ5 baja en estas especies.

Como conclusión general, la mejora de los conocimientos adquiridos, en esta tesis, sobre *H. tuberculata coccinea*: el desarrollo embriónario y larvario, los factores que afectan a los procesos de fijación y la nutrición temprana de post-larvas y juveniles, han llevado al desarrollo de mejoras en las técnicas de cría de esta especie, la producción experimental en masa de semillas y a la mejora del crecimiento de las post-larvas.