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Ecologia e Gestione Sostenibile delle Risorse Ambientali – ciclo XXIX

THE ROLE OF SEA URCHIN *PARACENTROTUS LIVIDUS* (LAMARCK, 1816) IN AN ENVIRONMENTALLY SUSTAINABLE REARING SYSTEM (IMTA)

s.s.d. BIO/07

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Abstract

In a Mediterranean context, where overfishing causes the depletion of the sea urchin *Paracentrotus lividus* in the wild, echinoculture is becoming a fundamental activity for bridging the gap between supply and demand. At the same time, aquaculture contributes to increasing the level of nutrients in the environment. For this reason, the sustainable Integrated Multi-Trophic Aquaculture (IMTA) system is being developed by scientists.

The general objective of this PhD was to enhance the hatchery production of the sea urchin *P. lividus*, thus promoting responsible and sustainable aquaculture (IMTA). This was done by integrating the sea urchin production system with a grey mullet *Mugil cephalus* hatchery system. With this in mind, the thesis can be divided in two main research areas: (i) the investigation of the reproduction and rearing of *P. lividus* (Chapters 2, 3 and 4); (ii) the cultivation of micro- and macroalgae in grey mullet *M. cephalus* wastewater (Chapters 5 and 6).

In Chapter 2, published in the scientific literature (*Aquaculture Research*, 2016, 1-11, doi:10.1111/are.12990), I studied the *P. lividus* larval rearing methods and compared the growth of larvae cultured using two different methods. This first is a "variable method" based upon a variable amount of feed (microalgae) and seawater exchange (30% or 50%), while the second is a "fixed method" characterized by a fixed amount of feed and seawater exchange. Data suggest that the use of the variable method improves the survival and development of larvae and reduces consumption of resources (phytoplankton, seawater and labour), which in turn could potentially improve the hatchery production of *P. lividus*.

The variable method was applied in Chapter 3 and the results were published in the scientific literature (*Aquaculture*, 2016, 465: 265-270). I assessed the effects of four phytoplankton diets [*Dunaliella tertiolecta*; *Isochrysis* aff. *galbana* (T-Iso) + *D. tertiolecta*; *Chaetoceros gracilis* + *D. tertiolecta*; T-Iso + *C. gracilis* + *D. tertiolecta*] on the survival of *P. lividus* larvae and post-larvae at 10, 20, 30, 100 and 180 days post-settlement. The data showed significant differences in the survival of larvae and post-larvae for the different dietary treatments. Only larvae fed with *D. tertiolecta* alone or

mixed with *C. gracilis* survived 180 days post-settlement, 1.5 ± 1.5% and 3.0 ± 2.0% (mean ± SE), respectively.

I aimed to improve the larval settlement and so, in a second experiment in Chapter 3, I compared the effects of the macroalga *Ulvella lens* to a natural biofilm of diatoms, and I evaluated the effect of the presence of conspecifics on metamorphosis rate, survival and growth of post-larvae for the first time. Data have shown that conspecifics increase the larval settlement of *P. lividus* (36 - 46%) and *U. lens* could represent an efficient alternative to natural biofilms as a metamorphosis-inducing factor and first feeding item.

The effect of stocking density on the somatic growth of juvenile sea urchins, fed *ad libitum* with fresh thalli of *Ulva* sp., was investigated in Chapter 4. As previously observed for other invertebrates, the somatic growth of *P. lividus* decreases with increasing density, resulting in a higher ($p < 0.001$) test diameter and wet weight when individuals were stocked at low density (100/m^2). According to our results, six-month-old individuals reach 15 mm in test diameter after about 5.5, 7 and 9.5 months when stocked at 100/m2, 250/m2 and 400/m2, respectively.

The production system of *P. lividus* was integrated with a grey mullet *M. cephalus* hatchery production system. I evaluated the capability of micro- and macroalgae to grow in *M. cephalus* wastewater and to assimilate dissolved nutrients.

The nutrient removal efficiency (nitrogen and phosphorous) and biomass yield of *I. galbana* and *D. tertiolecta* were evaluated in Chapter 5 and compared to another microalga species previously tested for bioremediation of aquaculture wastewater, *Tetraselmis suecica*. After 7 days, *D. tertiolecta* and *T. suecica* showed an efficient assimilation of nitrogen (~95%) and phosphorous (more than 90%) and a good biomass yield (0.38 ± 0.04 g/L for *D. tertiolecta*, 0.60 ± 0.03 g/L for *T. suecica*), thus confirming the possibility of employing *T. suecica* for wastewater bioremediation and identifying *D. tertiolecta* as a valid candidate species.

We also obtained satisfactory results on the efficiency of nutrient removal in *M. cephalus* wastewaters with *Ulva* sp. (Chapter 6). At the end of the experiment (10 days), *Ulva* sp. achieved ~69% and ~87% removal of nitrogen and phosphorous, respectively.

However, the cultivation system had a poor biomass yield (from 0.7 to 4.7 g dry weight/m^2/day), and needs to be improved to ensure a higher production.

This thesis project demonstrates that the production of juvenile sea urchins in laboratory-controlled conditions is feasible. Adopting the variable method and feeding larvae with suitable diets (*D. tertiolecta* alone or mixed with *C. gracilis*) ensures a good larval development and survival at competence. *U. lens* represents a valid alternative as a factor for inducing metamorphosis, and using adult conspecifics improves larval settlement. Finally, it is possible to obtain juvenile individuals in about 13 months when maintained at low density and fed with *Ulva* sp. It is possible to integrate the hatchery production of *P. lividus* with the hatchery production of *M. cephalus* in an environmentally sustainable IMTA system, by re-using the wastewaters to produce live feed for larvae (*D. tertiolecta*) and juvenile (*Ulva* sp.) sea urchins. Nevertheless, further studies would be needed to test the applicability of this system at greater volumes, with a higher number of both larvae and juveniles and a higher biomass yield of *Ulva* sp., to verify the applicability of these methods to a larger scale production system.

Riassunto

In un contesto come quello del Mar Mediterraneo, dove l'eccessivo sforzo di pesca causa un impoverimento delle popolazioni naturali di riccio di mare *Paracentrotus lividus*, l'echinocultura diventa un'attività fondamentale per colmare il divario esistente tra domanda e offerta. Tuttavia, l'acquacoltura contribuisce anche ad aumentare il livello di nutrienti nell'ambiente circostante, e per questo motivo si sta sviluppando il sistema sostenibile di Acquacoltura Multi Trofica Integrata (IMTA).

L'obiettivo generale di questo progettto di dottorato era quello di aumentare la produzione del riccio di mare *P. lividus* promuovendo un'acquacoltura responsabile e sostenibile (IMTA), attraverso l'integrazione del sistema di produzione di riccio di mare con una hatchery di cefalo da bottarga *Mugil cephalus*. Per questo scopo, la tesi può essere divisa in due principali aree di ricerca: (i) riproduzione e allevamento di *P. lividus* (Capitolo 2, 3 e 4); (ii) coltivazione di micro- e macroalge in acque reflue di *M. cephalus* (Capitolo 5 e 6).

Nel Capitolo 2, pubblicato nella letteratura scientifica (*Aquaculture Research*, 2016, 1-11, doi:10.1111/are.12990), è stato studiato il metodo di allevamento larvale di *P. lividus*, attraverso il confronto di due diverse metodologie: il "metodo variabile" basato su una quantità variabile di alimento (microalghe) e ricambio idrico (30% o 50%), e il "metodo fisso" caratterizzato da una quantità fissa di alimento e ricambio idrico. I dati hanno dimostrato che il metodo variabile aumenta la velocità di sviluppo e la sopravvivenza larvale, e riduce il consumo di risorse (fitoplancton, acqua di mare e lavoro manuale), migliorando in questo modo la produttivita in una hatchery di *P. lividus*.

Il metodo variabile è stato successivamente applicato nel Capitolo 3, pubblicato nella letteratura scientifica (*Aquaculture*, 2016, 465: 265-270), per valutare gli effetti di quattro diete a base di fitoplancton [*Dunaliella tertiolecta*; *Isochrysis galbana* (T-Iso) + *D. tertiolecta*; *Chaetoceros gracilis* + *D. tertiolecta*; T-Iso + *C. gracilis* + *D. tertiolecta*] sulla sopravvivenza di larve e post-larve di *P. lividus* dopo 10, 20, 30, 100 e 180 giorni dalla metamorfosi. I dati hanno mostrato differenze significative sulla sopravvivenza di larve e post-larve; in particolare, solo le larve alimentate con *D. tertiolecta* e *D.*

tertiolecta + *C. gracilis*, infatti, sono sopravvissute fino a 180 giorni dopo la metamorfosi, con una sopravvivenza pari a, rispettivamente, 1,5 ± 1,5% e 3,0 ± 2,0% (media ± ES).

Con l'obiettivo di aumentare la metamorfosi larvale, sempre all'interno del Capitolo 3, è stata testata la macroalga *Ulvella lens* come fattore di induzione alla metamorfosi larvale, ed è stata confrontata con un biofilm naturale di diatomee. Inoltre, per la prima volta, è stato valutato l'effetto di adulti conspecifici sul tasso di metamorfosi, la sopravvivenza e la crescita delle post-larve. I dati hanno dimostrato che gli adulti conspecifici aumentano il tasso di metamorfosi larvale di *P. lividus* (36-46%), mentre *U. lens* potrebbe rappresentare una valida alternativa al biofilm naturale di diatomee come fattore di induzione alla metamorfosi.

Nel Capitolo 4 è stato valutato l'effetto della densità sulla crescita somatica di giovanili di riccio di mare alimentati *ad libitum* con talli freschi di *Ulva* sp. Come già osservato per altri invertebrati, la crescita somatica di *P. lividus* diminuisce all'aumentare della densità; il diametro e il peso umido degli individui risulta essere maggiore ($p < 0,001$) quando vengono mantenuti a bassa densità (100/m^2). Secondo i nostri risultati, individui di 6 mesi di vita raggiungono la taglia di 15 mm di diametro dopo circa 5,5, 7 e 9,5 mesi, se mantenuti a densità di 100/m^2, 250/m^2 e 400/m^2, rispettivamente.

Il sistema di allevamento di *P. lividus* è stato integrato con una hatchery di muggine *M. cephalus*, valutando la capacità di specie micro- e macroalgali utilizzate come alimento per larve e giovanili di riccio di crescere nelle acque reflue di *M. cephalus* e assimilare i nutrienti disciolti.

L'efficienza di rimozione dei nutrienti (azoto e fosforo) dall'acqua reflua e la produzione di biomassa di *I. galbana* e *D. tertiolecta* sono stati valutati nel Capitolo 5, e confrontati con un'altra specie di microalga positivamente testata per il biorisanamento di acque reflue da acquacoltura, *Tetraselmis suecica*. Dopo 7 giorni, *D. tertiolecta* e *T. suecica* mostravano una buona efficienza di rimozione di azoto (~95%) e fosforo (oltre il 90%) e una buona resa in termini di biomassa prodotta (0,38 ± 0,04 g/L per *D. tertiolecta*, 0,60 ± 0,03 g/L per *T. suecica*), confermando la possibilità di impiegare *T. suecica* per il biorisanamento di acque reflue e identificando *D. tertiolecta* come valida alternativa.

Buoni risultati sull'efficienza di rimozione di nutrienti nelle acque reflue di *M. cephalus* sono stati ottenuti anche con la macroalga *Ulva* sp. (Capitolo 6). Alla fine dell'esperimento (10 giorni), *Ulva* sp. aveva rimosso ~69% di azoto e e ~87% fosforo, ma il sistema di coltivazione risultava piuttosto scarso in termini di biomassa prodotta (da 0,7 a 4,7 g di peso secco/m^2/giorno), per cui necessita di essere migliorato per garantire una maggiore produzione.

Questo progetto di tesi dimostra che l'allevamento di ricci di mare giovanili in condizioni controllate di laboratorio è piuttosto efficace. Adottando il metodo di allevamento variabile e alimentando le larve con diete adeguate (*D. tertiolecta* o *D. tertiolecta* + *C. gracilis*) determina un veloce sviluppo larvale e una buona sopravvivenza fino alla competenza. *U. lens* rappresenta una valida alternativa alle diatomee come fattore di induzione alla metamorfosi, mentre l'uso di adulti conspecifici aumenta la percentuale di individui metamorfosati. Infine, è possibile ottenere individui giovanili di riccio in circa 13 mesi se mantenuti a bassa densità e alimentati con *Ulva* sp. La hatchery di *P. lividus* è integrabile con una hatchery di *M. cephalus* in un sistema sostenibile come l'IMTA, attraverso il riutilizzo delle acque reflue per la produzione di alimento vivo per larve (*D. tertiolecta*) e giovanili (*Ulva* sp.). Sono tuttavia necessari ulteriori studi per verificare l'applicabilità di questo sistema a volumi maggiori, con un maggior numero di larve e giovanili e una maggiore produzione di *Ulva* sp.

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1 GENERAL INTRODUCTION

Humans have consumed sea urchins for a long time. In the Mediterranean Sea, this consumption dates from prehistory (Gutiérrez-Zugasti, 2011).

Nowadays, Japan is the most important market of sea urchin gonads in the world (*roe, uni* in Japanese). The Japanese consume about 60000 tons of fresh echinoids per year and represent more than 95% of the entire world market (Grosjean, 2001). *Strongylocentrotus intermedius*, *S. nudus*, *Heterocentrotus pulcherrimus*, *Pseudocentrotus depressus*, *Anthocidaris crassispina* and *Tripneustes gratilla* are the main species exploited (Fernandez, 1996; Hagen, 1996; Fuji and Kawamura, 1970; Fuji, 1967). Other species are imported from North America (*Strongylocentrotus droebachiensis* and *S. franciscanus*) (Sloan, 1985; Kato, 1972) and Chile (*Loxechinus albus*) (Lawrence et al, 1997; González et al, 1993).

Consuming around 100 tons of sea urchins per year, France is the second largest consumer of sea urchin roe in the world and one of the oldest (Le Direac'h, 1987; Le Gall, 1987). *Paracentrotus lividus* is the major species harvested and consumed in Brittany and in the Mediterranean, but *Psammechinus miliaris* and *Sphaerechinus granularis* are also harvested (Guillou and Michel, 1993; Le Gall, 1987).

Sea urchin fishing is a profitable and easy activity. Natural stocks are picked at low tide by hand or at shallow depths with simple equipment (Le Direac'h, 1987; Le Gall, 1987), and few rules exist to limit overexploitation (Grosjean, 2001). In addition to a high fishing pressure of these species (San Martin, 1995), a slow growth rate (Grosjean, 2001) and the harvesting of animals before they spawn cause a loss of recruitment and, consequently, a decline in wild populations (Le Gall, 1990). Moreover, removing adults has a negative effect on the survival of juveniles, because their spines protect juveniles from predation (Tegner and Dayton, 1977).

Although hatcheries were established for seed production and artificial feeding was done in harvested areas, a decline in natural sea urchin populations was recorded in Japan, Chile and the USA in the 1980s and 1990s (Saito, 1992). Europe has also suffered a sharp decline in wild stocks of *P. lividus* due to overfishing (Addis et al., 2009; Pais et al., 2007).

In the last decades, due to overexploitation and an increasing market demand of *P. lividus*, aquaculture has become a necessary alternative to sea urchin fishing (Pearce, 2010; Grosjean, 2001). The identification of suitable diets and rearing methods enables the production of high-quality gonads available on the market throughout the year (Carboni, 2013). On the other hand, the production of a large number of juveniles and their subsequent introduction into the natural environment could be a valid solution for the enhancement of natural populations (Andrew et al., 2002).

Nowadays, the echinoculture of *P. lividus* is limited by a high mortality rate during the larval and post-larval stages, a low percentage of metamorphosis and a low growth rate of juveniles. For this reason, increasing the success rate of echinoculture techniques during these production phases has become fundamental (Carboni et al., 2012; Daume et al., 2004).

Aquaculture is one of the fastest-growing food producing sectors in the world, providing almost 50% of all fish for human consumption. By 2030, this amount is projected to rise to 62% (FAO, 2014a). However, aquaculture represents one of the major contributors of the increasing levels of dissolved and particulate nutrients in the environment (Lamprianidou et al., 2015).

In an effort to reduce the impact of traditional aquaculture, several countries around the world are developing Integrated Multi-Trophic Aquaculture (IMTA) systems, which have the potential to contribute to the sustainability of aquaculture (Neori et al., 2004; Chopin et al., 2001). The IMTA system is characterized by the recycling of resources, such as water and energy, to produce other commercial species. In aquaculture, wastewater provides nutrients (ammonia, nitrite, nitrate and many-dissolved organic nitrogen) for the production of micro and macroalgae (Converti et al., 2006; Soletto et al., 2005; Abe et al., 2002). Primary biomass produced with bioremediation could be used as an energy source, as fertilizer, to produce fine chemicals or as feed in aquaculture (Mulbry et al., 2006; Vilchez et al., 1997).

IMTA is a promising technology and is not a new approach (Lalibert´e et al., 1994), at least as far as research devoted to macroalgae is concerned (Troell et al., 2003). In contrast, very few studies exist on the application of microalgae in integrated systems and these are focused on a few phytoplankton species used in aquaculture (Gao et al., 2016; Michels et al., 2014; Sirakov and Velichkova, 2014; Borges et al., 2005).

1.1 *Paracentrotus lividus*

1.1.1 Biology and Ecology

The edible sea urchin *P. lividus* (Lamarck, 1816) belongs to the Echinoidea Class. It is widespread throughout the Mediterranean Sea and along the northeastern Atlantic coast, from Scotland and Ireland to Southern Morocco and the Canary Islands (Boudouresque and Verlaque, 2007). *P. lividus* is particularly common in geographical zones with a seawater temperature of 10-15 °C in winter and 18-25 °C in summer. Salinity higher than 20 ppt and lower than 40 ppt is necessary to ensure the survival of this species (Le Gall et al., 1989; Pastor, 1971).

This is a typical subtidal species, which lives 10-20 m under the surface, even though isolated individuals have been found at depths of up to 80 m (Boudouresque and Verlaque, 2007). *P. lividus* colonizes two preferred habitats: intertidal rocks and seagrass meadows of *Posidonia oceanica* and *Zoostera marina* (Fernandez, 1996; Tortonese, 1965). On high rocks exposed to waves, *P. lividus* is able to create cup-shaped cavities where it lives permanently or temporarily, protected from predators.

Figure 1: Sea urchin *Paracentrotus lividus* in *Posidonia oceanica* meadow.

The somatic growth rate of *P. lividus* is correlated to seawater temperature (Fernandez, 1996); growth occurs up to 8 °C, reaches a peak at 18-22 °C, decreases from 22 °C and stops at 28 °C (Le Gall et al., 1990). Temperatures higher than 28 °C could be lethal, although it has been observed that *P. lividus* resists temperatures up to 30 °C in Mediterranean lagoons (Fernandez, 1996; Le Gall et al., 1990; Tortonese, 1965). In the Mediterranean Sea, the maximum growth rate of *P. lividus* occurs in spring (between 12-18 °C) and is minimal in winter (Shpigel et al., 2004; Turon et al., 1995).

Hydrodynamics and feed quantity are other factors which influence the growth of *P. lividus* (Gago et al., 2003). Lower growth rates and smaller individuals have been observed in exposed habitats characterized by a low abundance of feed than in deeper habitats where food is unlimited (Turon et al., 1995).

P. lividus is one of the most important herbivores in the Mediterranean Sea (Verlaque and Nedelec, 1983a). With its grazing activity, it influences the structure, the dynamics and the functioning of benthic sublittoral communities of hard substrates, affecting mainly erect species such as macroalgae and *P. oceanica* meadows (Benedetti-Cecchi et al., 1998). In suitable conditions, overgrazing causes a reduction in the algal biomass, sometimes going as far as to create barrens. These are areas which are totally lacking in erect plant covering and are dominated by encrusting coralline algae (Guidetti and Dulcic, 2007).

Feeding preferences of *P. lividus* vary on the basis of diversity and abundance of plant species and according to the size of individuals (Verlaque and Nedelec, 1983b). The highest algal selectivity occurs in conditions of high algal diversity, diminishes with increasing grazing activity and disappears completely when grazing becomes excessive (Gago et al., 2003).

Although macroalgae are the main feeding resource, P. lividus is considered a generalist opportunistic species, able to eat every trophic resource, especially when these are limited. Sponges, hydrozoans and copepods, have been found in their gut contents (Régis, 1978; Pastor, 1971; Tortonese, 1965). In high-density populations, cannibalism has been observed where juvenile sea urchins (2-3 cm in test diameter) are attacked and consumed by adults (Pastor, 1971).

1.1.2 External morphology and internal anatomy

P. lividus has a radially symmetrical "pseudo-spherical" body, constrained by an endoskeleton located under the epidermis. The endoskeleton is composed of calcareous ossicles and armed with mobile spines. Lengthwise, the body is divisible in two hemispheres: the aboral pole bears the anus while the opposite pole, the oral pole, includes the mouth and is directed towards the substratum (Figure 2). The mouth is called Aristotele's lantern and is a complex pentasymmetric structure composed of five pyramids with each one bearing a tooth. Behind the mouth there is a long digestive system, composed of the pharynx, esophagus, stomach, gut and anus (Ruppert and Barnes, 1994) (Figure 3).

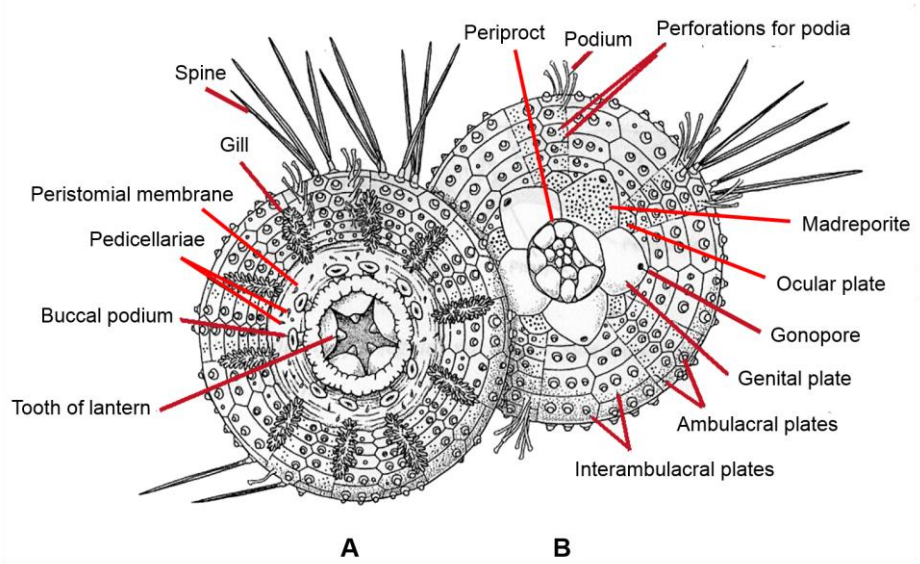

Figure 2: External anatomy of the sea urchin *Paracentrotus lividus*. A = Oral view. B = Aboral view (modified after Reid W.M., in: Ruppert and Barnes, 1994).

P. lividus has a locomotive water-vascular system composed of elongated, retractile tube-feet, equipped with a sucker-shaped tip that can glue and unglue itself to the substratum (Flammang et al, 1998). Sometimes, the tube-feet locomotion is supported by the movement of the spines (Grosjean, 2001).

1.1.3 Reproduction and life cycle

P. lividus has two distinct sexes, without sexual dimorphism, although cases of hermaphroditism have been observed (Byrne, 1990). Five gonads (red for females, yellow-orange for males) are disposed radially in the coelomic cavity and make up the reproductive organs. The gonads start developing when *P. lividus* reaches 4-6 mm in test diameter (Spirlet et al, 1994), but the animal becomes sexually mature at around 20 to 25 mm (Grosjean, 2001).

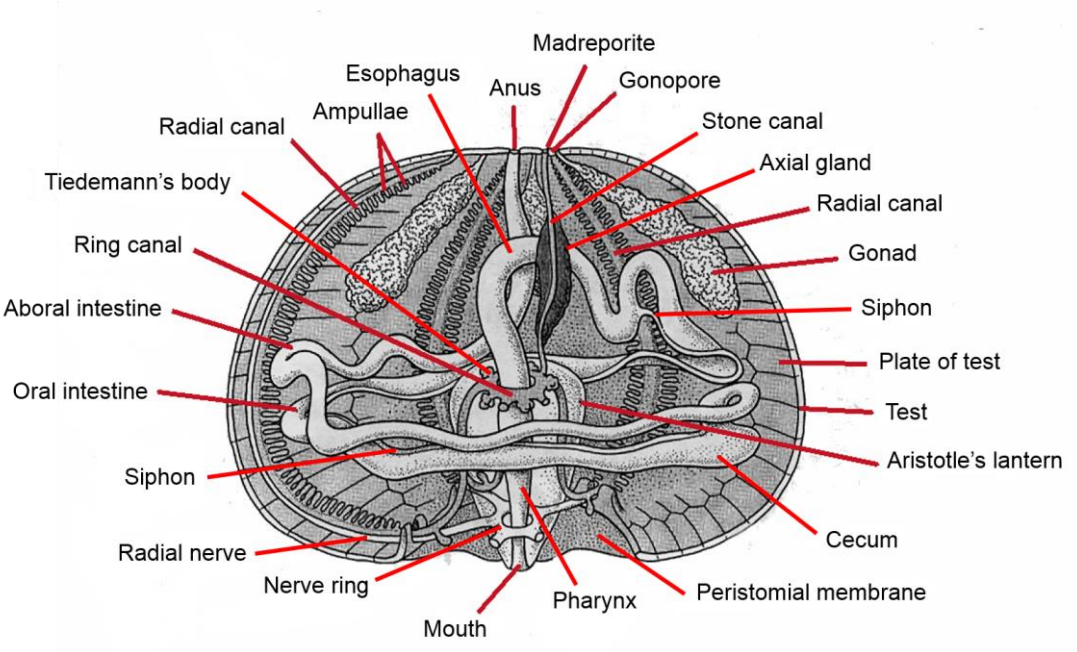

Figure 3: Internal anatomy of the sea urchin *Paracentrotus lividus* (modified after Reid W.M., in: Ruppert and Barnes, 1994).

The gonads are composed of somatic cells (or nutritive phagocytes) and germinal cells (oogonia and spermatogonia). Nutritive phagocytes store nutrients (proteins, lipids and carbohydrates) and make them available to the germinative cells during gametogenesis. Moreover, they absorb residual eggs and sperm at the end of the reproductive cycle (Walker et al., 2007).

The annual pattern of the reproductive cycle of *P. lividus* is highly variable (Guettaf et al., 2000), and differences in gonad growth have been observed between different populations, even when separated by relatively short distances. In the cold Atlantic seawater, numerous authors detected a unique reproductive cycle going from spring (April-May) to summer (July-August) (Spirlet et al., 1998; Byrne, 1990). In the

Mediterranean Sea, one or two annual reproductive cycles have been observed for this species. The main cycle coincides with the Atlantic cycle (from spring to summer) (Sellem and Guillou, 2007), while the second one occurs during autumn (Pedrotti, 1993). In some cases, however, mature individuals have been observed throughout the year, suggesting that *P. lividus* is able to produce larvae constantly, without a break (Soualili and Guillou, 2009). External fertilization occurs in the water column; during spawning, male and female gametes are released simultaneously by gonopores, following an external signal such as a change in seawater temperature or a mechanical disturbance (Spirlet, 1999; Spirlet et al., 1998).

A series of divisions leads the fertilized egg to the stages of morula, blastula and gastrula, after which the embryo assumes the typical shape of echinoid larvae, the echinopluteus at the 4-arm stage. The development of additional pairs of arms, 6-arm pluteus and 8-arm pluteus, characterizes the developmental stage of the larva, after which it forms echinoid rudiment and achieves competence for settlement. When rudiment is well developed, the larva is considered competent for settlement and seeks a solid substrate on which to metamorphose into a benthic animal (Carboni, 2013).

The growth of juveniles is influenced by temperature, feed availability and hydrodynamics (Gago et al., 2003; Fernandez, 1996; Turon et al., 1995). It has been observed that a high density of adult sea urchins can also negatively affect the growth of juveniles (Grosjean et al., 1996). Numerous studies have tested various methods and growth curves with the aim of evaluating the age and somatic growth of *P. lividus*, (e.g. Gompertz and von Bertalanffy, Turon et al., 1995). According to these studies, the growth of *P. lividus* resulted in about 10 mm (test diameter) at around 1 year. 20 mm individuals were two years old, while 40 mm individuals were 4 to 5 years old (Boudouresque and Verlaque, 2007). In the Gulf of Tunis, Sellem et al. (2000) reported that individuals with a diameter of 53 mm were about 8-years old, indicating that the growth of this species diminishes with age. This is consistent with results from other studies, according to which the longevity of *P. lividus* corresponded to 10 to 11 years on the Atlantic coast (Allain, 1978) and 7 to 8 years on the Mediterranean coast (Azzolina, 1987). However, the maximum size is about 70 mm in test diameter (Grosjean, 2001).

1.1.4 Echinoculture of Paracentrotus lividus

The rearing cycle of *P. lividus* is divisible in six steps: fertilization, larval rearing, metamorphosis, growth of post-larvae and juveniles, growth of subadults, and growth and conditioning of adults (Figure 4).

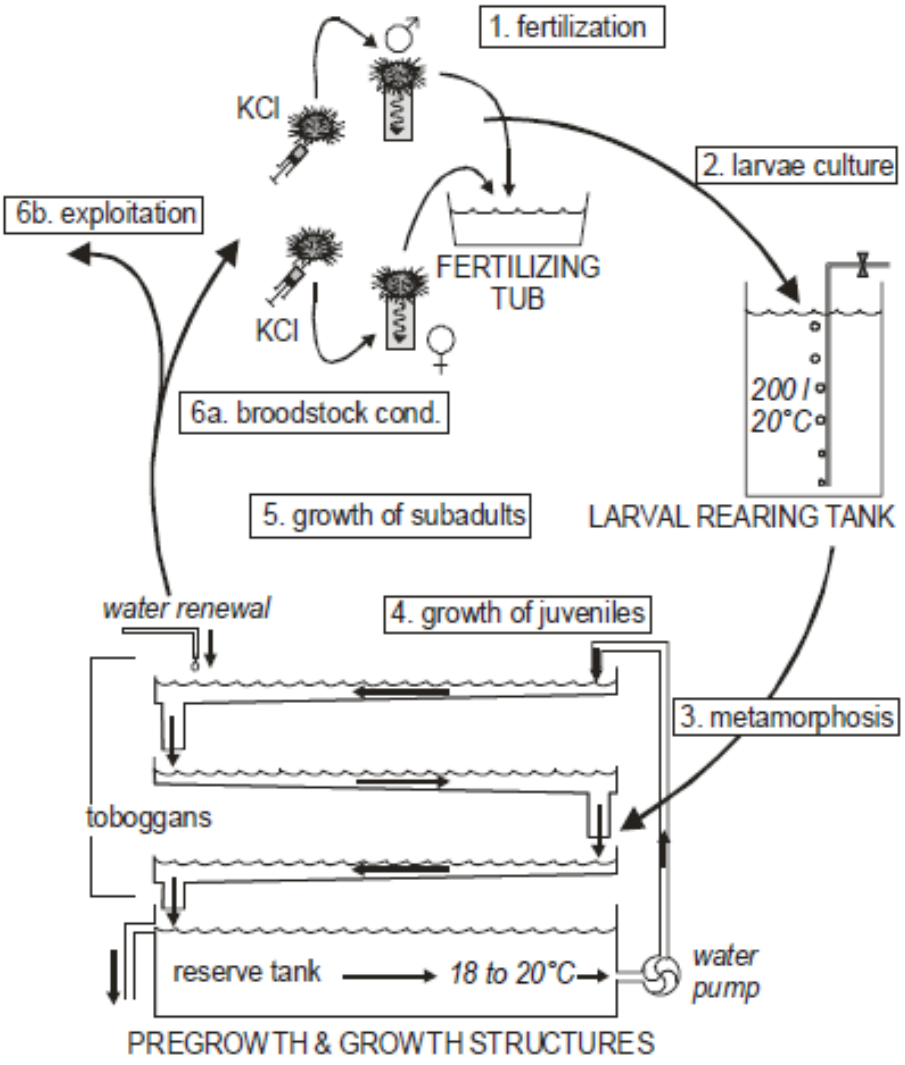

Figure 4: Overview of the closed-cycle process and devices used to produce sea urchins on land at a pilot scale (from Grosjean et al., 1998).

There are different methods for obtaining male and female gametes from adult sea urchins. One of the most known is the induction by potassium chloride (KCl) or acetylcholine (Ach). A solution of KCl or Ach, concentrated according to the body weight, is injected through the peristomial membrane; individuals naturally spawn gametes (Grosjean, 2001). Gametes can be also obtained directly from the gonads after

dissection of the animals. Female gametes are collected in previously filtered (1 μm cartridge filter) natural seawater (NSW), while male gametes are collected "dry" (Falugi and Angelini, 2000).

Eggs are fertilized within two minutes of the addition of the sperm. The fertilization is considered successful when at least 80% of the eggs acquires the fertilization membrane within 40-80 seconds (Falugi and Angelini, 2000). It has been demonstrated that the success rate of fertilization of sea urchins is higher when the eggs are exposed to sperm from more than one male (Evans and Marshall, 2005).

Larval rearing begins about 40 h post-fertilization, when the pelagic larvae, echinopluteus, is able to swim and eat. Stocking densities reported in the literature varied from 0.25 to 4 larvae/mL, but the best results were obtained at 1-2 larvae/mL (Azad et al., 2011; Privitera et al., 2011; Liu et al., 2007; Càrcamo et al., 2005; Kelly et al., 2000; Pedrotti and Lemée, 1999; Pedrotti and Fenaux, 1993; Fenaux et al., 1985).

A pyramidal shape (Figure 5A) and a length of about 250 μm (Carboni, 2013; Falugi and Angelini, 2000) characterize the echinopluteus at the 4-arm stage. Larvae develop additional pairs of arms, 6-arm pluteus (Figure 5B) and 8-arm pluteus (Figure 5C), until finally developing the rudiment echinoid on the stomach side (Figure 5D) and the tube-feet primordia on the opposite side. The larva is ready to metamorphose when the rudiment size is equal to or larger than the stomach (Figure 6A) and spines are visible (Carboni et al., 2012). Metamorphosis lasts about one hour and occurs following a chemical signal, which generally comes from the substrate (Gosselin and Jangoux, 1996). The post-larva looks like an adult but is endotrophic; it has neither mouth nor anus (Figure 6B) (Gosselin and Jangoux, 1998). Within seven days, post-larva becomes an exotrophic juvenile provided with a developed and functional digestive tract (Grosjean, 2001).

Traditionally, a static system is employed, which is characterized by a partial (50%) water exchange every two or three days (Carboni et al., 2014; Carboni et al., 2012; Swanson et al., 2012; Azad et al., 2011; Privitera et al., 2011; Gibbs et al., 2009; Càrcamo et al., 2005; Kelly et al., 2000; Pedrotti and Lemée, 1999).

Larval rearing ends within 18-25 days post-fertilization, when larvae reach competence for settlement and acquire the ability to metamorphose into a benthic animal (Falugi and Angelini, 2000). At this stage, in the presence of a suitable environmental stimulus, as

specifics macroalgae or adult conspecifics, larvae swim toward substrate and test their adaptability to benthic life (Carboni, 2013).

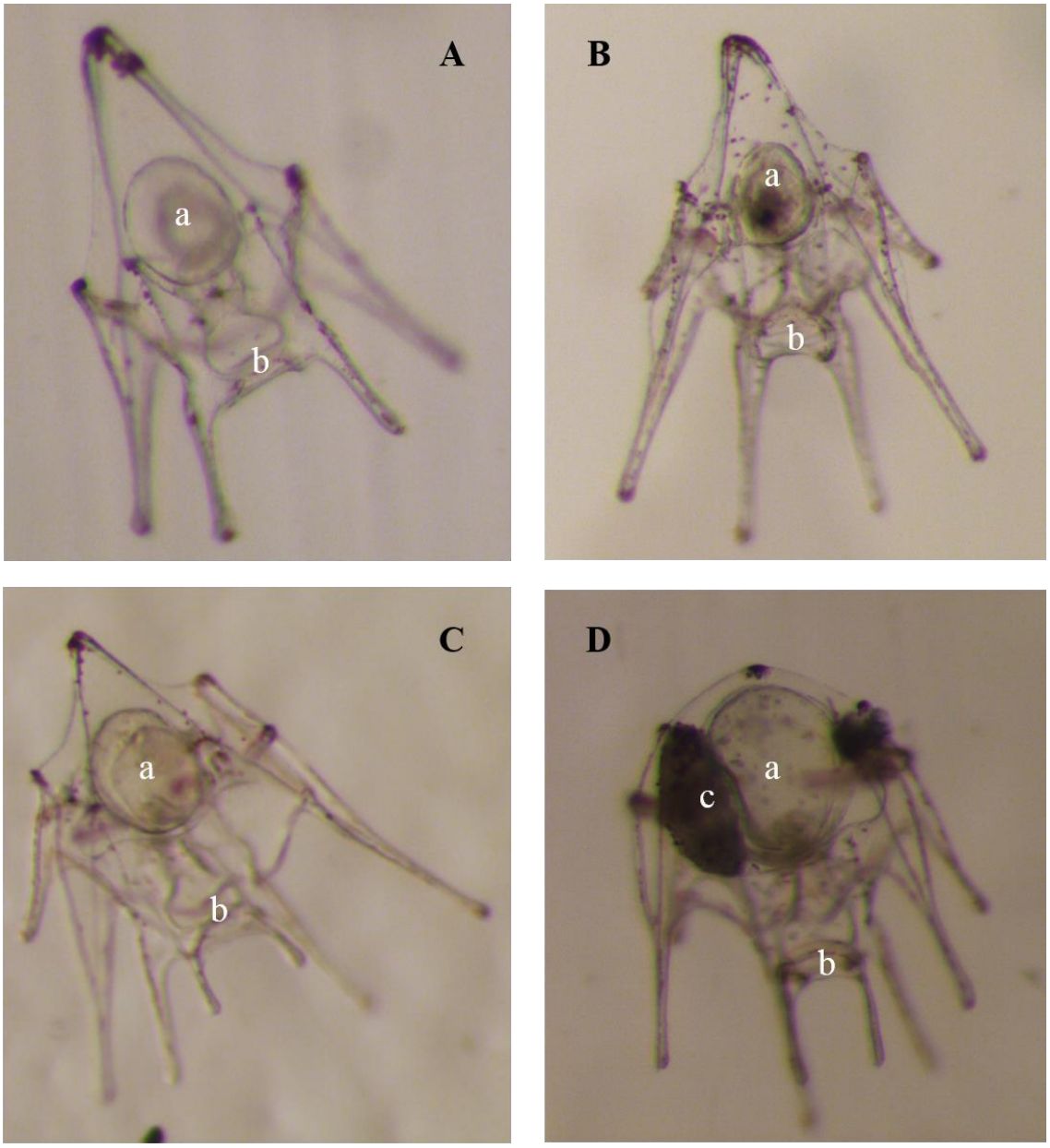

Figure 5: Development stages of *Paracentrotus lividus* larvae: 4-arms stage (A), 6-arms stage (B), 8-arms stage (C) and competent stage (D). a = stomach; b = mouth; c = echinoid rudiment.

In *P. lividus* hatcheries, competence for settlement may be tested daily starting sixteen days post-fertilization (Gosselin and Jangoux, 1998). A sample of randomly selected competent larvae are transferred into a beaker, Petri dish or Multiwell plate, containing filtered NSW and a metamorphosis-inducing factor (e.g. a natural biofilm of diatoms).

Metamorphosis lasts for about one hour (Burke, 1987), and metamorphosed larvae are counted after 24, 48 and 72 h. Settlement rate is calculated as the ratio between metamorphosed larvae and the initial number of larvae stocked. When at least 75% of the stocked larvae have metamorphosed, the whole larval culture is transferred into settlement tanks containing filtered NSW and the metamorphosis-inducing factor (Carboni, 2013; Carboni et al., 2012; Liu et al., 2007; Kelly et al., 2000).

For about eight days after settlement, post-larvae sessile are not able to eat, because they still have to develop their digestive system. The post-metamorphic period is crucial for survival as mortality rates higher than 90% can occur (Buitrago et al., 2005; Rahim et al., 2004; Grosjean et al., 1998; Shimabukuro, 1991).

The juvenile stage starts after the opening of mouth and anus, which then become functional, as well as the digestive system. At this point, individuals are exotrophic and start to graze the biofilm used as the metamorphosis-inducing factor (Carboni, 2013). Caboni (2013) suggests stocking the juveniles in NSW at 36.5 ± 1 ppt salinity, 20 ± 2 °C temperature, 12 h light photoperiod and a stocking density of 400 individuals/m^2. Juveniles can be reared in moving water through a re-circulating system supplied with biological and mechanical filters; an exchange of 100% water daily should be ensured (Carboni et al., 2013). When individuals have a test diameter of 3-4 mm, the biofilm diet can be integrated with macroalgae such as *Enteromorpha* spp., *Ulva* spp., *Saccharina* spp. and *Laminaria* spp.

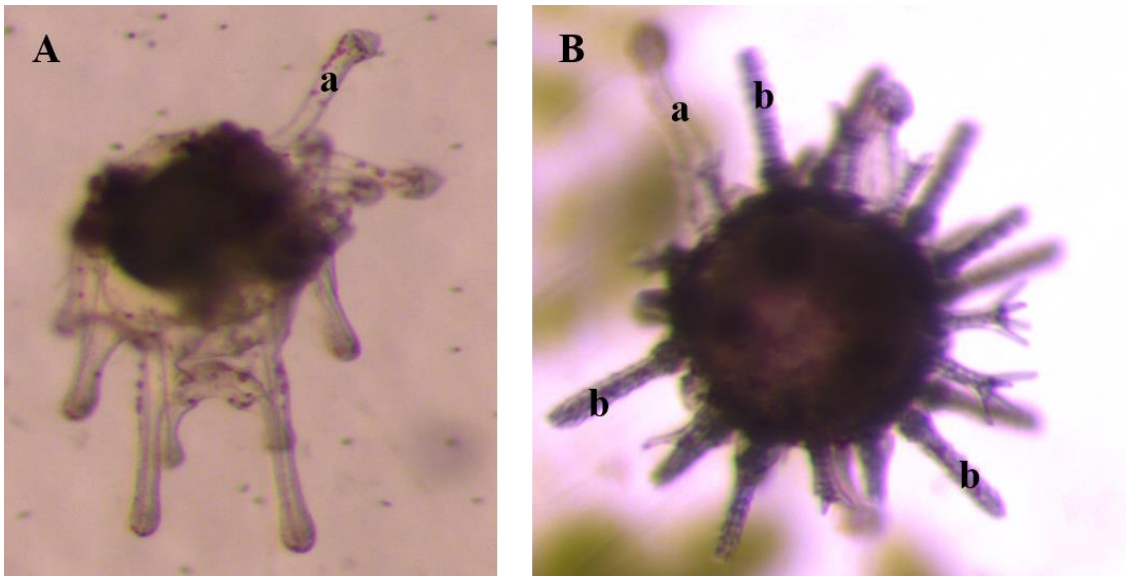

Figure 6: Echinopluteus pre- (A) and post-metamorphosis (B). a = primary podia; b = spines.

Juveniles are then sorted in size and individuals bigger than 10 mm in test diameter are classified as subadults and transferred to other tanks. Subadults are fed *ad libitum* with macroalgae until they reach 40 mm in size. During this stage, it is important to promote the somatic growth of individuals despite gonadic development, while trying to optimize food allocation to the soma. It is important that there be good water circulation in the tanks, as well as the elimination of the solid waste produced (Grosjean, 2001).

When sea urchins reach 40 mm in test diameter, they are conditioned to prepare their gonads for market. During conditioning, urchins are fed *ad libitum* with feed rich in proteins (Klinger et al, 1998) and stored at a minimum of 16 °C temperature for two or three months, depending on the feed quality (Grosjean, 2001).

1.1.5 Limitations of echinoculture

The major bottleneck limiting the aquaculture production of various marine species is the high mortality rate during larval and post-larval stage (Dhert et al., 2001). This occurs even for the sea urchin *P. lividus* (Carboni et al., 2014; Carboni et al. 2012; Mos et al., 2011). In an attempt to improve larval survival, previous studies focused on the identification of suitable rearing methods (Carboni et al., 2014), diets and feed rations (Carboni et al., 2012; Liu et al., 2007; Cárcamo et al., 2005; Kelly et al., 2000; Pedrotti and Fenaux 1993).

Traditionally, larval production was performed using static systems characterized by gentle aeration and complete water exchange (Carboni et al., 2013; Carboni et al., 2012; Azad et al., 2011; Dworjanyn and Pirozzi, 2008; Liu et al., 2007; Kelly et al., 2000) or partial water exchange (Privitera et al., 2011; Cárcamo et al., 2005; Pedrotti and Lemée, 1999; Pedrotti and Fenaux, 1993). Recently, Carboni et al. (2014) obtained a higher larval survival at competence by using a flow-through system (21.2 ± 3.3%) in comparison with traditional systems (6.7 ± 1.9%). According to Carboni et al. (2014), the reduction in handling during manual water exchanges reduces stress and physical damage to the larvae, increasing larval survival. Starting from the 4-arm pluteus, larvae were fed with live microalgae.

It is known that each echinoderm species has specific dietary needs and requires specific fatty acids (Carboni et al., 2012). Live microalgae are typically used to feed sea

urchin larvae even though they are labour intensive and costly to produce. Alternatively, formulated feeds and algal paste diets were tested as feed for echinoderm larvae. For instance, Liu et al. (2007) obtained a complete development of *P. lividus* larvae fed with artificial feed, even though they performed worse than larvae fed with live microalgae.

Previous studies tested various phytoplankton species as live diets for the production of sea urchin larvae. The results obtained differed in terms of larval survival and development at competence, according to the phytoplankton species, the sea urchin species, the stocking density of the larvae, the volume adopted and the seawater exchange. Good survival rates were obtained employing *Cricosphaera elongata*, *Dunaliella tertiolecta*, *Pleurochrysis carterae*, *Chaetoceros* spp. and *Isochrysis* spp. (Table 1). Specifically, *P. carterae* and *C. elongata* were able to improve the survival rate and development of sea urchin larvae (Carboni et al., 2012), while *D. tertiolecta* supported the larval rearing cycle of *P. lividus* (Liu et al., 2007; Carboni et al., 2012) and other echinoid species (Azad et al., 2011; George et al., 2004; Sewell et al., 2004; Kelly et al., 2000; Pearce and Scheibling, 1994, 1991, 1990a; Hart and Scheibling, 1988; Hinegardner, 1969).

Azad et al. (2011) demonstrated that feed rations also influence the survival rate and growth of larvae. In particular, he showed that low feed rations decrease the survival rate and larval growth of *Strongylocentrotus purpuratus*, while high feed levels negatively influence growth and development. Moreover, Càrcamo et al. (2005) observed that frequent feedings increase the survival rate and the size of the echinoid rudiment and post-larvae.

For these reasons, echinopluteus are generally fed every 2 or 3 days (Carboni et al., 2014; Carboni et al., 2012; Jimmy et al., 2003) with small quantities of microalgae. However, the amount of feed increases according to the larval development stage and varies based on phytoplankton cell-size (Table 2). The cell-size of microalgae plays a key role for the palatability of echinopluteus, as they are able to feed on microalgae with about 20 μm in diameter (Càrcamo et al., 2005). Carboni (2013) found that *P. lividus* at 4-arm larval stage, with a length of about 200-250 μm, fed on phytoplankton with a range in volume of 200-400 μm^3.

Table 1: Investigations on the production of sea urchin larvae and characteristics of the rearing methods: Microalgae diet, Density (larvae/mL), Volume (L) adopted, seawater (SW) exchange (% and frequency), larval Development and Survival at competence (Cp), metamorphosis (Mt), or days post-fertilization (DPF).

Author	Sea urchin	Microalgae diet	Density	Volume	SW exchange	Development	Survival
Carboni et al., 2015	*Paracentrotus lividus*	*Dunaliella tertiolecta*	4/mL	80 L	100%, 3 days	5.2% (Cp)	
		Tetraselmis suecica	4/mL	80 L	100%, 3 days	0%	
		Pleurochrysis carterae	4/mL	80 L	100%, 3 days	5.7% (Cp)	
		Cricosphaera elongata	4/mL	80 L	100%, 3 days	14.4% (Cp)	
Paredes et al., 2015	*Paracentrotus lividus*	*T. suecica* + *Isochrysis* spp. + *Chaetoceros gracilis* + *Phaeodactylum tricornutum*	1/mL	100 L	100%, 2/3 days	40.5% (Cp)	
Azad et al., 2011	*Strongylocentrotus purpuratus*	*D. tertiolecta*	1/mL	15 L	100%, 2 days	<28 DPF (50% Cp)	42.2% (28 DPF)
		Chaetoceros muelleri	1/mL	15 L	100%, 2 days	>28 DPF (50% Cp)	27.8% (28 DPF)
		Isochrysis spp.	1/mL	15 L	100%, 2 days	>28 DPF (50% Cp)	~37% (28 DPF)
		D. tertiolecta + *C. muelleri*	1/mL	15 L	100%, 2 days	>28 DPF (50% Cp)	~37% (28 DPF)
		D. tertiolecta + *Isochrysis* spp.	1/mL	15 L	100%, 2 days	<28 DPF (50% Cp)	44.4% (28 DPF)
		C. muelleri + *Isochrysis* spp.	1/mL	15 L	100%, 2 days	>28 DPF (50% Cp)	~37% (28 DPF)
		D. tertiolecta + *C. muelleri* + *Isochrysis* spp.	1/mL	15 L	100%, 2 days	>28 DPF (50% Cp)	~37% (28 DPF)
		D. tertiolecta + *Isochrysis* spp. (Low ration)	1/mL	15 L	100%, 2 days	>28 DPF (50% Cp)	17.8% (28 DPF)
		D. tertiolecta + *Isochrysis* spp. (Normal ration)	1/mL	15 L	100%, 2 days	>28 DPF (50% Cp)	25.6% (28 DPF)
		D. tertiolecta + *Isochrysis* spp. (Standard ration)	1/mL	15 L	100%, 2 days	<28 DPF (50% Cp)	53.3% (28 DPF)
		D. tertiolecta + *Isochrysis* spp. (Medium ration)	1/mL	15 L	100%, 2 days	>28 DPF (50% Cp)	47.8% (28 DPF)
		D. tertiolecta + *Isochrysis* spp. (High ration)	1/mL	15 L	100%, 2 days	>28 DPF (50% Cp)	45.6% (28 DPF)
Liu et al., 2007	*Paracentrotus lividus*	*D. tertiolecta*	1.5/mL	60 L	100%, 2 days	18 DPF (Cp)	67.8-75.6%
Càrcamo et al., 2005	*Loxechinus albus*	*C. calcitrans*	0.7/mL	20 L	30/50%, 2 days		48.8±16.9%
		C. calcitrans + *I. galbana*	0.7/mL	20 L	30/50%, 2 days		58.3±14.4%
Càrcamo, 2004	*Loxechinus albus*	*C. gracilis* + *I. galbana* (trial 1)	0.6/mL	2 L	50%, 2 days	20 DPF (Before Mt)	58.5% (Before Mt)
		C. gracilis + *I. galbana* (trial 2)	0.6/mL	2 L	50%, 2 days	22 DPF (Before Mt)	42.1% (Before Mt)
		C. gracilis + *I. galbana* (trial 3)	0.6/mL	2 L	50%, 2 days	21 DPF (Beforfe Mt)	43.8% (Before Mt)
		C. gracilis + *I. galbana* (trial 4)	0.6/mL	2 L	50%, 2 days	21 DPF (Before Mt)	55.0% (Before Mt)
George et al., 2004	*Lytechinus variegatus*	*D. tertiolecta*	0.5/mL	1 L	90%, 2 days		85±4% (14 DPF)
Jimmy et al., 2003	*Echinus esculentus*	*D. tertiolecta*	1/mL	40 L	100%, 3 days		59,17% (21 DPF)
		P. tricornutum	1/mL	40 L	100%, 3 days		55,00% (21 DPF)
		D. tertiolecta + *P. tricornutum*	1/mL	40 L	100%, 3 days		57,92% (21 DPF)
		D. tertiolecta (Standard ration)	1/mL	40 L	100%, 3 days		51.67% (16 DPF)
		D. tertiolecta (High ration)	1/mL	40 L	100%, 3 days		55.00% (16 DPF)
Kelly et al., 2000	*Psammechinus miliaris*	*P. elongata* (Low ration)	1/mL	60 L	100%, 2 days	Fail	
		P. elongata (Optimum ration)	1/mL	60 L	100%, 2 days	Normal morphology	
		P. elongata (High ration)	1/mL	60 L	100%, 2 days	Irregular morphology	
		P. elongata	1/mL	60 L	100%, 2 days		46% (17 DPF)
		D. tertiolecta	1/mL	60 L	100%, 2 days		53.8% (17 DPF)
		P. carterae + *D. tertiolecta*	1/mL	60 L	100%, 2 days		44.7% (17 DPF)
		P. carterae	1/mL	60 L	100%, 2 days	20 DPF (Cp)	61.5% (17 DPF)
		D. tertiolecta (trial 2)	1/mL	60 L	100%, 2 days	20 DPF (Cp)	48.9% (17 DPF)
Pedrotti and Lemée, 1999	*Paracentrotus lividus*	*C. elongata*	2/mL	1 L	80%, 2 days	11 DPF (rudiment)	94.9% (18 DPF)
Zamora and Stotz, 1994	*Loxechinus albus*	*C. gracilis* + *I. galbana*	1.26/mL	500 L	40%, 2 days		58.7% (Before Mt)
Bustos et al., 1990	*Loxechinus albus*	*C. gracilis*	0.5/mL	185 L	~75%, 2 days		37.5% (Before Mt)

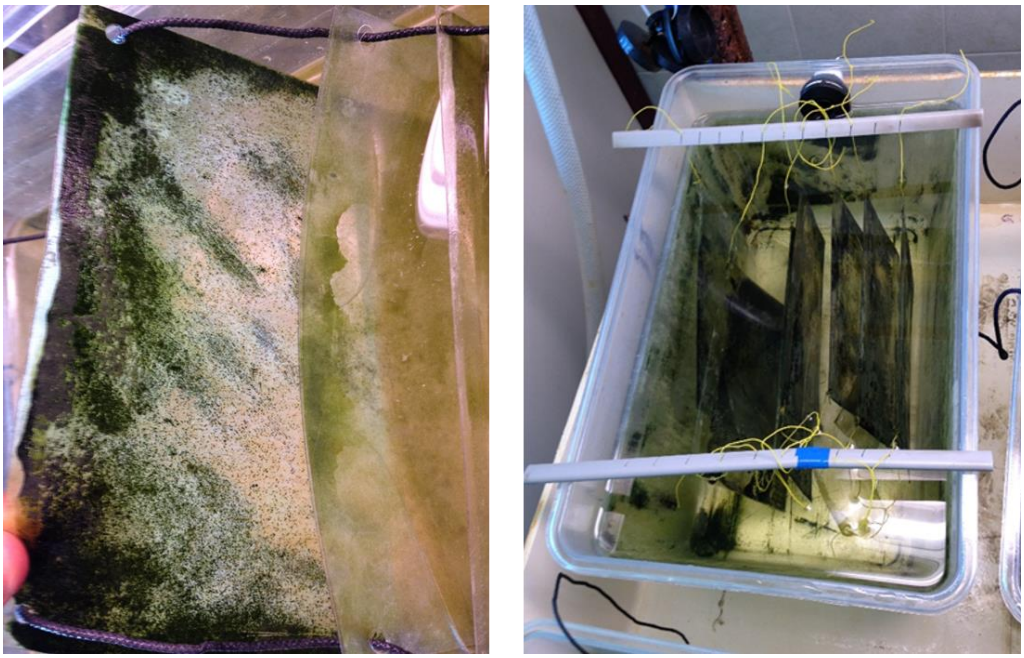

Figure 7: Cultivation of *Ulvella lens* under laboratory controlled conditions.

Settlement represents another critical phase in the culture of sea urchins. Metamorphosis rates have been recorded from 0 to 90% (Huggett et al., 2006; Buitrago et al., 2005; Rahim et al., 2004; Grosjean et al., 1998; Gosselin and Jangoux, 1996; Pearce and Scheibling, 1991; Cameron and Hinegardner, 1974) and mortality rates have been noted higher than 90% within the first weeks of benthic life (Buitrago et al., 2005; Rahim et al., 2004; Grosjean et al., 1998; Shimabukuro, 1991). Numerous studies indicate that marine larvae actively participate in the transition from planktonic to benthic life, reacting to physical, chemical and biological stimuli (Berntsson et al., 2004). In production, sea urchin larvae are typically induced to settle on biofilms (mainly diatoms) cultured on vertical plates (McBride, 2005; Harris et al., 2003; Shimabukuro, 1991). However, laboratory experiments positively tested a wide range of cues, such as macroalgae (Mos et al., 2011; Dworjanyn and Pirozzi, 2008; Huggett et al., 2006; Gosselin and Jangoux, 1996; Pearce and Scheibling, 1991, 1990a), adult conspecifics (Mos et al., 2011; Dworjanyn and Pirozzi, 2008) and bacteria (Mos et al., 2011; Huggett et al., 2006).

Nonetheless, Swanson et al. (2012) demonstrated that some species of sea urchins (*Holopneustes purpurascens* and *H. inflatus*) metamorphosed in absence of any substrate, stimulated only by histamine. Recently, the green macroalgae *Ulvella lens*

was positively tested as a metamorphosis-inducing factor for invertebrate species. In particular, *U. lens* improves larval settlement and represents an initial feed for juveniles of abalones (Daume et al., 2004, 2000; Takahashi and Koganezawa, 1988), sea cucumbers (Matsuura et al., 2009) and sea urchins (Hannon et al., 2015, 2014; Takahashi et al., 2002).

Table 2: Microalgae species used as feed for the production of echinoderm larvae, volume cell-size (µm^3) and amount of feed (number of cells/mL) given according to development stage.

Author	Species	Cell-size	4-arms	6-arms	8-arms
Azad et al., 2011	*Chaetoceros muelleri*	71.4	7,000	8,750	8,750
Azad et al., 2011	*D. tertiolecta*	180	2,000	4,500	7,500
Carboni et al., 2012	*D. tertiolecta*	180	1,500	4,500	7,500
Kelly et al., 2000	*D. tertiolecta*	180	3,000	9,000	15,000
Liu et al., 2007	*D. tertiolecta*	180	1,500	4,500	7,500
Azad et al., 2011	*Isochrysis* spp.	62	8,000	10,000	12,000
Carboni et al., 2012	*P. carterae*	380	750	2,250	3,250
Kelly et al., 2000	*P. carterae*	380	1,000	3,000	5,000
Carboni et al., 2012	*P. elongata*	380	750	2,250	3,250
Pedrotti and Lemée, 1999	*P. elongata*	380	1,000		3,000
Carboni et al., 2012	*Tetraselmis suecica*	180	1,500	4,500	7,500

Post-settlement survival is considered the second most important bottleneck in the production of sea urchins in aquaculture (Carboni, 2013). Environmental variables such as temperature are important in promoting post-larval survival (Mos et al., 2011). Larval diet and feed rations also influence the survival and test diameter of various species of sea urchin post-larvae (Liu et al., 2007; Jimmy et al., 2003; Kelly et al., 2000; Meidel et al., 1999; Hart and Strathmann, 1994). However, the effects of diet on the survival of juvenile sea urchins has received little attention.

1.2 Integrated Multi-Trophic Aquaculture

Aquaculture is one of the fastest-growing food producing sectors in the world and, nowadays, it represents a fundamental sector for sustaining human consumption of fish products (FAO, 2014b).

In 2012, global aquaculture production resulted in 90.43 million tons. 73.7% of this was represented by fish for human consumption (fin fishes, crustaceans, molluscs, amphibians, freshwater turtles, sea cucumbers, sea urchins, sea squirts and edible jellyfishes), while the remaining 26.3% was algae (mostly marine macroalgae/seaweeds) (FAO, 2014a). Aquaculture's contribution to global fish production reached 42.2% in 2012, up from 25.7% in 2000. Asia is the only continent capable of producing more fish from aquaculture (54%) than from captures (46%), and increases in production occured in all other continents. For example, Europe saw an increase of 18%, while the production of other contintents increased by less than 15% (FAO, 2014a).

Traditional aquaculture systems are designed to produce a single commercial species, fed with formulated feeds and using a continuous supply of natural seawater. Often, aquaculture is very expensive in terms of labour and energy costs. In addition, it is subject to limitations, such as the availability of suitable sites and the different uses (e.g. maritime traffic and tourism) of marine space (Cataudella and Spagnolo, 2011). Moreover, aquaculture wastewater is rich in residues of feed, fecal matter and dissolved nutrients (ammonia, nitrite, nitrate, phosphate), and therefore represents one of the major contributors to the increasing levels of dissolved and particulate nutrients in the environment (Lamprianidou et al., 2015).

In order to reduce the impact of traditional methods of aquaculture, several countries around the world are developing integrated aquaculture systems, which involve the producing of different commercial species while sharing resources such as water, feed and labor (Lorkowski et al., 2012).

Among the various integrated systems, the Integrated Multi-Trophic Aquaculture (IMTA) is the most renowned (Lorkowski et al., 2012). The term "multi-trophic" refers to various aquatic species of different trophic or nutritional levels being reared in the same system (Neori et al., 2007; Chopin, 2006).

The IMTA systems are set up in such a way that nutrient and energy fluxes of a main production can be easily used for the production of secondary species. The fluxes of nutrients and energy are channelled throughout active (pumping water) or passive (water currents) transport systems, from main production tanks to secondary production tanks, where they are used for the growing and/or proliferation of secondary species (Lorkowski et al., 2012). In this way, the IMTA promotes the re-use of resources such as water, feed, energy and space for the production of other commercial species (Chopin et al., 2001).

IMTA are flexible and adaptable systems, and they can be open-water, semi-closed or land-based systems, characterized by different water salinities: marine or freshwater. Moreover, different combinations of species and trophic levels can be developed, according to the geographic area and market demand (Barrington et al., 2009; Troell, 2009; Chopin, 2006; Neori et al., 2004). Barrington et al. (2009) presented the situation and the potential of IMTAs in the world's marine temperate waters, identifying the genera of specific interest and higher potential, among which:

- *Laminaria*, *Saccharina*, *Sacchoriza*, *Undaria*, *Alaria*, *Ecklonia*, *Lessonia*, *Durvillaea*, *Macrocystis*, *Gigartina*, *Sarcothalia*, *Chondracanthus*, *Callophyllis*, *Gracilaria*, *Gracilariopsis*, *Porphyra*, *Chondrus*, *Palmaria*, *Asparagopsis* and *Ulva* (seaweeds).
- *Haliotis*, *Crassostrea*, *Pecten*, *Argopecten*, *Placopecten*, *Mytilus*, *Choromytilus* and *Tapes* (molluscs).
- *Strongylocentrotus*, *Paracentrotus*, *Psammechinus*, *Loxechinus*, *Cucumaria*, *Holothuria*, *Stichopus*, *Parastichopus*, *Apostichopus* and *Athyonidium* (echinoderms).
- *Nereis*, *Arenicola*, *Glycera* and *Sabella* (polychaetes).
- *Penaeus* and *Homarus* (crustaceans).
- *Salmo*, *Oncorhynchus*, *Scophthalmus*, *Dicentrarchus*, *Gadus*, *Anoplopoma*, *Hippoglossus*, *Melanogrammus*, *Paralichthys*, *Pseudopleuronectes* and *Mugil* (fish).

Generally, the main interesting species for the IMTA, usually fish or shrimp, are fed with formulated feeds and reared according to traditional methods, in the conventional production containments (ponds, tanks or net cages). Feed which is not ingested and faeces produced by these species are eaten by detritivorous and filter feeders. In turn, the main dissolved nutrients (ammonia, nitrite, nitrate and phosphorous) are assimilated by the primary producers, micro and macro-algae. In this way, primary producers play a double role (both ecological and productive) in the IMTA, reducing waste given off from a fish farm and introducing new energy into the system in the form of organic carbon (Lorkowski et al., 2012).

Detritivorous, filter feeders and algae can be used for direct human consumption, providing an economic diversification of the product and helping to reduce economic risks (Barrington et al., 2009). Moreover, raw materials contained in some species of micro and macroalgae can be used for pharmaceutics or other biotechnical processes (e.g. production of biogas and biofuel) (Lorkowski et al., 2012).

However, primary biomass can be re-used in the system as a feed supplement for other cultured species. Phytoplankton is widely used for the production of rotifers, crustaceans, mollusks and larval stages of fishes and echinoderms, while macroalgae represent a natural feed for the production of echinoderms such as sea cucumbers and sea urchins, which are excellent candidate species for IMTAs (Barrington et al., 2009).

1.2.1 Microalgae

Microalgae, or phytoplankton, are simple microscopic heterotrophic and/or autotrophic photosynthetic organisms, ranging from unicellular to multi-cellular forms (Zhu, 2014).

Due to the enormous biodiversity (more than 40,000 species of microalgae identified according to Fuentes-Grunewald et al., 2009), microalgae are widely used for human consumption, the production of biodiesel, formulated feeds and integrators, as well as biofertilizer and wastewater bioremediation (Tredici, 2006; Pulz and Gross, 2004).

Microalgae are the main primary producers in the aquatic ecosystem (Becker, 2004). Through photosynthesis, they can absorb substantial amounts of CO_2 to produce oxygen and sugars, which can be converted in lipids, proteins and carbohydrates (Field et al., 1998).

Like other microorganisms, the growth of microalgae goes through four phases: lag phase, exponential phase, stationary phase and death (or lysis) phase (Figure 8). The growth of microalgae is influenced by biotic and abiotic factors, the more important of which are light, temperature, salinity and pH (Zhu, 2014). Light is one of the key variables influencing the growth, as it is their energy input source. At low levels of light intensity, for instance, there is no net growth; growth increases as the light intensity increases, until reaching the light saturation point, where the photosynthesis rate is at its maximum (Ye et al., 2012).

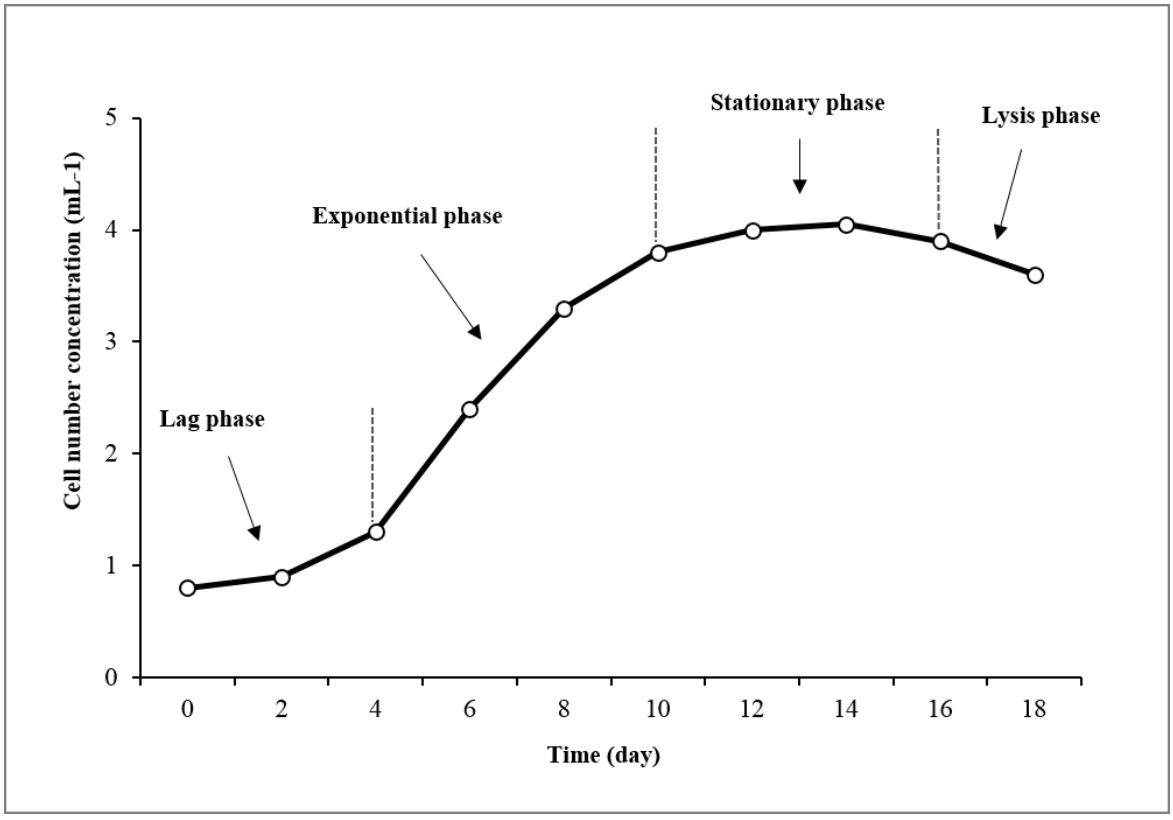

Figure 8: Algal growth phases (modified by Moazami et al., 2012).

Temperature is another key limiting factor that influences growth. Each algal species is characterized by an optimal growing temperature (Chisti, 2007); generally, this ranges between 20-30 °C (Madigan et al., 2000). Although some algae species are very tolerant of a broad range of pH values, the optimum range for algal cultures is between 6 and 8. Of course, their level of tolerance is species-dependent (Zeng et al., 2011).

In addition to sunlight and CO_2, water, nitrogen and phosphorous are the major inputs for algae growth. However, other macro- (Si, Mg, Na, Ca, and K) and micro-nutrients (Mo, Mn, B, Co, Fe and Zn) are required (Zhu, 2014). Effluents from intensive fish farms are enriched in dissolved nutrients, mainly in the form of inorganic nitrogen and phosphorus (Pillay, 1992), representing a suitable culture medium for the growth of microalgae (Shpigel, 2013). For this reason, microalgae have been used widely for nutrient removal in wastewater (Borges et al., 2005), but their use in the treatment of aquaculture wastewater has been neglected (Sirakov and Velichkova, 2014).

Due to their high nutritional values, *Tetraselmis* spp. and *Nannochloropsis* spp. are widely employed as live feed in the aquaculture industry for hatchery-grown herbivores and filter-feeding larvae (Michels et al., 2014). For this reason, they are the main species tested for bioremediation of fish farm wastewaters (Michels et al., 2014; Sirakov and Velichkova, 2014; Borges et al., 2005). Results obtained by previous studies showed that *Tetraselmis* spp. and *Nannochloropsis* spp. remove a high amount of nitrogen and phosphorus and grow well when cultured in aquaculture wastewater (Michels et al., 2014; Sirakov and Velichkova, 2014; Borges et al., 2005). However, a more efficient assimilation was observed when the N:P ratio was correct (Michels et al., 2014; Borges et al., 2005; Lefebvre et al., 1996). For instance, Michels et al. (2014) reported N and P removal efficiencies of ~50% and 99%, respectively, when *Tetraselmis suecica* was cultured in turbot wastewater. By contrast, when extra phosphate was added to the wastewater to adjust the N:P ratio, a removal efficiency was obtained of 95.7% for N and 99.7% for P.

Therefore, for fish farm bioremediation, it is important to know the nutrient composition of the wastewater, because it differs according to the type of fish feed used, the fish species farmed, the size of the fish and the rearing temperature (Lemarié et al., 1998).

1.2.2 Macroalgae

Macroalgae (or seaweeds) are marine or freshwater species containing more carbohydrates and lower lipids in comparison to microalgae (Boonstra, 2014). These are unicellular or multicellular algae capable of growing up to 70 m in length (Brennan et al., 2012).

On the basis of pigment type, macroalgae are divided into green algae (Chlorophytes), red algae (Rhodophytes) and brown algae (Phaeophytes) (Roesijadi et al., 2008). Pigments affect the light requirement and capture active radiation photosynthetically at wavelengths ranging between 400 and 700 nm (Florentinus et al., 2008).

The production of macroalgae is widespread in the world because they can be used in different sectors and are versatile in their applications: food for human consumption, the production of hydrocolloids, fertilizers, the production of biofuel, animal feed, platform chemicals, pigments and medical and pharmaceutical applications (Boonstra, 2014). For this reason, macroalgae have a large global market and they are widely cultivated (Table 3).

Table 3: Most important cultivated macroalgae species in the world (modified by Boonstra (2014)).

Species/Genus	Group	Countries producing in 2010	Quantity 2010 (ktonnes)	Average value per kg (US$/kg)
Saccharina japonica	brown	4	5147	0.06
Eucheuma spp.	red	12	3748	0.31
Kappaphycus alvarezii	red	6	1875	0.14
Gracilaria spp.	red	9	1717	0.31
Porphyra spp.	red	3	1648	0.71
Undaria pinnatifida	brown	4	1537	0.43
Sargassum spp.	brown	1	78	0.46
Ulva spp.	green	1	4	0.81
Caulerpa spp.	green	1	4	0.59

Like for microalgae, the growth of macroalgae is affected by many factors such as chemical nutrient balance, the temperature and salinity of seawater, solar irradiation, currents and waves (Titlyanov and Titlyanova, 2010).

Often, seaweeds are cultivated in natural water areas using only naturally available light, heat, water motion energy and nutrients (extensive cultivation). While microalgae are mostly cultivated in land-based systems (like photobioreactors or raceway ponds) and more easily controlled growing conditions than macroalgae (FAO, 2009), the cultivation of macroalgae is a more complicated practice (Boonstra, 2014).

1.2.2.1 *Ulva* spp.

Ulva spp. are among the few species of green macroalgae to be cultivated. The species belonging to this genus are typically ephemeral algae, with a relatively undifferentiated thallus and a high area to volume ratio of the thallus (Littler and Littler, 1980).

They are employed in pharmaceutical and chemical applications, as well as in the production of biofuel (van der Wal et al., 2013). Furthermore, they are also consumed by human populations (commonly named "sea lettuce", FAO, 2013), and used in aquaculture as fresh feed for herbivores (Robertson-Andersson, 2003).

Ulva spp. are characterized by a rapid reproduction and fast growth in suitable environmental conditions (Littler and Littler, 1980). Often, they have a high nitrogen demand (Barr and Rees, 2003), therefore their growth is enhanced by pollution due to sewage (Arevalo et al., 2007), sedimentation (Eriksson and Johansson, 2005) and urbanisation (Mangialajo et al., 2007). For these reasons, *Ulva* spp. are targeted for the bioremediation of nitrogen and phosphorous from aquaculture (Copertino et al., 2009; Neori et al., 2003).

It is well demonstrated that *Ulva* spp. effectively uptake nutrients dissolved in aquaculture wastewater derived from fish (Shpigel, 2013; Neori et al., 2004) or shrimp farms (Copertino et al., 2009). Previous studies have tested the employment of the genus *Ulva* for the bioremediation of wastewater in integrated systems, showing a high nutrient removal efficiency (from 40 to 90%) and biomass yield (40–100 g of $DW/m^2/day$) (Neori et al., 2003, 2000, 1998, 1991; Schuenhoff et al., 2003; Martínez-Aragón et al., 2002; Jiménez del Río et al., 1994; Shpigel et al., 1993; Vandermeulen and Gordin, 1990). However, these values may vary between species, characteristics of the cultivation system employed and local environmental conditions such as salinity, turbidity, photoperiod and temperature (Copertino et al., 2009). The growth rate of *Ulva lactuca*, for instance, is predominantly linked to water temperature and light conditions (Neori et al., 1996; Vandermeulen and Gordin, 1990), so its biomass production is highly seasonal (Neori et al., 1998).

Copertino et al. (2009) demonstrated that the growth of *Ulva clathrata* in shrimp (*Litopenaeus vannamei*) aquaculture water can be enhanced by optimizing the cultivation method. According to these authors, growth rate and biofiltering efficiency can be improved at a salinity higher than 30 ppt, as suggested by Friedlander (1991) and

Kamer et al. (2001). Having a suitable seaweed density for the wastewater nutrient load and maintaining the algae culture under high water movement and aeration can also be useful as reported by Lüning and Pang (2003).

The water flow is considered a key factor for seaweed nutrient assimilation. A lower biofiltering efficiency and biomass yield has been observed when *Ulva* spp. is cultivated in lower rather than higher water flow rate systems (Copertino et al., 2009; Msuya and Neori, 2008; Martinéz-Arágon et al., 2002; Ale et al., 2011; Hurd, 2000).

1.2.3 Grey mullet Mugil cephalus

Worldwide, Asia is the biggest market of Mugilidae with 70% of the total capture, followed by Africa (14%), the Americas (10%), Europe (2%) and Oceania (1%) (FAO, 2015).

In the mullet family, *Mugil cephalus* (Linnaeus, 1758) is an important food species and its *roe* is a high-priced product in Mediterranean markets (Aizen et al., 2005). Due to the high market demand, the total world landings of *M. cephalus* increased significantly between 1990 and 2000, but a decrease in yield has been recorded since 2004 (Whitfield et al., 2012). On the contrary, as often happens when coastal fishery yield declines, the production of Mugilidae in aquaculture has been showing an increase in the total harvest since 1994 (Whitfield et al., 2012). In Sardinia, for example, although *M. cephalus* is traditionally reared in coastal lagoons with the application of extensive aquaculture techniques, its productivity has decreased during the last decades due to overfishing and a reduced migration of juveniles into the lagoons (Cabras Fishermen's Association, personal communication).

In this context, aquaculture could represent a valid way to limit negative effects caused by overfishing, through the production of juvenile individuals to employ in reseeding activities. However, although the ecological aspects have been studied previously, the rearing methods for the production of *M. cephalus* still need to be improved. A pilot hatchery system for the reproduction and rearing of *M. cephalus* has been developed at the International Marine Centre - IMC Foundation with the aim of enhancing aquaculture techniques, (Torregrande, Oristano).

1.2.3.1 The hatchery system

The *M. cephalus* hatchery was equipped with a broodstock system, a larval rearing system and a live-feed (phyto- and zooplankton) production system.

Broodstocks were collected by hand at the Cabras (39°54'31" N; 8°29'34" E) and Mistras (39°54'08" N; 8°27'59" E) lagoons and transported to the IMC laboratories in fish transport bags. There they were held in a recirculating aquaculture system (RAS) consisting of two 2500 L fiberglass tanks. One female and four males were in each tank. Natural seawater was at 35 ± 1 ppt salinity and 22.5 ± 1.0 °C temperature. A natural photoperiod was maintained (14/10 light and dark).

Larvae were reared in a RAS system of four circular fiberglass tanks of 2000 L volume. All systems, broodstock and larval rearing, were equipped with a biological and a mechanical cartridge filter (10 µm), UV lamp, protein skimmer, gentle aeration and temperature regulation system.

Larvae were reared in seawater with 36 ± 1 ppt salinity and 23 ± 1 °C temperature. From two days post-hatching, larvae were fed with a 1:1 ratio of *Nannochloropsis oculata* and *Isochrysis galbana*, at a density of 3 million cells/mL. The phytoplankton diet was integrated with the rotifer *Brachionus plycatilis* (4.5/mL) from 3 to 35 days post-hatching, and *Artemia salina* nauplii (2/mL) from 16 to 45 days post-hatching. When *M. cephalus* juveniles were at 0.35 ± 0.43 g (mean ± SD) body weight they were stocked at an average density of 0.5 g body weight/L and fed with formulated feed supplied by Skretting SpA (PERLA LARVA): 62% crude protein, 11% crude oils and fats, 9% crude ash, 0.8% crude fiber and 1.2% crude phosphorus.

Uneaten feed and faeces were siphoned out twice a week to maintain good water quality; a 30% seawater exchange was performed weekly and a part of this 30% was employed as a medium culture for the cultivation of live micro- and macroalgae to use as feed for *P. lividus*.

1.3 Aims of the thesis

The general objective of this PhD project was to enhance the hatchery production of the sea urchin *P. lividus* and the micro- and macroalgae on which it feeds, in an Integrated

Multi Trophic Aquaculture (IMTA) system. We aimed to obtain juvenile individuals while promoting responsible and sustainable aquaculture (Figure 9).

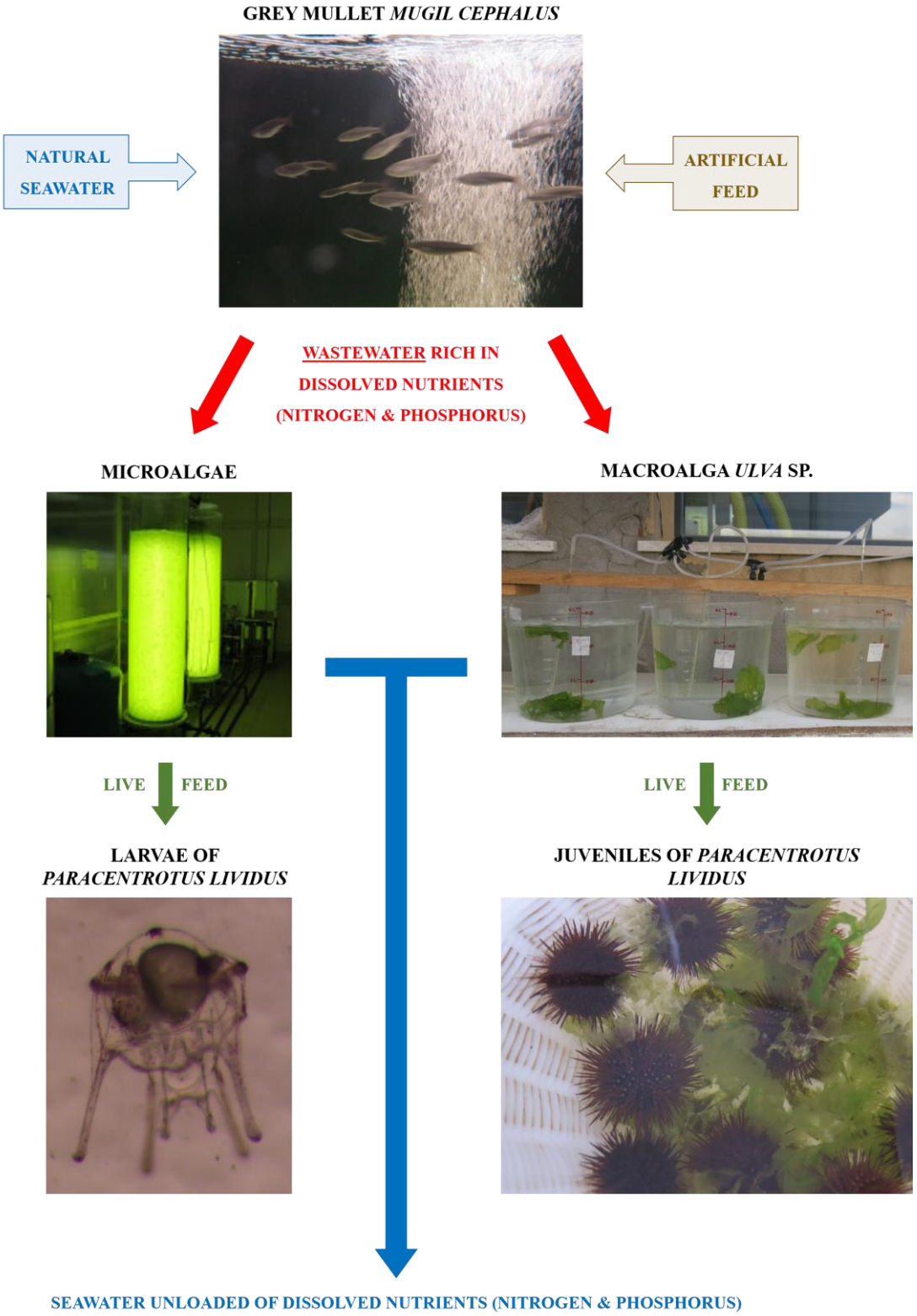

Figure 9: Integrated Multi Trophic Aquaculture system proposed in this study.

Specifically, my objectives were:

1) to identify a suitable larval rearing method;

2) to identify suitable diets for the production of *P. lividus* juveniles;

3) to evaluate the growth and feed consumption of juveniles at different stocking densities;

4) to verify the possibility of producing larval feed (microalgae) using the hatchery wastewater of *M. cephalus*;

5) to verify the possibility of producing juvenile feed (macroalgae) using the hatchery wastewater of *M. cephalus*.

My attempts to reach these specific objectives were divided into five experiments, or chapters of the thesis.

*1.3.1 Effects of on-demand feeding on sea urchin larvae (*Paracentrotus lividus*; Lamarck, 1816), development, survival and microalgae utilization*

Although previous studies focused on the identification of suitable diets aimed at reducing the mortality rate during larval rearing of *P. lividus*, little attention has been given to the rearing methods adopted in aquaculture.

Traditionally, larval production is performed using static systems characterized by gentle aeration and a complete or partial water exchange, carried out every 2 or 3 days, in order to maintain good seawater quality which is free of debris and settled materials. Alternatively, positive results have been obtained using a flow-through larviculture system characterized by a complete daily water exchange. However, some studies have revealed that routine maintenance operations like siphoning, bubbling, sieving and filtration can be stressful to the larvae and could damage the larvae in suspension.

In this experiment, I tested the hypothesis that an alternative static breeding system characterized by a low level of physical disturbance improves the survival and development of *P. lividus* larvae. I assessed the growth of larvae cultured using a variable method, characterized by a variable amount of feed and seawater exchange, established according to the larval consumption of phytoplankton. Results obtained with

the variable method were compared to those of a fixed method, characterized by a definite amount of feed and seawater exchange.

*1.3.2 Effects of larval diet and metamorphosis cue on survival and growth of sea urchin post-larvae (*Paracentrotus lividus*; Lamarck, 1816)*

The echinoculture production of *P. lividus* could be increased by improving the survival of larvae and post-larvae and the settlement rate. These represent the main bottleneck limiting this activity.

It is well known that different phytoplankton species used as feed have different effects on the survival and development of *P. lividus* larvae. It has also been demonstrated that diets and feed rations influence the growth of various species of sea urchin post-larvae. However, the effects of larval dietary treatments on the survival and growth of juveniles have received little attention in scientific researches. Indeed, few studies have focused on the survival and growth of various species of sea urchin post-larvae, but these investigations were carried out just 10 days post-settlement.

In hatcheries, the transition from the planktonic to benthic period is typically promoted by plates colonized with diatoms. Recently, it has been demonstrated that the larval settlement of sea urchins, abalones and sea cucumbers is improved by the green macroalgae *U. lens*, as well as by the presence of conspecifics.

In my first experiment, I assessed the effects of four phytoplankton diets [*D. tertiolecta*; *Isochrysis* aff. *galbana* (T-Iso) + *D. tertiolecta*; *Chaetoceros gracilis* + *D. tertiolecta*; T-Iso + *C. gracilis* + *D. tertiolecta*] on the survival of *P. lividus* larvae and post-larvae at 10, 20, 30, 100 and 180 days post-settlement. In a second experiment, I tested the effects of two different settlement substrates, *U. lens* and a natural biofilm of diatoms on survival, and I evaluated the presence of conspecifics on larval settlement and post-larval survival and growth of *P. lividus* for the first time.

1.3.3 Stocking density influences the growth of juvenile sea urchins, Paracentrotus lividus *(Lamarck, 1816)*

The growth rate of *P. lividus* is quite low and it is affected by environmental conditions, among which are salinity and temperature. Benthic individuals require about three years to reach market size (50 mm in test diameter) and this has often been considered a barrier for the commercial uptake of sea urchin aquaculture.

Stocking density is an important factor for the growth of aquatic animals, particularly during the earlier culture stages, and it could influence the somatic growth rate. For instance, it is well known that increasing stocking density negatively influences the somatic growth of fishes and invertebrates. It has been demonstrated, moreover, that a high stocking density increases the risk of mortality due to disease outbreaks and cannibalism and has a negative effect on the survival and gonad growth of sea urchins. However, studies examining the effects of stocking density on the cultivation of sea urchins are scarce and, to our knowledge, there are no published studies on the effects of stocking density on the somatic growth of juvenile *P. lividus*.

In this experiment, I evaluated the effects of three different stocking densities on the somatic growth, feed intake, absorption efficiency and food conversion ratio of juvenile *P. lividus* sea urchins.

1.3.4 Bioremediation of aquaculture wastewater from Mugil cephalus *(Linnaeus, 1758) with different microalgae species*

Aquaculture represents one of the major contributors to the increasing levels of dissolved and particulate nutrients in the environment. Several countries around the world are developing Integrated Multi-Trophic Aquaculture (IMTA) systems with the aim of reducing the impact of traditional aquaculture. IMTA systems are characterized by the re-use of wastewater for the growth of microalgae employed as an energy source, a fertilizer, fine chemical production or as fresh feed in aquaculture.

In this experiment, I evaluated and compared the capabilities of the phytoplankton species tested as live feed for *P. lividus* larvae in the removal of dissolved inorganic nutrients (nitrogen and phosphorous) and biomass yield in aquaculture wastewater.

1.3.5 Nutrient assimilation and growth of Ulva *sp. (Linnaeus, 1753) in grey mullet (*Mugil cephalus*) wastewater*

Ulva sp. is one of the algae consumed the most by sea urchin species and it is widely employed as fresh feed in the stocking and rearing activities of *P. lividus*. Due to its versatility and variety of different applications, the production of *Ulva* sp. is widely carried-out all over the world. However, its cultivation is a complicated practice because it is often cultivated in natural water areas and environmental conditions, using only naturally available light, heat, water motion and nutrients.

Ulva sp. has a high nitrogen demand and grows well in conditions of sewage, sedimentation and urban pollution. For this reason, it is considered a target macroalgae for the bioremediation of nutrients (nitrogen and phosphorous) in aquaculture wastewater. The growth rate and nutrient removal efficiency, however, are influenced by many factors. Examples of these are the characteristics of the cultivation system used, the balance of chemicals and nutrients in the seawater, as well as the temperature, salinity, solar irradiation, currents and waves. Therefore, the biomass yield and the nutrient removal efficiency of *Ulva* sp. vary highly according to locations and seasons.

In this experiment, I evaluated the efficiency of the cultivation of *Ulva* sp. in natural environmental conditions in Sardinia (Italy) during the spring, and I compared the biomass yield and removal efficiency of dissolved inorganic nutrients (nitrogen and phosphorous) in two culture media, namely natural estuarine water and aquaculture wastewater.

2 EFFECTS OF ON-DEMAND FEEDING ON SEA URCHIN LARVAE (*PARACENTROTUS LIVIDUS*; LAMARCK, 1816), DEVELOPMENT, SURVIVAL AND MICROALGAE UTILIZATION

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Keywords:

Sea urchin, *Paracentrotus lividus*, larviculture, survival, development.

Contribution:

The author designed and conducted the trial, collected the samples, carried out all laboratory and statistical analysis and wrote the manuscript.

2.1 Abstract

This study compared the growth of sea urchin *Paracentrotus lividus* larvae cultured using two different rearing methods: a variable method based upon a variable amount of feed (microalgae) and seawater exchange (30% or 50%) established according to the phytoplankton concentration in the larval cultures, and a fixed method characterized by a fixed amount of feed and seawater exchange. Three microalgae diets, *Isochrysis* spp. (Tahitian strain, T-Iso), *Chaetoceros gracilis* and a 50:50 mixed diet were tested with both rearing methods. Larval development and survival were assessed at the 6-arm pluteus stage (P6), competence (Cp) and metamorphosis (Mt). Data showed that the variable method reduced the requirements for phytoplankton and seawater exchange. Indeed, through the optimization of feed rations it was possible to reduce the production of debris and settled phytoplankton, minimizing the need for water exchanges. Higher larval survival resulted at Cp and Mt stages for those reared with the variable method as opposed to the fixed one. Survival and development were also influenced by the tested dietary treatments: at Mt stage the mixed diet resulted in a higher larval survival (63.3 ± 8.9%) than T-Iso (19.7 ± 12.1%) and *C. gracilis* (23.4 ± 15.1%) ($p < 0.05$). These results suggest that the use of the variable method improves the larval survival and development and also it reduces resource consumption (phytoplankton, seawater use and work effort), which in turn could potentially improve the hatchery production of *P. lividus*.

2.2 Introduction

Echinoderms, like sea cucumbers and sea urchins, are commercially relevant resources worldwide. In Europe, France is the most important market of sea urchins as *Paracentrotus lividus, Psammechinus miliaris* and *Sphaerechinus granularis* are the main exploited and commercialized species (Grosjean, 2001).

Paracentrotus lividus (Lamarck) plays an important role in the Mediterranean coastal ecosystems. Its life cycle is characterized by two stage: larval planktonic and adult benthic. During the planktonic stage the larva, echinopluteus, is able to swim and consume phytoplankton. In the benthic stage the organism mostly eats macroalgae. Its grazing activity influences the structure of the macroalgae communities: low densities of the organism often result in the substantial growth of macroalgal forests, while high densities overconsume erect algae and cause formation of barrens, bare rocks with encrusting algae (Agnetta et al., 2015; Boudouresque and Verlaque, 2007).

Paracentrotus lividus is also relevant for commercial purposes as it is the most consumed sea urchin in Europe (Carboni et al., 2012). Due to the high market demand for its gonads in both Mediterranean and non-Mediterranean areas (Pais et al., 2007), natural populations are exposed to over fishing in many European coastal areas. This often cause a sharp decline in the abundance of wild stocks (Addis et al., 2009; Boudouresque and Verlaque, 2007; Pais et al., 2007).

Scientific research aims to bridge the gap between supply and demand of aquaculture products (Pearce, 2010). Adults with excellent quality gonads could be available for the market throughout the whole year, while juveniles bred in controlled conditions could be employed in restocking activities (Carboni, 2013).

Nowadays, the larval planktonic stage represents one of the key commercial bottlenecks for echinoculture (Carboni et al., 2014, 2012). The planktonic stage lasts for about 25 days, during which the echinopluteus goes through a series of morphological transformations until they achieve competence. The competent larva metamorphoses into a benthonic organism when it finds a suitable substrate. Under culture conditions, a high larval mortality rate can represent significant limitations for the production of juvenile sea urchins (Carboni et al., 2014, 2012).

With the aim of reducing larval mortality and improving hatchery productivity, previous studies focused on the identification of suitable diets, generally based on microalgal species, and on the optimisation of feed rations (Carboni et al., 2012; Liu et al., 2007; Cárcamo et al., 2005; Kelly et al., 2000; Pedrotti and Fenaux, 1993). Indeed, evident negative effects on larval development and survival have been demonstrated to be produced by low feed rations (Azad et al., 2011; Sewell et al., 2004; Kelly et al., 2000; Fenaux et al., 1994; Strathmann et al., 1992; Boidron-Metairon, 1988), and high ones (Azad et al., 2011; Kelly et al., 2000).

In order to maintain a good quality of water, free of debris and settled materials, larval production was traditionally performed using static systems characterized by gentle aeration and complete water exchange (Carboni et al., 2013, 2012; Azad et al., 2011; Dworjanyn and Pirozzi, 2008; Liu et al., 2007; Kelly et al., 2000) or partial water exchange (Privitera et al., 2011; Cárcamo et al., 2005; Pedrotti and Lemée, 1999; Pedrotti and Fenaux, 1993), carried out every 2 or 3 days. As an alternative larviculture system, a flow-through system characterized by a 100% water exchange daily and a Banjo filter (40 µm mesh size) to prevent larvae loss can be very beneficial to larval survival (Carboni et al., 2014). Furthermore, some studies revealed that routine maintenance operations like siphoning and bubbling in traditional static systems, and sieving and filtration in alternative flow-through systems can be stressful and could damage the larvae in suspension (Carboni et al., 2014; Russell, 2000; Strathmann, 1978).

In the present study, we tested the hypothesis that an alternative static breeding system characterized by a low level of physical disturbance could improve the survival and development of *P. lividus* larvae.

2.3 Materials and methods

Adult sea urchins (test diameter > 40 mm) were collected from a 5 m depth at the "Penisola del Sinis-Isola di Mal di Ventre" Marine Protected Area (39°89'N 8°41'W, western Sardinia, Italy) and transported to the laboratory in thermal insulated and refrigerated containers.

More than one individual was used to increase the egg fertilisation rate in reproductive trials (Evans and Marshall, 2005), and ten specimens (5 males and 5 females) were used for gamete strip-spawning. Gametes were obtained after the dissection of adult sea urchins. Each individual was hemisected with metal scissors, the mouth and the stomach were removed. Male gonads were collected and kept dry at 4 °C, while female gonads were kept in a beaker containing filtered (0.47 µm) natural seawater (NSW). Four drops of diluted (5:100) sperm were added to the eggs and the solution was gently stirred in order to facilitate fertilization.

A sample was taken from the starting solution and the number of fertilized eggs was determined as result of 5 replicates, using a tubular plankton chamber and a Leica MZ8 Stereo Microscope. The fertilization rate was verified by the presence of the fertilization membrane (Azad et al., 2011; Liu et al., 2007; Grosjean et al., 1998).

Embryos were reared under static conditions at a density of 20/mL until they reached the echinopluteus stage (approximately 40 h after fertilization took place). Echinoplutei were reared in previously filtered (0.47 µm) NSW, 36.5 ± 0.2 gL^{-1} salinity, constantly kept in motion by motor-driven rotation without aeration and in continuous light with OSRAM Natura type 50 cm below the water's surface. The temperature was maintained at 19.0 ± 2.0 °C.

2.3.1 Experimental design

Two different larval breeding methodologies were tested, here defined as *variable* and *fixed.* For each method, three microagal diets were tested: two monospecifics, *Isochrysis* spp. (Tahitian strain: T-Iso), *Chaetoceros gracilis* (Cha) and a mixture of both species (Mix). Larvae were stocked at a density of 1.5/mL into 5 L cylindrical tanks made of white plastic material. There were 5 replicates for each treatment in a total of 30 tanks.

In the fixed method the larvae were fed every 3 days with a fixed amount of feed adjusted according to larval development. This corresponded to 8000 cells/mL during the first 10 days post fertilization (DPF) and 16000 cells/mL after 10 DPF. In the mixture combined the two species in equal quantities (50:50 ratio). The number of algal cells was determined using a Leica DMRB microscope and a Neubauer counting chamber (Guillard and Sieracki, 2005). Before each feeding, a partial (50%) NSW

exchange was done to siphon out settled materials and dead cells; a 100 µm mesh size filter was used to avoid larval loss.

In the variable method, the amount of feed was established every 3 days and adjusted according to larval consumption. The first feed supply (2 DPF) was characterized by a fixed quantity (8000 cells/mL), while the subsequent administrations were established after measuring the residual phytoplankton concentration in larval tanks. Feed was not given when cells were abundant in the rearing tanks (more than 8000 and 16000 cells/mL before and after 10 DPF, respectively). In the mixed diet treatment, T-Iso and *C. gracilis* were counted and supplied separately. No feed was supplied when the microalgae concentration within the tanks was higher than 4000 and 8000 cells/mL, before and after 10 DPF, respectively for both species. The amount of NSW exchange was established according to the phytoplankton concentration inside the tanks. A 30% exchange was done when the microalgae concentration resulted higher than 12000 cells/mL, within 10 DPF, and 24000 cells/mL, after 10 DPF. A 50% exchange was done when the microalgae concentration resulted higher than 16000 cells/mL, within 10 DPF, and 32000 cells/mL, after 10 DPF.

2.3.2 Phytoplankton cultures

We chose to use *Isochrysis* spp. and *C. gracilis* as feed for sea urchin larvae because they were previously used in numerous other studies (Paredes et al., 2015; De La Uz et al., 2013; Azad et al., 2011; Cárcamo, 2004; Miller and Emlet, 1999; Zamora and Stotz, 1994; Bustos et al., 1992). They were provided by the Agency for Agricultural Research in Sardinia (AGRIS) and sourced from the Culture Collection for Algae and Protozoa (CCAP: Oban, Scotland).

Cultures were maintained in batch lines at 25 °C, exposed to a 16 h L/8 h D photoperiod and supplied with gentle aeration. The 30 gL^{-1} salinity seawater was pre-filtered (1 µm filter paper), enriched with modified Guillard f/2 and autoclaved at 121 °C for 30 min.

The phytoplankton was supplied to the larvae during the exponential growth phase.

2.3.3 Larval development and survival

Larval development was evaluated by observation of larval structures (number of arms, presence and size of the rudiment) under the microscope, and development stages were defined according to previous studies (Carboni et al., 2012; Liu et al., 2007). For these observations, a minimum of 10 randomly sampled larvae were used. The different development stages were considered achieved when at least 75% of the sampled larvae were considered to be at that stage. Competence for settlement was considered achieved when the rudiment was equal in size or larger than the stomach (Carboni et al., 2012).

Larval survival was assessed volumetrically and the mean value of each measurement was then used to calculate the number of larvae in the tanks. Survival at each development stage was finally expressed as percentage of the initial number of larvae stocked.

Metamorphosis tests were done when larvae reached competence. A stock of 50 larvae was transferred into a 50 mL volume beaker containing filtered NSW and a 50x50 mm PVC layer colonized by the macroalgae *Ulvella lens*. This macroalgae was used as a metamorphosis inducing factor for many organisms in previous studies (De Viçose et al., 2012; Daume et al., 2004; Taniguchi et al., 1994). PVC layers were set up according to the method described in Daume et al. (2004).

The number of metamorphosed individuals was counted at 24, 48 and 72 h post exposure to settlement media; Mt was considered achieved when at least 75% of the larvae were metamorphosed.

2.3.4 Statistical analysis

Data were analyzed by Statistica 6.1 StatSoft, Inc. (2004). The amount of feed supplied and seawater exchanges, as well as the effects of rearing systems and algal diets on larval development and survival were assessed using a two-way analysis of variance (ANOVA). Shapiro Wilk's W test was used to verify the normality of the data distribution and Levene's test was used to verify the homogeneity of variances. The General Linear Model (GLM) was used when normality and homogeneity were significant, and also when the experimental design was unbalanced. Tukey's honestly-

significant difference (HSD) test was used to evaluate all pair-wise treatment comparisons ($p < 0.05$).

2.4 Results

2.4.1 Water exchange and feeding regime

The amount of NSW employed for the total experimental period significantly differed ($p < 0.01$) for the two rearing methods studied, and for the different diets used. Indeed, 23.8 ± 0.7 L (mean ± se) of NSW were consumed in the fixed method, whilst 9.6 ± 0.7 L were employed for the variable method. For the variable method, a lower amount of NSW was required when T-Iso was used as the larval feed when compared to the Cha treatment (Figure 10).

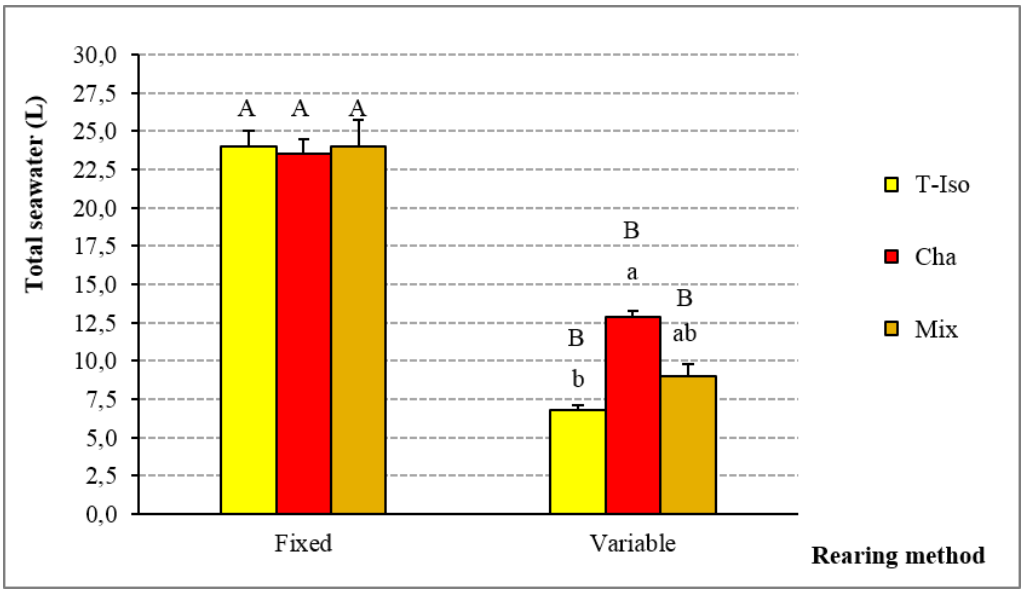

Figure 10: Total seawater (L) consumed during the whole larval rearing. *Isochrysis* spp. (Tahitian strain, T-Iso), *Chaetoceros gracilis* (Cha), 50:50 mixture of the same species (Mix). Capital superscripts indicate significant differences between rearing methods; lowercase superscripts indicate significant differences among diets. Values are expressed as mean ± SE (n=5).

Furthermore, the variable method required a significantly ($p < 0.001$) lower amount of *C. gracilis* in both the Cha ($40.0 \pm 0.0 \cdot 10^6$) and the Mixed diet ($20.0 \pm 0.0 \cdot 10^6$)

treatments, than the fixed method (respectively 552.0 ± 32.0·10^6 and 284.0 ± 27.0·10^6 cells). A lower ($p < 0.01$) consumption of T-Iso resulted in the Mixed diet treatment when using the variable method (123.0 ± 20.9·10^6) than when the fixed method was used (284.0 ± 27.1·10^6). However, a 24.6% increase ($p < 0.01$) of T-Iso was observed when this microalgae was used as a monospecific diet. This was due to longer rearing cycle required by the variable method (38 days) compared to the fixed one (26 days) (Figure 11).

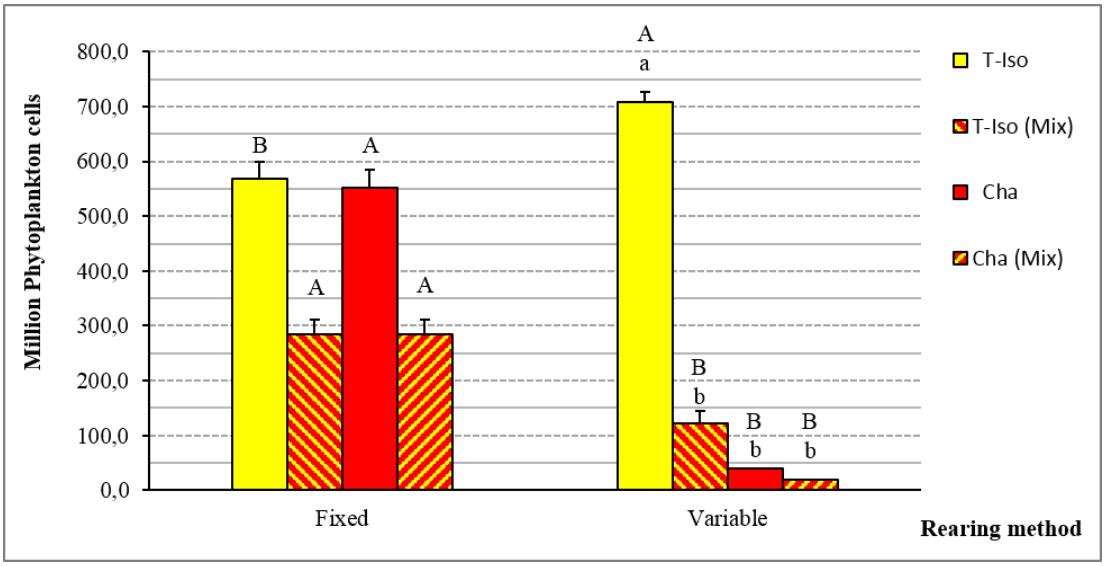

Figure 11: Total phytoplankton (million of cells) supplied during the whole larval rearing. *Isochrysis* spp. (Tahitian strain T-Iso), *Chaetoceros gracilis* (Cha), *Isochrysis* spp. (T-Iso (Mix)) and *C. gracilis* (Cha (Mix)) in the mixture. Capital superscripts indicate significant differences between rearing methods; lowercase superscripts indicate significant differences among diets. Values are expressed as mean ± SE (n=5).

2.4.2 Rearing method effects on larval development and survival

Our results show that larval development and survival are significantly influenced by diets and rearing methodology. In fact, the variable method resulted in a significantly ($p < 0.001$) higher larval survival at the Cp stage than the fixed method. Also the larvae which were fed T-Iso achieved significantly ($p < 0.01$) higher survival rates compared to the larvae fed with the Cha treatments, respectively 88.5 ± 4.4 (mean ± se) and 43.8 ± 10.1 (Figure 12).

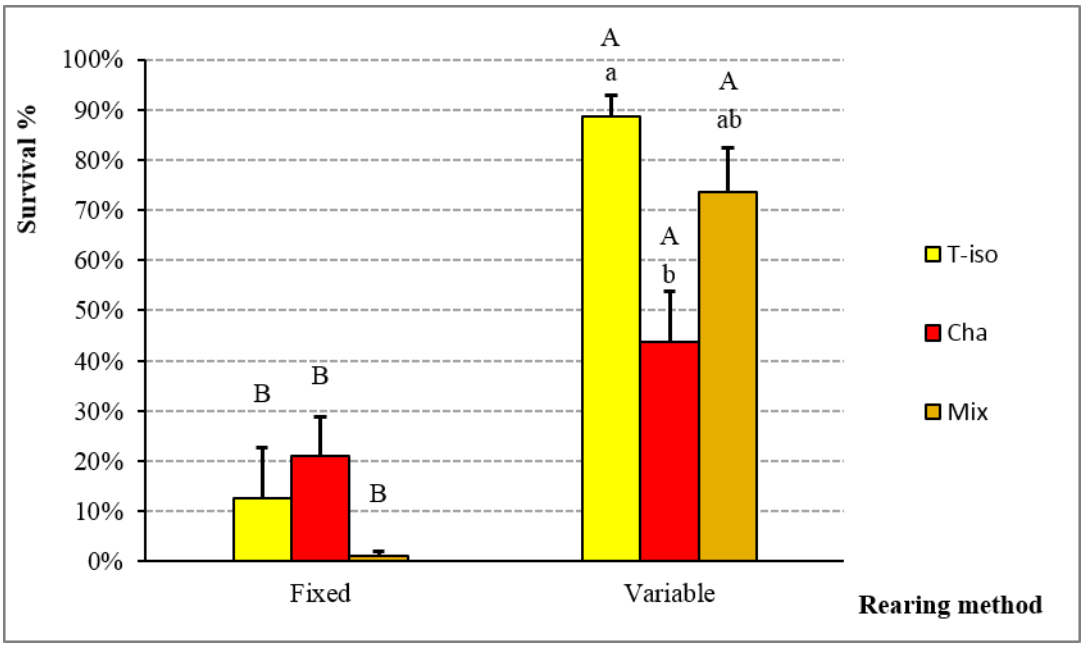

Figure 12: Larval survival with variable and fixed rearing method at the Competence stage (Cp). *Isochrysis* spp. (T-Iso), *Chaetoceros gracilis* (Cha), mixture of T-Iso and *C. gracilis* (Mix). Capital superscripts indicate significant differences between rearing methods; lowercase superscripts indicate significant differences among diets. Values are expressed as mean ± SE (n=5).

Feeding T-Iso resulted in a significantly ($p < 0.01$) faster development up to the P6 stage regardless of the method used. At 14 DPF, in fact, 84.9 ± 7.3% (mean ± se) of the larvae fed T-iso achieved this stage, whilst only 28.7 ± 10.9% and 46.9 ± 16.6% of the larvae fed Cha and Mix respectively achieved this stage. Similar results were observed for the variable method where 92.6 ± 6.0% of the larvae fed T-Iso achieved P6 whilst 9.2 ± 5.8% and 55.9 ± 8.9% of the larvae were at this stage when fed with Cha and Mix treatments respectively. By 17 DPF, all the larvae fed T-Iso in the variable methods were observed to be at the P6 stage, whilst 88.0 ± 7.4% of them was at this stage under the fixed rearing regime. Nonetheless, larvae fed T-Iso were significantly more advanced ($p < 0.01$) than larvae fed with Cha regardless of the rearing method used. Interestingly, a significant difference ($p < 0.001$) between the fixed and the variable methods was observed in the Mix diet treatment at 17 DPF. Indeed, significantly more larvae achieved P6 when reared under variable conditions than under the fixed method (Figure 13).

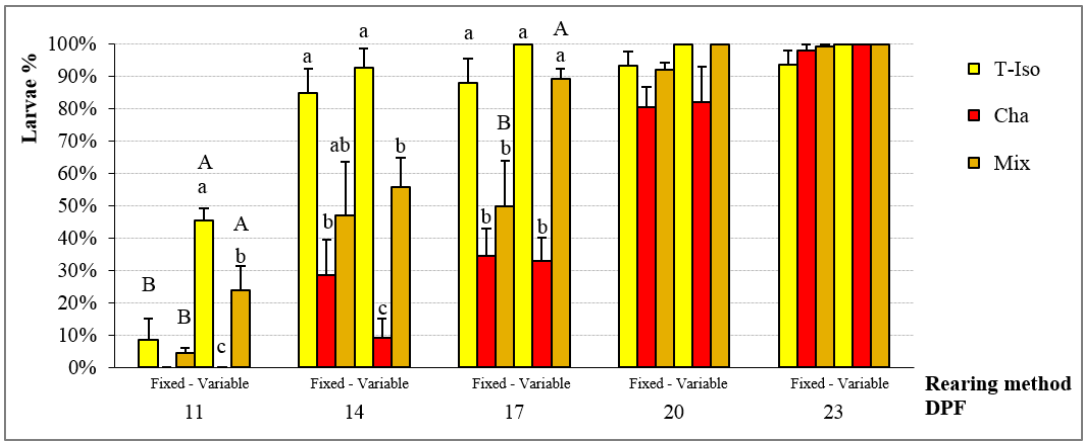

Figure 13: Larval development at the 6-arm stage (P6). Days post fertilization (DPF), *Isochrysis* spp. (T-Iso), *Chaetoceros gracilis* (Cha), mixture of T-Iso and *C. gracilis* (Mix). Capital superscripts indicate significant differences between rearing methods; lowercase superscripts indicate significant differences among diets. Values are expressed as mean ± SE (n=5).

Competence was achieved by all surviving larvae in the variable method by 29 DPF. However, larvae fed the Mix diet achieved competence significantly faster ($p < 0.01$) than those reared under fixed conditions. In the former method, 34.6 ± 10.2% were considered competent to settle at 17 DPF, whilst none of them were ready in the latter. By 20 DPF, 99.0 ± 1.0% and 9.6 ± 8.2% achieved competence in the variable and fixed methods respectively. At 23 DPF, all larvae fed the Mix diet in the variable method achieved competence, whilst only 9.6 ± 8.2% were found to be at this stage in the fixed method. Between 23 and 29 DPF, however, 44.8 ± 22.4% of the larvae fed this diet in the fixed method achieved the competence stage. No significant differences were observed between the two tested rearing methods when larvae were fed the other diets, with the only exception of larvae fed Cha at 20 DPF where, once again, larvae under the variable method outperformed those in the fixed one. Within the variable method, significant differences were nonetheless observed between diets. A faster development occurred in larvae fed with the Mix diet than T-Iso at 20 DPF (99.0 ± 1.0% and 41.0 ± 19.2%, respectively), 23 DPF (100% and 58.2 ± 13.7%, respectively) and 26 DPF (100% and 72.0 ± 10.2% respectively) (Figure 14).

After achieving the competence stage, a delay was observed before competent larvae could undergo metamorphosis. A significant difference was observed in the percentages of successfully metamorphosed larvae for the different dietary treatments and rearing

methods. Indeed, the fixed method did not yield any metamorphosed larvae whilst 35.5 ± 8.4% of larvae successfully metamorphosed under the variable method. Furthermore, higher survival rates resulted for larvae fed with Mix (63.3 ± 8.9%) than with T-Iso (19.7 ± 12.1%) and Cha (23.4 ± 15.1%) treatments ($p < 0.05$) (Figure 15).

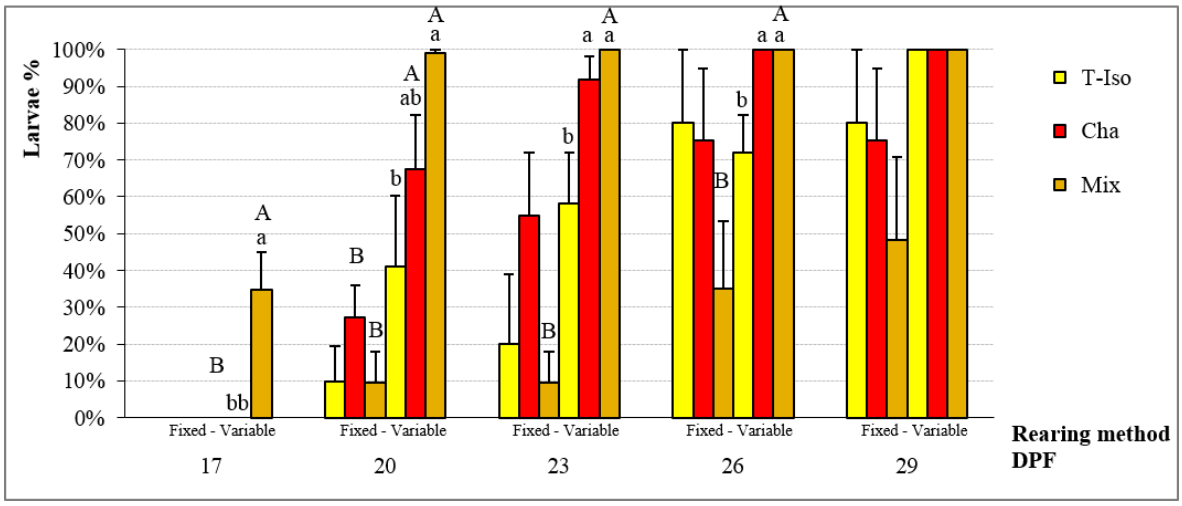

Figure 14: Larval development percentage at the competence stage (Cp). Days post fertilization (DPF), *Isochrysis* spp. (T-Iso), *Chaetoceros gracilis* (Cha), mixture of T-Iso and *C. gracilis* (Mix). Capital superscripts indicate significant differences between rearing methods; lowercase superscripts indicate significant differences among diets. Values are expressed as mean ± SE (n=5).

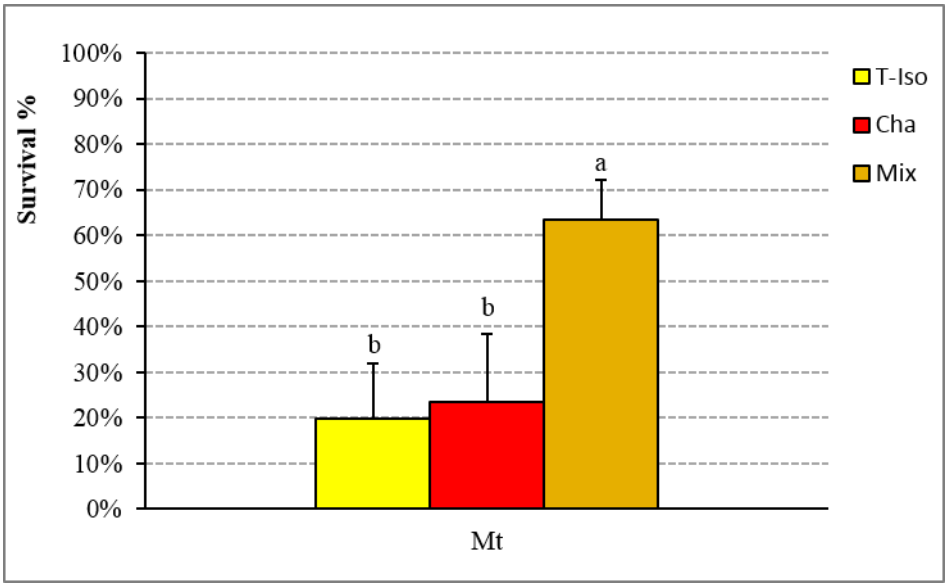

Figure 15: Larval survival at the Metamorphosis stage (Mt). *Isochrysis* spp. (T-Iso), *Chaetoceros gracilis* (Cha), mixture of T-Iso and *C. gracilis* (Mix). Fixed method is not show due to 0% survival. Lowercase superscripts indicate significant differences among diets. Values are expressed as mean ± SE (n=5).

2.5 Discussion

The alternative rearing system tested in this study (variable method) improved survival and development rates in *P. lividus* larvae up to metamorphosis. Indeed, by giving a variable amount of microalgae it was possible to optimize the feed ration, according to larval consumption. Moreover, a visible decrement in the production of debris and settled phytoplankton on the bottom of the tanks was observed, and this led to less maintenance of larval tanks and therefore reduced larval handling and water replacement.

Our results clearly show faster larval development and higher survival with the variable method *versus* the fixed one. More specifically, larvae fed with a variable amount of feed reached competence faster than those reared with the fixed method. Moreover, regardless of the dietary treatment applied, all larvae reared with the variable method achieved the metamorphosis stage. Instead, when a fixed amount of feed was supplied larval development decreased progressively and, regardless of the feed used, no larvae reached metamorphosis. More importantly and regardless of the method employed, a lower larval survival rate at metamorphosis compared to competence, and a delay between competence and successful metamorphosis was observed. This could indicate that the use of the rudiment size, equal or larger than the stomach, may not be the best morphological indicator of larval readiness for settlement, as reported by numerous authors (Carboni et al., 2012; Fenaux et al., 1994).

The variable method involved supplying a feed ration directly proportional to the larval consumption, thereby minimizing the phytoplankton in excess. In traditional static systems, the fixed feed ration causes a high amount of debris and phytoplankton to settle on the bottom of the tanks, so siphoning and water exchange are crucial for maintaining water quality and for achieving successful larval production (Azad et al., 2011; Cárcamo, 2004). Nevertheless, manual water exchange can be counterproductive as it is stressful and can damage the larvae in suspension (Russell, 2000). This was also suggested by Carboni et al. (2014), who obtained better results with a flow-through system (characterized by the absence of manual water exchange) than with a traditional static water exchange system. Nonetheless, the flow-through system described by Carboni et al. (2014) still required significant amount of microalgae as the continuous water exchange applied increased the risk of feed losses. With our proposed variable

method we have also reduced larval handling and associated potential causes for mortality and, at the same time, reduced the requirements for microalgae and water replacement. The survival rate achieved with the variable method regardless of the diets was significantly higher than that of the fixed method. Moreover, the larval survival is comparable with that achieved by other authors that used the same diets and similar initial stocking density and higher than that obtained by previous studies that employed higher stocking densities (Carboni et al., 2014, 2012).

It is possible that this difference is due to the small volumes used in the present experiment. It is known, in fact, that a laboratory scale results in higher survival rates, but these techniques are not always applicable on a large-scale (Carboni et al., 2012; Liu et al., 2007; George et al., 2004; Kelly et al., 2000; Pedrotti and Fenaux, 1993; Fenaux et al., 1985). The highest survival of *P. lividus* larvae fed with phytoplankton were obtained by Pedrotti and Lemée (1999) in 1 L volume tanks, with a 95% survival at 18 days post-fertilization with *Cricosphaera elongata*. Using higher culture volumes, Liu et al. (2007) and Paredes et al. (2015) reported lower survival than Pedrotti and Lemèe (1999); respectively 68-76% in 60 L (*Dunaliella tertiolecta)* and 40% in 100 L (mix of *Tetraselmis suecica*, T-Iso, *C. gracilis*, *Phaeodactylum tricornutum* and *Cylindrotheca closterium*).

In 80 L tanks Carboni et al. (2012) obtained even lower survival rates: 0% with *T. suecica*, ~5% with *D. tertiolecta*, ~6% with *Pleurochrysis carterae* and ~14% with *C. elongata*. These low survival percentages could be due to the high larval stocking densities (4/mL) adopted by Carboni et al. (2012), as that investigation focused on the application of the *P. lividus* echinoculture on a commercial-scale. Conversely, work by Paredes et al. (2015), Liu et al. (2007) and Pedrotti and Lemèe (1999) used lower densities: 2, 1.5 and 1 larva/mL, respectively. In laboratory experiments, low densities improve larval survival. However, the use of higher stocking densities in commercial settings offsets the lower larval survival at the competence stage by increasing the final output of competent larvae.

In this study, larval development and survival were also influenced by diets. *C. gracilis* resulted in a slower development up to the P6 than T-Iso. According to previous studies, the *I. galbana* diet is generally considered inferior to other microalgae for echinoderm larval rearing (Schiopuet al., 2006; Pechenik, 1987) as its use resulted in poorer growth

and survival for *P. lividus* larvae (Fenaux et al., 1988) and other marine invertebrates such as *Crassostrea gigas*, *Venerupis philippinarum* and *Pecten maximus* (Marshall et al., 2010). At the end of the rearing cycle, the larvae fed with the Mix diet presented the fastest development and the highest survival at competence when the variable methodology was employed. Indeed, the fact that invertebrate larvae grow better on a mixture of phytoplankton species is established knowledge (Pedrotti and Fenaux, 1993), as the lack of biomolecules supplied by one algae species could be provided by the other species in the mix (Schiopu et al., 2006; Pechenik, 1987; Strathmann, 1971).

The effects of feeding rations on sea urchin larval development and survival are well documented in the literature. Indeed, supplying an appropriate feed ration promotes growth rate, enhances the size of the larvae and the post-metamorphic survival in different sea urchin species, *P. lividus* (Vaïtilingon et al., 2001), *Loxechinus albus* (Cárcamo et al., 2005), *Dendraster excentricus* (Hart and Strathmann, 1994), *P. miliaris* (Kelly *et al*. 2000), *Strongylocentrotus purpuratus* (Azad et al., 2011; Miller and Emlet, 1999) and *S. droebachiensis* (Meidel et al., 1999). By using the variable method, this study describes a protocol to further optimize feed rations for optimal larval development and survival. As observed by Azad et al. (2011), the larvae of *S. purpuratus* displayed a typical development and the highest survival percentage when fed with a standardized ration (1500-4000 cells/mL, increasing according to larval developmental stage and stocking density). A low ration (500 cells/mL), on the other hand, had negative effects on larval development (failure to metamorphosis) and survival. Nonetheless, high feed levels may also have detrimental effects on morphometric larval development and survival. In their study, Azad et al. (2011) also observed that larval development and survival of *S. purpuratus* differed according to the microalgal diets used, although their results showed slower development and survival when compared with the results described in this report. Moreover, Jimmy et al. (2003) analysed the morphology, the development and the metamorphic rate of *Echinus esculentus* larvae fed with a standard ration (1000, 3000, and 5000 cells/mL according to the developmental stage) and high ration (3000, 9000, and 15000 cells/mL) of *D. tertiolecta*. Indeed, a standard ration promoted larval growth (length, width and rudiment length), a shorter development time (from 21-23 to 16 days), a higher number of larvae metamorphosed and larger juveniles at 5 months post-settlement. Similar results have been reported for *P. miliaris* larvae (Kelly et al., 2000).

In our work, different amounts of microalgae species were supplied in the tanks. The tanks fed with T-Iso needed more constant and larger amounts of feed than the other tanks, due to their higher cell consumption. On the contrary, larvae fed with Cha were supplied with microalgae only when the larvae achieved the echinopluteus stage (2 DPF) as a high amount of unconsumed *C. gracilis* was recorded during the whole rearing cycle. In the tanks fed with the Mix diet, a higher amount of *C. gracilis* was consistently observed along with the almost total absence of T-Iso. The different microalgae species consumption observed in this study was probably due to cell dimension, especially during the initial developmental stages. Two pairs of arms echinoplutei consume phytoplankton cells with a volume of 200-400 μm^3 (Carboni, 2013). The lack of a cell wall and the small dimension (5 ± 1 μm in diameter) make *Isochrysis* spp. readily digestible by small larval invertebrates (Cordoba-Matson et al., 2013), while *Chaetoceros* spp. is larger than *Isochrys* spp., with a diameter of 7.3 ± 0.8 μm (Herawati et al., 2013), and may therefore be less preferable as food if an alternative microalgae species is also offered. This observation highlights the complexity of feed choices for different larval stages, and suggests that the nutritional profile of a given microalgae species does not always dictate larval preferences.

Our results demonstrate for the first time that a fixed method characterized by continuous seawater replacement may not be essential for the production of *P. lividus* larvae if there is constant monitoring and replacement of the consumed microalgae cells. Variable amounts of feed improved larval survival and development, avoiding the need for water exchanges and minimizing phytoplankton requirements for the hatcheries. This variable method requires more technical labour and expertise in order to make assessment every three days regarding the amount of microalgae to be fed to the larvae, but it has the potential to reduce manual labour, water volumes and phytoplankton requirements in a sea urchin hatchery.

The survival results obtained in this study could be further improved by applying other microalgae diets, which have been demonstrated to promote higher survival and development of *P. lividus* larvae, such as *C. elongata* and *P. carterae*. Nevertheless, further studies would be needed to test the variable method at greater volumes and at a higher stocking density of larvae, in order to verify the applicability of this method to

larger scale production systems and for other commercially important marine invertebrate species.

2.6 Acknowledgments

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3 EFFECTS OF LARVAL DIET AND METAMORPHOSIS CUE ON SURVIVAL AND GROWTH OF SEA URCHIN POST-LARVAE (*PARACENTROTUS LIVIDUS*; LAMARCK, 1816)

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Keywords:

Sea urchin, *Paracentrotus lividus*, Settlement, Post-larvae, Survival.

Contribution:

The author designed and conducted the trial, collected the samples, carried out all laboratory and statistical analysis and wrote the manuscript.

3.1 Abstract

In this study, we present the results of two experiments; in the first one, we evaluated the effects of four larval dietary treatments on the survival and growth of the sea urchin *Paracentrotus lividus*, larvae and post-larvae. In the second experiment, we have measured the effects of two different settlement substrates, combined with the presence of conspecifics, on metamorphosis, survival and growth of post-larvae. The microalgae dietary treatments consisted in: *Dunaliella tertiolecta* (Duna); 50% mixture of *Isochrysis galbana* and *D. tertiolecta* (ID); 50% mixture of *Chaetoceros gracilis* and *D. tertiolecta* (CD); 33% mixture of *I. galbana*, *C. gracilis* and *D. tertiolecta* (ICD). Although all dietary treatments resulted in a good survival at competence, significant difference in postlarval survival was observed between treatments, and indeed, only larvae fed Duna and CD survived to 180 days post settlement (DPS).

In the second experiment, the settlement substrates consisted in a film of cultured *Ulvella lens* or a naturally developing biofilm of diatoms, and the employed rearing water was either natural seawater or seawater previously exposed to *P. lividus* adults. At 10 DPS, larger ($p < 0.05$) post-larvae were observed in the natural biofilm treatment, whilst the presence of conspecifics significantly increased larval settlement in both substrates ($p < 0.01$). These results indicate that it is important to consider the survival of post-larvae and juveniles to establish the efficiency of the dietary treatment on the hatchery production of *P. lividus*. Furthermore, it suggests that improved settlement protocols, such as the use of conspecifics, could contribute to increase hatchery outputs. Finally, it confirms the suitability of *U. lens* as settlement cue but also highlights that further research is required to establish its effectiveness for post-larvae first feeding.

3.2 Introduction

Paracentrotus lividus (Lamarck, 1816) is the most consumed sea urchin species in Europe (Carboni et al., 2012). Due to the high market demand for its gonads, natural populations are exposed to overfishing in many Mediterranean and non-Mediterranean coastal areas (Pais et al., 2007), causing a sharp decline of the stock (Boudouresque and Verlaque, 2007; Pais et al., 2007; Addis et al., 2009).

This decrease is driving the development of echinoculture methods that started with *Pseudocentrotus depressus* by Yamabe (1962). These culture methodologies could represent a solution to limit the damages caused by wild stock overfishing and to protect natural populations (Mos et al., 2011; Carboni, 2013).

In the culture of sea urchins the transition from planktonic larvae to benthic juveniles represents a critical phase. Indeed, laboratory experiments report variable larval settlement and metamorphosis rates from 0 to 90% (Huggett et al., 2006; Buitrago et al., 2005; Rahim et al., 2004; Grosjean et al., 1998; Gosselin and Jangoux, 1996; Pearce and Scheibling, 1991) with post-settlement periods characterized by mortality rates higher than 90% within the first weeks of *P. lividus* benthic life (Buitrago et al., 2005; Rahim et al., 2004; Grosjean et al., 1998; Shimabukuro, 1991). The echinoculture production could therefore be increased by improving settlement rates and post-larvae survival, which currently represent the main bottleneck limiting this activity (Mos et al., 2011).

Several studies focused on microalgae diets and feed ration (Carboni et al., 2012; Azad et al., 2011; Liu et al., 2007; Cárcamo et al., 2005; Kelly et al., 2000; Pedrotti and Fenaux, 1993) and have identified several microalgae species, such as *Dunaliella tertiolecta*, which supports the rearing cycle and improve the larval survival and development of *P. lividus* (Carboni et al., 2012; Liu et al., 2007) and other echinoid species (Azad et al., 2011; George et al., 2004; Sewell et al., 2004; Kelly et al., 2000; Pearce and Scheibling, 1994, 1991, 1990a; Hart and Scheibling, 1988; Hinegardner, 1969). Many authors reported that *D. tertiolecta* is capable of producing healthy larvae because it is easily ingested (i.e. it has an appropriate cell size) and it is quickly digested (Basch, 1996; Cameron and Hinegardner, 1974; Strathmann, 1971). Moreover, it has an appropriate fatty acid profile for the larval growth (Carboni et al., 2012).

Although the effects of dietary treatments on *P. lividus* larval survival and development have been investigated, and it is well known that larval diet and feed ration influence survival and test diameter of various species of sea urchin post-larvae (Liu et al., 2007; Jimmy et al., 2003; Kelly et al., 2000; Meidel et al., 1999; Hart and Strathmann, 1994). However, their effects on determining the survival of juveniles has received little attention. Indeed, few studies focused on the survival and test diameter of various species of sea urchin post-larvae, but these investigations has been carried out just within 10 days post-settlement (Liu et al., 2007; Kelly et al., 2000; Meidel et al., 1999; Hart and Strathmann, 1994). Only Jimmy et al. (2003) evaluated the influence of three microalgal diets on the test diameter of *Echinus esculentus* at 6 months postsettlement.

In aquaculture settings, the transition from planktonic to benthic period is typically promoted by plates colonized with diatoms, which are believed to provide a good settlement cue and represent the initial feed for the juveniles (Cárcamo et al., 2005; McBride, 2005; Harris et al., 2003; Shimabukuro, 1991). However, laboratory experiments demonstrate that the settlement of sea urchin larvae is improved by a wide range of cues, among which the presence of conspecifics (Mos et al., 2011; Dworjanyn and Pirozzi, 2008). Indeed, Dworjanyn and Pirozzi (2008) reported for the first time that the sea urchin *Tripneustes gratilla* preferentially settled in response to the presence of conspecifics and seawater previously exposed to conspecifics and their faeces.

Recently, plates colonized by the green macroalgae *Ulvella lens* have been shown to improve larval settlement and represent the initial feed for juveniles sea urchin (Hannon et al., 2015, 2014; Takahashi et al., 2002) and sea cucumber (Matsuura et al., 2009). However, it has been recognized that some sea urchin species such as *S. intermedius* prefers to feed on diatoms rather than *U. lens* (Kawamura et al., 1983).

In the present study, we reported results of two experiments; in the first one, we evaluated the effects of four phytoplankton diets on survival of *P. lividus* larvae at competence and post-larvae at different days post-settlement. In the second experiment, we compared two different settlement substrates, *U. lens* or a natural biofilm, and, although this topic has been investigated in other sea urchin species, such as *T. gratilla* (Mos et al., 2011; Dworjanyn and Pirozzi, 2008), we evaluated for the first time the effects of the presence of conspecifics on larval settlement and post-larval survival and growth of the sea urchin *P. lividus*.

3.3 Materials and methods

For both experiments, embryos of *P. lividus* were produced in the International Marine Centre - IMC laboratory (Oristano, Sardinia, Italy) from adult sea urchins (diameter larger than 45 mm) following published methods (Liu et al., 2007). Broodstock was collected from 5 m depth at the "Penisola del Sinis-Isola di Mal di Ventre" Marine Protected Area (39°89′N8°41′W). Ten specimens (5male, 5 female) were used for the gametes production.

The presence of the fertilization membrane was used to verify the fertilization rate, observed by using a tubular plankton chamber and a Leica DMRB Microscope (100× enlargement) (Azad et al., 2011; Grosjean et al., 1998; Liu et al., 2007). Embryos were stocked at a density of 20/mL until they reached the echinopluteus stage, about 40 h after fertilization took place. Subsequently, the echinoplutei were stocked at density 1.5/mL into 5 L cylindrical white plastic tanks. Cultures were constantly kept in motion by motor-driven rotation. Both embryos and echinopluteus were reared in filtered (0.47 μm) natural seawater (NSW), with a salinity of 36.5 ± 1.0 ppt, without aeration, in continuous light at 31 μmol photons/m2/s and at a temperature of 19.0 ± 2.0 °C.

3.3.1 Experiment 1: effect of microalgae diets on larvae and juveniles, development, growth and survival

Four microalgae diets were tested during larval rearing: a single species diet of *D. tertiolecta* (Duna), a two species mixed diet (50% number of cells) of *Isochrysis* aff. *galbana* (T-Iso) and *D. tertiolecta* (ID), a two species mixed diet (50%) of *Chaetoceros gracilis* and *D. tertiolecta* (CD), a three species mixed diet (33%) of T-Iso, *C. gracilis* and *D. tertiolecta* (ICD). Although the three phytoplankton species tested in this study have a different cell size (T-Iso 40–50 μm^3, *C. gracilis* 80 μm^3, *D. tertiolecta* 170 μm^3, FAO, 2004) and dry weight (T-Iso 29.7 pg/cell, *C. gracilis* 74.8 pg/cell, *D. tertiolecta* pg/cell, FAO, 1996), we administered an equal number of microalgae cells to the larvae. Adopting the rearing method tested by Brundu et al. (2016), every three days we restored the amount of phytoplankton consumed by the larvae, guaranteeing constant ad-libitum feeding.

Phytoplankton cultures were maintained in batch lines at 25 °C, exposed to a 16/8 h (L/D) photoperiod at 63 μmol photons/m^2/s and supplied with gentle aeration. The 30 ppt salinity seawater was pre-filtered (1 μm filter paper), enriched with modified Guillard f/2 and autoclaved at 121 °C for 30 min.

The larvae were fed with microalgae cultures in their exponential growth phase, T-Iso 3.7 ± 0.2 million cells/mL, *C. gracilis* 3.3 ± 0.2 million cells/mL, *D. tertiolecta* 4 ± 0.4 million cells/mL. Larvae were reared in a total of 20 tanks, 5 replicates for each dietary treatment. Larval development was assessed every three days by observation of larval structures (number of arms, presence and size of the rudiment), according to previous studies (Carboni et al., 2012; Liu et al., 2007). For these purposes, a minimum of 10 randomly sampled larvae from each replicate were placed in a tubular plankton chamber and they were observed under a Leica MZ8 Stereomicroscope (15× enlargement). Competence of the culture was considered achieved when at least 75% of the sampled larvae were considered to be at this stage.

Larval survival was assessed volumetrically in each replicate and the mean value of each measurement was used to calculate the number of larvae in the tanks. Survival was expressed as percentage of the initial number of larvae stocked.

Metamorphosis tests were conducted when larvae reached competence for settlement. 50 larvae, from each replicate of each treatment, were transferred into shading beakers containing 50 mL of filtered NSW and a 50 × 50 mm polycarbonate layer colonized by the macroalgae *U. lens*, according to the methods described in Daume et al. (2004). The number of larvae undergoing metamorphosis was counted after 24, 48, and 72 h.When at least 75% of the larvae were metamorphosed, the entire larval culture was considered ready to settle and was transferred to 20 rectangular tanks with a volume of 20 L, maintaining the same experimental design adopted for the larval rearing (five replicates by four dietary treatments). Each tank contained NSW and a 20 × 18 cm polycarbonate layer colonized by the macroalgae *U. lens*. The animals were kept in a seawater static system for a month, with 36.5 ± 1.0 ppt salinity, without aeration and a 50% seawater exchange was performed twice per week. Recorded temperature was 19.0 ± 2.0 °C during the trial period and 14 h light photoperiod at 22 μmol photons/m^2/s was applied using fluorescent lamps. After the one month, the tanks were connected to a recirculating system, provided with biological and mechanical filtration (10 μm).

The number of post-larvae in each replicate was recorded at 10, 20, 30, 100 and 180 days post-settlement (DPS) and survival rate was calculated as percentage of the initially stocked competent larvae. Moreover, at 180 DPS, 200 randomly sampled juveniles were placed on a water proof graph paper and photographed with a Canon PowerShot G15 digital camera. Image Processing Analysis in Java (ImageJ 1.49V) was calibrated appropriately for image analysis and measurement. A length of 1 mm was measured via a ruler, saved and then used as the standard for individual growth measurement, in terms of test diameter; the widest part of the sea urchin body was measured. To simplify these operations, a solution of KCl 1% was used to induce paralysis and detachment of juveniles from the tanks, as tested by Hagen (2003) for *S. droebachiensis*.

3.3.2 Experiment 2: effects of substrates and presence of conspecifics on larval settlement and post-larvae survival and growth

In a second experiment, we tested the effectiveness of two substrates on the induction of metamorphosis in competent larvae. Larvae were exposed to polycarbonate plastic layers coated with either *U. lens* or a natural biofilm. *U. lens* was cultivated in laboratory under controlled conditions according to the method described by Daume et al. (2004). Natural biofilm, instead, was naturally cultivated submerging the layers in tanks containing estuarine water for 30 days (Cárcamo, 2004). Natural biofilmis widely employed as larval settlement substrate for various species of sea urchins (Mos et al., 2011) and other invertebrates (Daume et al., 2004; Knauer et al., 1996; Searcy-Bernal et al., 1992; Leighton, 1989).

Furthermore, we tested the effects of the presence/absence of conspecifics on metamorphosis, post-settlement survival and growth of settled juveniles. This trial, therefore, consisted of four treatments: *U. lens*+conspecifics; *U. lens*; natural biofilm+conspecifics; natural biofilm. A total of 28 tanks (7 replicates by four treatments) were employed for this experiment.

Larvae used for this experiment were reared as previously described in experiment 1 and fed with a 50% mixture of *C. gracilis* and *D. tertiolecta*. Once competence was

achieved metamorphosis test was conducted as described above and larvae were then randomly distributed in equal concentration (1 larvae/mL) between the four treatments.

Importantly, larvae were never in direct physical contact with adult conspecifics but, instead, the water used for the two conspecific treatments was previously passed through a 20 L tank hosting 5 wild-harvest *P. lividus* adults (diameter larger than 45 mm) and then used for the trial. Conspecifics were starved and hosted for a week in previously filtered (1 μm) NSW, in static condition and with gentle aeration.

The number of larvae undergoing metamorphosis was counted after 24, 48, and 72 h. Survival was assessed at 10 DPS by observing the movement of spines and tube feet of the settled individuals under a Leica MZ8 Stereomicroscope (60× enlargement); growth was assessed at 10 DPS by measuring the individuals' test diameter with a Leica DMC2900 digital camera connected to a Leica MZ8 Stereomicroscope (30× enlargement) and using an image analysis software (Leica Application Suite LAS V4.5).

3.3.3 Statistical analysis

Data were analyzed by Statistica 6.1 StatSoft, Inc. (2004). The normality and homogeneity of the data distribution were assessed with Shapiro Wilk's W and Levene's tests, respectively. Where required, data on larval development and survival were analyzed by the nonparametric Kruskal-Wallis test; otherwise, one-way analysis of variance (ANOVA) was employed. Post-larvae survival was analyzed using repeated-measures ANOVAwith survival as a factor and DPS as a repeated factor. Tukey's honestly-significant difference (HSD) test was used to evaluate all pair-wise treatment comparisons ($p < 0.05$). For comparison of the juvenile growth, a size-class distribution was constructed. Size-classes were determined as 1 mm increments in diameter; Kolmogorov-Smirnov test was used to compare size-class distributions and to test the prediction that all size-classes occurred in similar proportions among dietary treatments.

Metamorphosis percentage was analyzed using repeated-measures ANOVA with inducing factor and seawater as factors and time as a repeated factor, while the effects on post-larvae survival and test diameter were assessed by a two-way ANOVA. Tukey's

honestly-significant difference (HSD) test was used to evaluate all pair-wise treatment comparisons ($p < 0.05$).

3.4 Results

3.4.1 Experiment 1: effect of microalgae diets on larvae and juveniles, development, growth and survival

Larval development was significantly influenced by microalgae diets. ICD resulted in a faster ($p < 0.01$) development than all other diets, and at 10 days post-fertilization (DPF) 79.9 ± 7.8% of the larvae fed ICD achieved the competent stage, whilst only 7.9 ± 3.4%, 6.1 ± 3.7% and 30.1 ± 13.4% of the larvae fed Duna, ID and CD, respectively, achieved this stage. Nonetheless, larvae in all treatments reached the competence by 22 DPF and no significant difference were observed after 10 DPF (Table 4).

Table 4: *Paracentrotus lividus* larvae (%) at competence stage (Cp). Days post fertilization (DPF), *Dunaliella tertiolecta* (Duna), mixture of T-Iso and *D. tertiolecta* (ID), mixture of *Chaetoceros gracilis* and *D. tertiolecta* (CD), mixture of T-Iso, *C. gracilis* and *D. tertiolecta* (ICD). The different letters indicate significant differences among diets (= $p < 0.01$). Values are expressed as mean ± SE (n= 5).**

Diet	10 DPF**	13 DPF	16 DPF	19 DPF	22 DPF
Duna	7.9 ± 3.4% [b]	88.3 ± 8.3%	90.3 ± 6.3%	93.9 ± 4.0%	100%
ID	6.1 ± 3.7% [b]	62.8 ± 18.1%	70.9 ± 17.5%	71.7 ± 16.4%	78.8 ± 16.2%
CD	30.1 ± 13.4% [b]	95.3 ± 4.7%	98.1 ± 1.9%	100%	100%
ICD	79.9 ± 7.8% [a]	88.8 ± 3.2%	97.0 ± 3.0%	100%	100%

No significant differences between treatments were recorded in larval survival up until the competent stage (Table 5). However, larvae fed the ICD diet presented a significantly lower ($p < 0.001$) larval survival at metamorphosis (4.2 ± 3.5%) than other treatments, Duna (65.0 ± 14.9%), ID (61.8 ± 16.5%) and CD (78.4 ± 10.2%) (Table 5).

All post-larvae in ICD treatment died before 10 days post settlement. Repeated-measures ANOVA showed a gradual decreasing of post-settlement survival from 10 to

100 DPS ($p < 0.001$) in all other treatments with no significant difference between treatments. Post-settlers resulting from the ID treatment did not survive past 100 DPS; at the end of the experiment, 180 DPS, a survival of 1.5 ± 1.5% and 3.0 ± 2.0%, respectively for Duna and CD, was recorded (Figure 16).

Table 5: Survival (%) of *Paracentrotus lividus* larvae at competence and metamorphosis. *Dunaliella tertiolecta* (Duna), mixture of T-Iso and *D. tertiolecta* (ID), mixture of *Chaetoceros gracilis* and *D. tertiolecta* (CD), mixture of T-Iso, *C. gracilis* and *D. tertiolecta* (ICD). The different letters indicate significant differences among diets (* = $p < 0.001$). Values are expressed as mean ± SE (n= 5).**

Diet	Competence (Cp)	Metamorphosis (Mt)
Duna	82.8 ± 10.6%	65.0 ± 14.9% [a]
ID	82.0 ± 14.8%	61.8 ± 16.5% [a]
CD	84.6 ± 9.9%	78.4 ± 10.2% [a]
ICD	72.8 ± 10.6%	4.2 ± 3.5% [b]

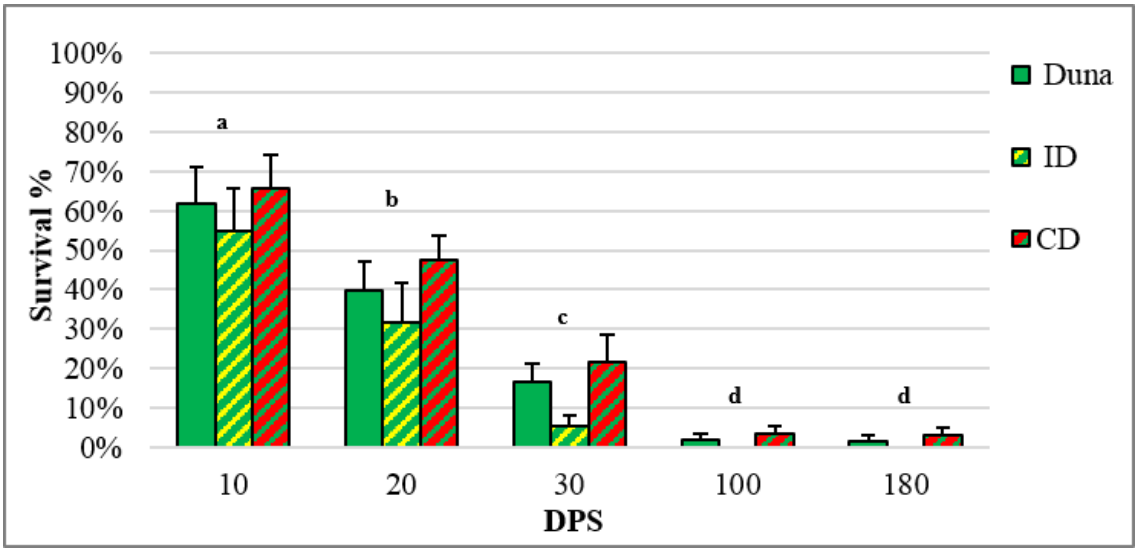

Figure 16: Survival rate of *Paracentrotus lividus* post-larvae at 10, 20, 30, 100 and 180 days post-settlement (DPS). *Dunaliella tertiolecta* (Duna), mixture of T-Iso and *D. tertiolecta* (ID), mixture of *Chaetoceros gracilis* and *D. tertiolecta* (CD). Superscripts indicate significant differences among DPS ($p < 0.001$). Values are expressed as mean ± SE (n=5).

At 180 DPS, survivors from both, Duna and CD treatments, had a test diameter from 1.1 mm to 10+ mm, but no significant differences were highlighted by Kolmogorov-

Smirnov test on the size-classes distribution, as well as by ANOVA on the mean test diameter, 6.4 ± 0.2 mm and 6.6 ± 0.2 mm for Duna and CD treatment, respectively (Figure 17).

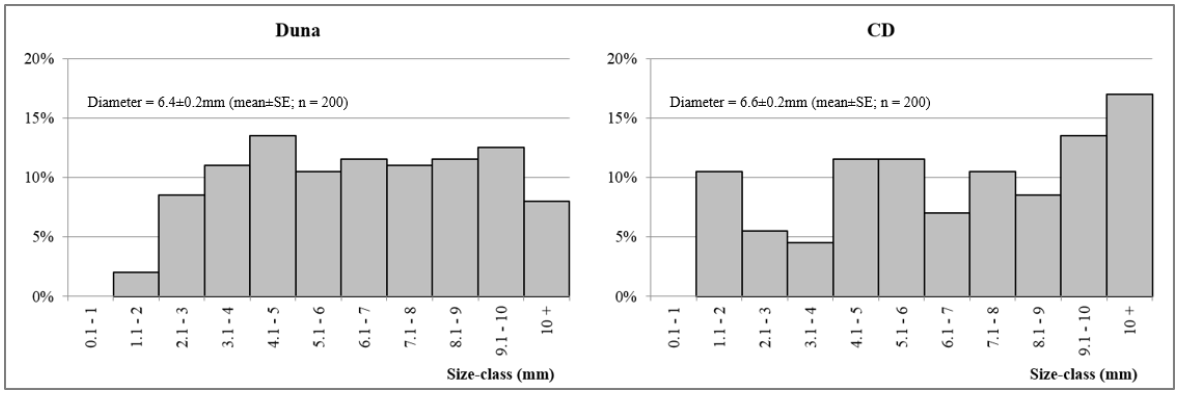

Figure 17: Size-class distributions (diameter in 1 mm intervals) of *Paracentrotus lividus* post-larvae resulting from the Duna and CD treatments, at 180 days post-settlement (DPS).

3.4.2 Experiment 2: effects of substrates and presence of conspecifics on larval settlement and post-larvae survival and growth

Metamorphosis was significantly influenced by the presence of conspecifics. Indeed, individuals exposed to seawater treated with conspecifics showed a significantly higher metamorphosis rate throughout the observation period ($p < 0.01$), whilst no significant differences were recorded between substrates (Figure 18).

At 10 DPS survival rate did not differ between treatments. Significant differences ($p < 0.05$) in the juveniles' test diameter, however, were observed between substrates and a significantly larger test diameter was, in fact, measured for juveniles feeding on natural biofilm (Figure 19).

3.5 Discussion

3.5.1 Experiment 1: effect of microalgae diets on larvae and juveniles, development, growth and survival

All dietary treatments tested in this study resulted in a better larval development and survival at competence, in comparison with monospecific diets of T-Iso (competence at

29 DPF and 88.5 ± 4.4% survival) and *C. gracilis* (competence at 23 DPF and 43.8 ± 10.1% survival), and a 50% mixture of the same species (competence at 20DPF and 73.7 ± 8.7% survival) previously tested (Brundu et al., 2016). The survival rate at competence obtained in this study by using *D. tertiolecta* (82.8 ± 10.6%) is higher than previously reported in the literature (Carboni et al., 2012; Azad et al., 2011; Liu et al., 2007; George et al., 2004; Jimmy et al., 2003; Kelly et al., 2000), suggesting that this species can indeed support larval development of *P. lividus*.

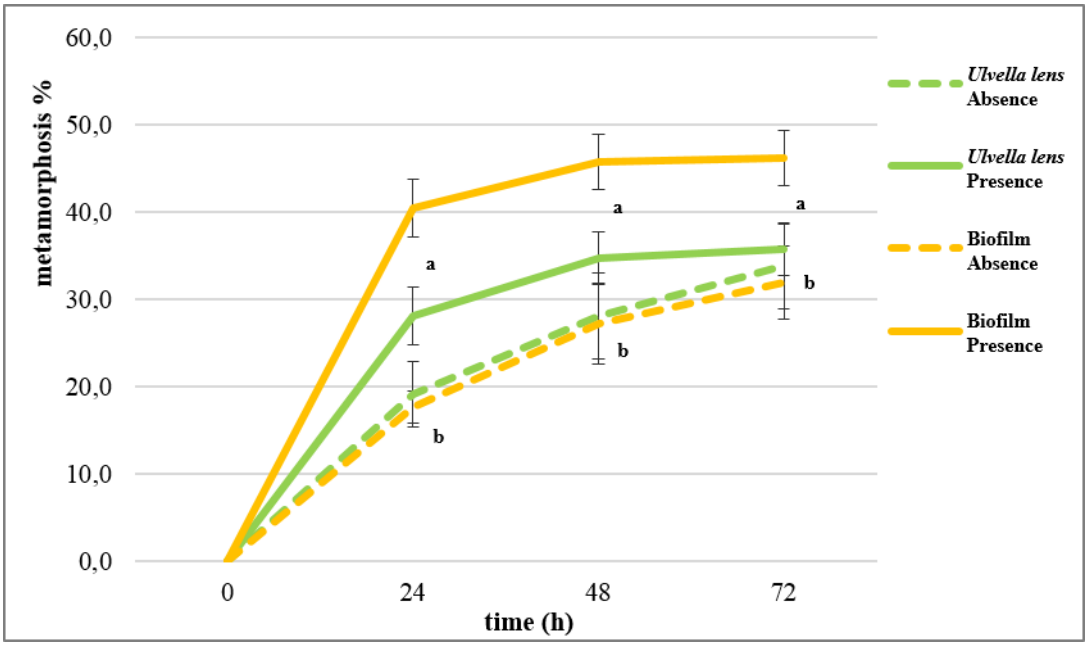

Figure 18: Metamorphosis rate of competent larvae of *Paracentrotus lividus* exposed to two substrate types, *Ulvella lens* and natural biofilm, and to the presence or absence of adult conspecifics. Superscripts indicate significant differences between substrates ($p < 0.01$). Values are expressed as mean ± SE (n= 7).

Liu et al. (2007) obtained a 65% survival and a normal development of *P. lividus* larvae fed with *D. tertiolecta*; moreover, a greater final body width and a faster development resulted with this species (settlement by day 18 post-fertilization) than microencapsulated formulated feeds (settlement by day 20 post-fertilization). Normal development of larvae fed *D. tertiolecta* was also obtained by Carboni et al. (2012), even though with a much lower survival rate. The difference between this and previous studies could be explained by the smaller tank volumes and lower larval densities

adopted. Nonetheless, our results on the larval survival of *P. lividus* are comparable with those obtained for other sea urchin species fed *D. tertiolecta* monospecific diet.

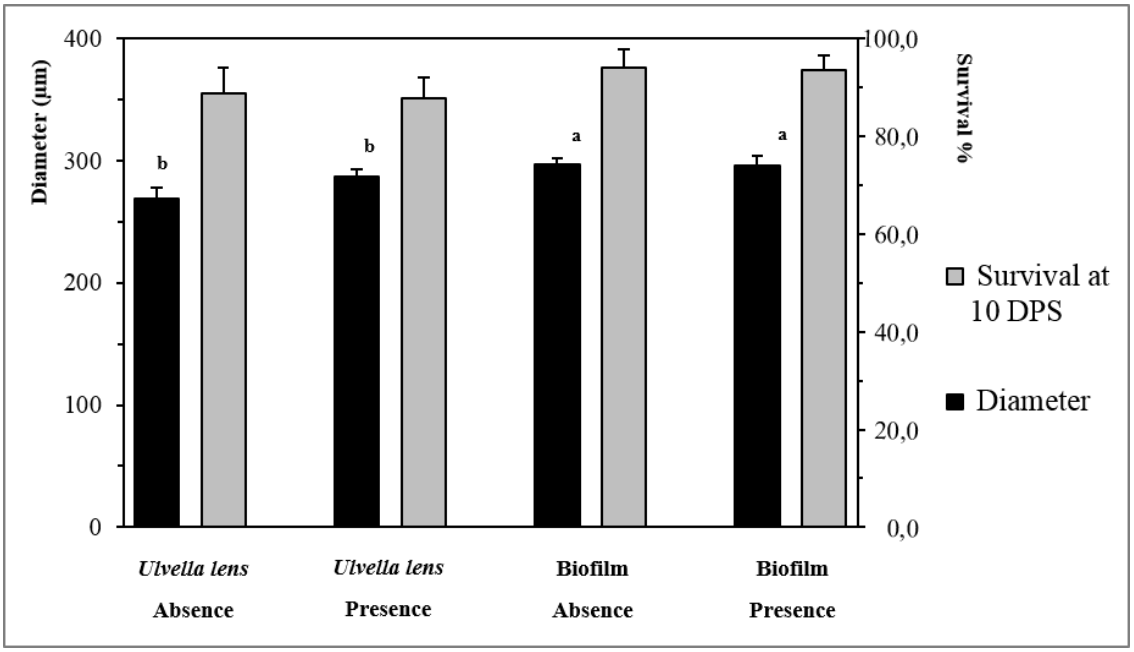

Figure 19: Test diameter and survival of *Paracentrotus lividus* post-larvae at 10 days post-settlement. Superscripts indicate significant differences between treatments ($p < 0.05$). Values are expressed as mean ± SE (diameter, n = 28; survival, n= 7).

As previously reported by Brundu et al. (2016), a delay between competence and metamorphosis completion was also observed in this study, potentially causing the unexpectedly mortality observed in larvae fed ICD. It is well known that mixed-species algal diets provide a more balanced nutrient profile than a single-algal diet (Azad et al., 2011; Pechenik, 1987) and they promote better results than single algal diets on the growth of echinoderm larvae (Azad et al., 2011; Cárcamo et al., 2005; Basch, 1996; González et al., 1987) and other marine invertebrates (Marshall et al., 2010). On the contrary, other studies observed allelopathy processes among phytoplankton species, which refers to the release of secondary metabolites by plants, microorganisms, viruses and fungi, which could result in competition among organisms (Gross, 2003). It has been observed, for instance, that *Prymnesium parvum* and *Alexandrium* spp. influence negatively the growth rate of other microalgae species, causing lysis of algal cells, and they can change the natural community structure (Fistarol et al., 2004; Fistarol et al.,

2003). Nonetheless, this phenomenon has never been described for the microalgae species used in this study which are, instead, commonly used in combination for aquaculture operations.

The lower larval survival rate of larvae fed the ICD treatment was also reflected in the population crash at 10 DPS, when none of the individuals survived. ICD tertiary combination, therefore, can be considered as an appropriate diet during the larval development stages up to the competence, but it failed to support survival during settlement and post-settlement stages. On the contrary, larvae fed Duna and CD showed significantly higher survival rates during early development and postmetamorphosis. The survival rates at 10 DPS of post-larvae treated with *D. tertiolecta* alone and mixed with *C. gracilis* are similar to the values reported in the literature for *P. lividus* (Liu et al., 2007) and other sea urchin species (Jimmy et al., 2003; Kelly et al., 2000). The observed differences between dietary treatments are probably due to a different proximal composition of the phytoplankton, as well as to the different cell size. It has been previously reported that *D. tertiolecta* is capable of producing healthy larvae because it has an appropriate cell size (Basch, 1996; Cameron and Hinegardner, 1974; Strathmann, 1971) and fatty acid profile for the larval growth of *P. lividus* (Carboni et al., 2012). On the contrary, T-Iso and *C. gracilis* have a smaller cell size and dry weight in comparison with *D. tertiolecta* (FAO, 2004, 1996), and contain a lower amount of protein, carbohydrate and lipid (FAO, 1996).

Larvae fed Duna, ID and CD showed a gradual decrease in the survival of post-larvae from10 DPS onwards; ID failed to survive past 100 DPS, while Duna and CD showed a similar survival at 100 and 180 DPS, as well as in the test diameter at the end of the experiment. This suggests that larval dietary treatment influences the survival for the first part of post-metamorphic life, but does not influence the growth of juveniles, as observed for *E. esculentus* by Jimmy et al. (2003). Moreover, the average test diameters at 180 DPS reported by this author, 6.83 ± 1.6 mm with *D. tertiolecta* and 6.55 ± 1.9 mm with a mixture of *D. tertiolecta* and *P. tricornutum*, are similar to our results, 6.4 ± 0.2 mm and 6.6 ± 0.2 mm with Duna and CD, respectively.

The growth of juveniles at 180 DPS was very heterogeneous regardless to the treatment; the large variation recorded in test diameter (from 1.1 mm to 10+ mm) was probably due to competition between individuals of different sizes, as previously observed in

other studies (McCarron et al., 2009; Grosjean et al., 1996; Guillou and Michel, 1993; Ebert, 1973).

In a population of 133 juveniles of *L. albus* (76 days old), González et al. (1987) recorded a size ranging between 0.5 and 2.7 mm. In another experiment, the same authors compared two populations (62 days old) treated with *D. tertiolecta* and a mixture of *D. tertiolecta* and T-Iso, obtaining a larger size-class distribution for the population fed the mixture than *D. tertiolecta*.

3.5.2 Experiment 2: effects of substrates and presence of conspecifics on larval settlement and post-larvae survival and growth

Echinoderm species, such as sea cucumber *Psolus chitonoides* (Young and Chia, 1982) and sand dollars *D. excentricus* (Burke, 1984) and *Echinarachnius parma* (Pearce and Scheibling, 1990b) are known to settle in response to conspecifics, as well as other marine invertebrate larvae (Hadfield and Paul, 2001; Pawlik, 1992; Crisp, 1974). Among echinoids, only *T. gratilla* was observed to settle in response to the presence of adults and particularly of their faeces, maybe because of a conspecific bacteria mediated signal (Mos et al., 2011; Dworjanyn and Pirozzi, 2008). Our results show, for the first time, that settlement of *P. lividus*, regardless of the employed substrate, is improved by the presence of adult conspecifics. The underlying mechanisms (e.g. bacteria, chemical signals) for the improved metamorphosis rate are however unclear.

Although it has been shown that *U. lens* improves larval settlement of *P. lividus* (Hannon et al., 2015, 2014) and abalones (Daume et al., 2000; Krisinich et al., 2000; Takahashi and Koganezawa, 1988), our study did not confirm that *U. lens* is better than natural biofilm as metamorphosis inducing factor. Before themetamorphosis tests, the polycarbonate plastic layers coated by *U. lens* were carefully washed and wiped clean to remove any unwanted biofilm, which could play an important role for the settlement cue, as previously hypothesized by Daume et al. (2004). In this way, we excluded a potential effect on metamorphosis of biofilm developed on the *U. lens* substrate.

Hannon et al. (2015) reported a higher larval settlement of *P. lividus* on *U. lens* than *Amphora* spp., a commonly used diatom for abalones settlement (Gordon et al., 2006; Daume et al., 2000). The mean settlement obtained by Hannon et al. (2015) on *U. lens*

(50%) is higher than our results on the same substrate, but it is similar to our result on natural biofilm + conspecifics (46.2 ± 3.2%), suggesting that both substrates, *U. lens* and natural biofilm, are good inducing factor for metamorphosis in *P. lividus*.

Similarly to our results, Daume et al. (2000) found that settlement of abalone *H. rubra* is higher on *U. lens* than diatoms, but growth rates were higher on diatoms than *U. lens*. Seki (1997) reported that *U. lens* sustained growth of post-larval *H. discus hannai*, but growth rates were improved by the inoculation of cultured diatoms. According to Kawamura et al. (1983), *U. lens* has been used in aquaculture centers in Japan for the production of the sea urchin *S. intermedius*, as they suggest that newly metamorphosed juveniles feed on diatoms first to then switch to *U. lens*.

Although no differences in survival at 10 DPS was recorded between settlement substrates, the higher diameter of post-larvae settled on the natural biofilm compared to *U. lens* strengthens the importance of diatoms for the growth and survival of *P. lividus* juveniles. This is particularly true as larger settlers are less susceptible to post-settlement mortality (Meidel et al., 1999).

3.6 Conclusions

This study could represent an improvement in the rearing methods of *P. lividus*. All diets tested in this study supported the larval development of *P. lividus*, but only juveniles resulting from the Duna and CD treatments survived to 180 DPS. This indicates that it is important to consider the survival of post-larvae and juveniles to establish the efficiency of the dietary treatment on the production of *P. lividus* juveniles.

Seawater previously “conditioned” by the presence of conspecifics increases the larval settlement rate of *P. lividus*. Furthermore, although *U. lens* provides an appropriate settlement substrate for competent larvae, higher growth rate of post-metamorphic individuals was achieved on a natural biofilm. Nevertheless, considering that dominant species in a natural biofilm change throughout the season and between locations, *U. lens* could represents an efficient and more reliable alternative to natural biofilms as metamorphosis inducing factor and first feeding item, but further investigations are required to ascertain its nutritional value for *P. lividus* post larvae.

3.7 Acknowledgments

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4 STOCKING DENSITY INFLUENCES THE GROWTH OF JUVENILE SEA URCHINS, *PARACENTROTUS LIVIDUS* (LAMARCK, 1816)

Not submitted

4.1 Abstract

In this study, we compared the effects of stocking density on the somatic growth, feed intake, absorption efficiency and food conversion ratio of juvenile sea urchins *Paracentrotus lividus*. Three stocking densities were tested: Low, 100 individuals/m^2; Intermediate, 250 individuals/m^2; High, 400 individuals/m^2. Sea urchins were stocked in nine containers floating in a 600 L rectangular tank, and fed *ad libitum* with fresh thalli of *Ulva* sp. Test diameter and wet body weight were measured at 30, 60 and 90 days and specific growth rate was calculated. As expected, test diameter and wet weight increase were higher ($p < 0.001$) when individuals were stocked at Low than other treatments. Stocking density influenced also the wet feed intake; individuals stocked at Low density, indeed, recorded the highest wet feed intake ($p < 0.001$). This study describes for the first time how stocking density influences somatic growth, feed intake and absorption efficiency of juvenile *P. lividus*.

4.2 Introduction

Stocking density is an important factor for the grow-out of aquatic animals, particularly during the earlier culture stages and it could influence their somatic growth rate. It is well known, indeed, that increasing stocking density influences negatively the somatic growth of fishes (Vijayan and Leatherland, 1990) and invertebrates (Pei et al., 2012; Dong et al., 2010; Lloyd and Bates, 2008; Park et al., 2008; Li et al., 2006). High stocking density, moreover, increases the risk of mortality due to disease outbreaks and cannibalism (Jobling, 1994). Siikavuopio et al. (2007) demonstrated that the increasing in stocking density fosters injuries and has a negative effect on survival and gonad growth of sea urchin *Strongylocentrotus droebachiensis*.

However, studies examining the effects of stocking density on the cultivation of sea urchins are scarce (Siikavuopio et al., 2007; Le Gall, 1990) and, to our knowledge, there are not published studies on the effects of stocking density on the somatic growth of juvenile *Paracentrotus lividus*.

The aim of this study was, therefore, to evaluate the effects of three different stocking densities on the somatic growth, feed intake, absorption efficiency and food conversion ratio of juveniles *P. lividus*.

4.3 Materials and methods

Juvenile individuals of *P. lividus*, 6-month-old and 6.9 ± 0.1 mm (mean ± SD) in test diameter, were reared at the International Marine Centre-IMC laboratory (Oristano, Sardinia, Italy) under controlled conditions. Larvae were fed with a 50% mixture (number of cells) of *Dunaliella tertiolecta* and *Chaetoceros gracilis*. The macroalgae *Ulvella lens* was used as inducing metamorphosis factor and as first feed for the post-metamorphic individuals; the diet was integrated with *Ulva* sp. after three months post-settlement.

4.3.1 Experimental design

The growth of sea urchins was investigated for 90 days at three stocking densities (three replicates per treatment): Low, Intermediate and High density. Low density was established as ¼ of the usually stocking density (400 individuals/m^2) reported by Carboni (2013) for the grow-out of juveniles. Intermediate density was extrapolated by previous studies on the base of their mean stocking densities adopted (Tomsic et al., 2015; Cook and Kelly, 2007; Siikavuopio et al., 2007) and High density was fixed as the value reported by Carboni (2013) (Table 6).

Sea urchins were stocked in nine truncated cone plastic containers with a volume of 2.9 L and a surface of 0.085 m^2; the number of individuals per container is indicated in Table 6. One additional container (length: 500, width: 310, height: 170 mm) was used as reserve; in the event of mortality, dead urchins were removed from the containers and substituted to maintain the starting density. Reserve individuals were fed *ad libitum* with *Ulva* sp. and maintained at the same conditions of those used in the experimental containers.

Table 6: Densities treatments and corresponding abundances of individuals per container.

	Low	*Intermediate*	*High*
Density	100/m^2	250/m^2	400/m^2
Individuals/container	9	21	34

The containers were perforated throughout the entire surface, as to allow the seawater flow inside them. The outside bottom of each container was upholstered with a plankton mesh (100 μm) as to avoid faeces dispersion.

As adopted in previous experimental designs (Cárcamo, 2015; Siikavuopio et al., 2007), all the containers floated in a unique rectangular tank with a volume of 600 L (length: 3850, width: 180, height: 850 mm), containing filtered (1 μm) natural seawater at 36.3 ± 0.1 ppt (mean ± SE) temperature of 20.0 ± 0.2 °C, pH of 8.4 ± 0.1 and dissolved oxygen of 87.5 ± 1.2%. The tank was supplied with a recirculating system (flow of 5 L/min) and equipped with biological and mechanical filter (10 μm); 50% seawater exchange

was done once a week. Gentle aeration and 14 h light photoperiod (22 μmol photons/m^2/s) were applied.

Prior to the experiment, individuals were starved and held for 2 weeks in the experimental laboratory system to acclimatize and standardize their nutritional condition (Minor and Scheibling, 1997).

4.3.2 Growth and mortality

The somatic growth of sea urchins was estimated at 30, 60 and 90 days by measuring the individual's test diameter (mm) and the wet body weight (mg). Individuals were placed on a waterproof graph paper and photographed with a Canon PowerShot G15 digital camera. Image Processing Analysis in Java (ImageJ 1.49V) was calibrated appropriately for image analysis and measurement: a length of 1 mm was measured via a ruler, saved and then used as the standard for individual growth measurement, in terms of test diameter; the widest part of the sea urchin body was measured. With the aim to simplify these operations, a solution of KCl 1% was used to induce paralysis and detachment of juveniles from the tanks (Hagen, 2003).

Specific growth rate (SGR) was calculated for test diameter $((LnTD_2 - LnTD_1) / (t_2 - t_1) \times 100)$ and wet weight $((LnWW_2 - LnWW_1) / (t_2 - t_1) \times 100)$, where TD_2 and TD_1 are test diameter at time 2 (t_2) and time 1 (t_1), respectively, and WW_2 and WW_1 the wet weight at time 2 and 1, respectively (Manuel et al., 2013).

Sea urchins in both containers and reserves were fed *ad libitum* every two days with fresh thalli of *Ulva* sp. Thalli were harvested in the nearby Mistras lagoon (39°90'N, 8°46'W), carefully washed with fresh water to remove epiphytes and frozen at -20 °C. Prior to the administering, *Ulva* sp. was defrosted and weighed (wet weight).

Uneaten feed was removed and weighed (wet weight). Five samples of uneaten feed were dried in an oven at 60 °C for 3 days and weighed (Cárcamo, 2015). The average ratio dry weight/wet weight (0.187) was used to calculate the dry weight of feed intake (McCarron et al., 2009). Faeces were siphoned every two days, dried at 60 °C for 3 days and weighed (dry weight) (Cárcamo, 2015).

4.3.3 Feed intake, absorption efficiency and food conversion ratio

The wet feed intake (gr/urchin/day) was calculated as difference between the total amount of wet feed provided and the total amount of wet feed uneaten, divided for the days of experimentation. The absorption efficiency was calculated by (average dry feed intake per individual – average dry faeces siphoned per individual)/average dry feed intake per individual ×100 (Hammer et al., 2004). The food conversion ratio (FCR) for each treatment was calculated as total dry feed intake (gr dry weight/urchin)/total wet body weight gain (gr wet weight/urchin) (Hammer et al., 2004).

4.3.4 Statistical analysis

Data were analyzed by Statistica 6.1 StatSoft, Inc. (2004). Analysis of variance ANOVA was used to test for differences among densities in the SGR of diameter and wet weight at 30, 60 and 90 days, as well as in the total increase diameter and wet weight, wet feed intake, absorption efficiency and FCR. The normality and homogeneity of the data distribution were assessed with Shapiro Wilk's W and Levene's tests, respectively. Where required, data were analyzed by the non-parametric Kruskal-Wallis test. Tukey's honestly-significant difference (HSD) test was used to evaluate all pair-wise treatment comparisons ($p < 0.05$).

4.4 Results

4.4.1 Growth and mortality

No mortality was recorded during all the experimental period and for all density treatments. Stocking density influenced significantly the SGR of diameter and total increase. Individuals showed a higher ($p < 0.001$) SGR of test diameter at 30 days (from 0.8 ± 0.1% to 0.9 ± 0.1%, mean ± SE) in comparison with 90 days (from 0% to 0.3 ± 0.1%), and significant differences were also showed among stocking densities, resulting in a higher SGR at Low than High treatment ($p < 0.05$) (Figure 20).

At 90 days, Low density (4.6 ± 0.4 mm, mean ± SE) had a higher total diameter increase than High treatment (2.7 ± 0.3 mm), while no significant differences were observed for Intermediate (3.6 ± 0.1 mm) ($p < 0.05$) (Figure 21).

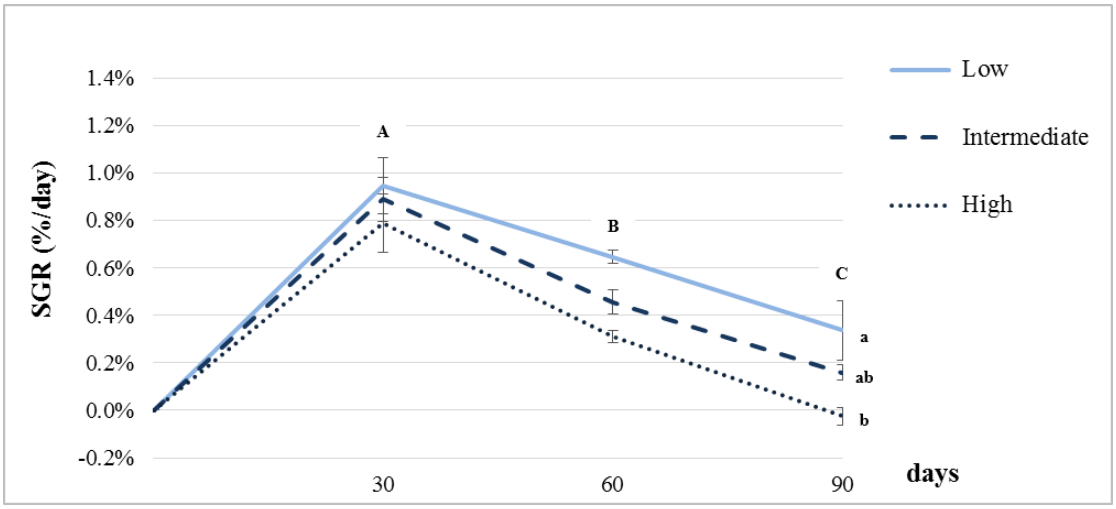

Figure 20: Test diameter Specific Growth Rate (%/day) of *Paracentrotus lividus* at 30, 60 and 90 days, stocked at three densities: Low, Intermediate and High. Capital superscripts indicate significant differences among days; lowercase superscripts indicate significant differences among densities. Values are expressed as mean ± SE (n=3).

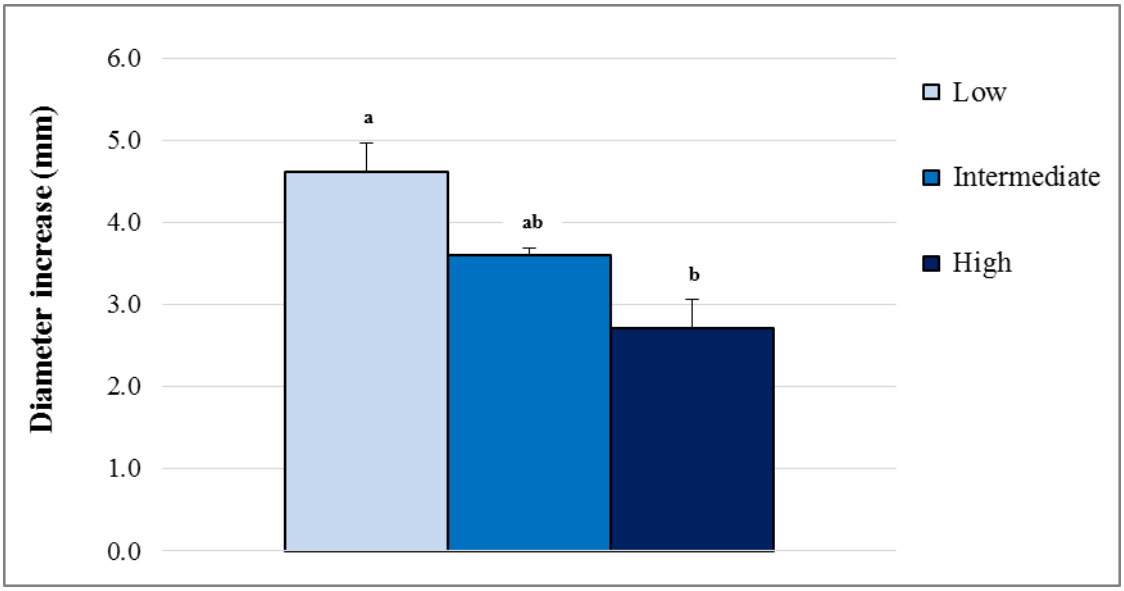

Figure 21: Total diameter increase (mm) of *Paracentrotus lividus* at 90 days stocked at three densities: Low, Intermediate and High. Lowercase superscripts indicate significant differences among densities. Values are expressed as mean ± SE (n=3).

At the end of the experiment, juveniles resulted in a final test diameter of 10.7 ± 0.2 mm, 10.1 ± 0.1 mm and 10.1 ± 0.1 mm, for Low, Intermediate and High density, respectively; no significant differences resulted among treatments (Figure 22).

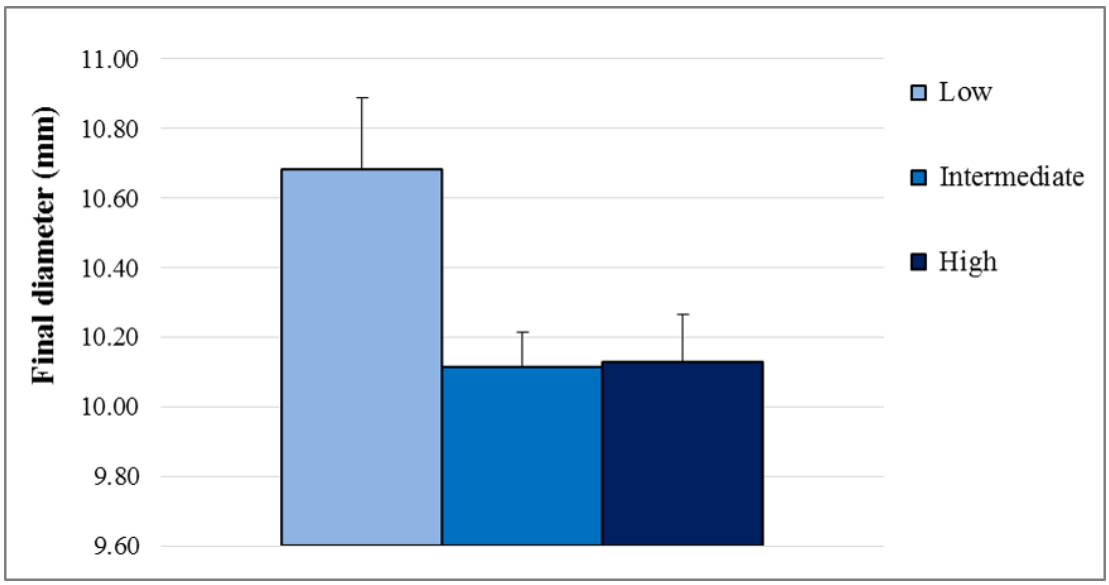

Figure 22: Final test diameter (mm) of *Paracentrotus lividus* at 90 days stocked at three densities: Low, Intermediate and High. Values are expressed as mean ± SE (n=3).

Wet weight increase showed a similar pattern to the diameter increase, resulting significantly influenced by stocking density. SGR of wet weight resulted in a higher value at 30 days (from 2.0 ± 0.2% to 2.7 ± 0.1%) in comparison with 90 days (from 0.2 ± 0.1% to 0.7 ± 0.1% ($p < 0.001$). Moreover, individuals showed significant differences among densities at 60 days, resulting in a higher SGR when stocked at Low (2.1 ± 3%) than Intermediate (0.7 ± 0.0%) and High treatment (0.7 ± 0.1%) ($p < 0.05$) (Figure 23).

Low density resulted in a higher total weight increase (324 ± 21 mg) than High treatment (234 ± 210 mg) ($p < 0.01$), while no significant differences were observed for Intermediate (275 ± 5 mg) (Figure 24).

No significant differences among density treatments were showed in the final wet weight; Low, Intermediate and High recorded a final wet weight of 437 ± 27 mg, 395 ± 5 mg and 395 ± 13 mg, respectively (Figure 25).

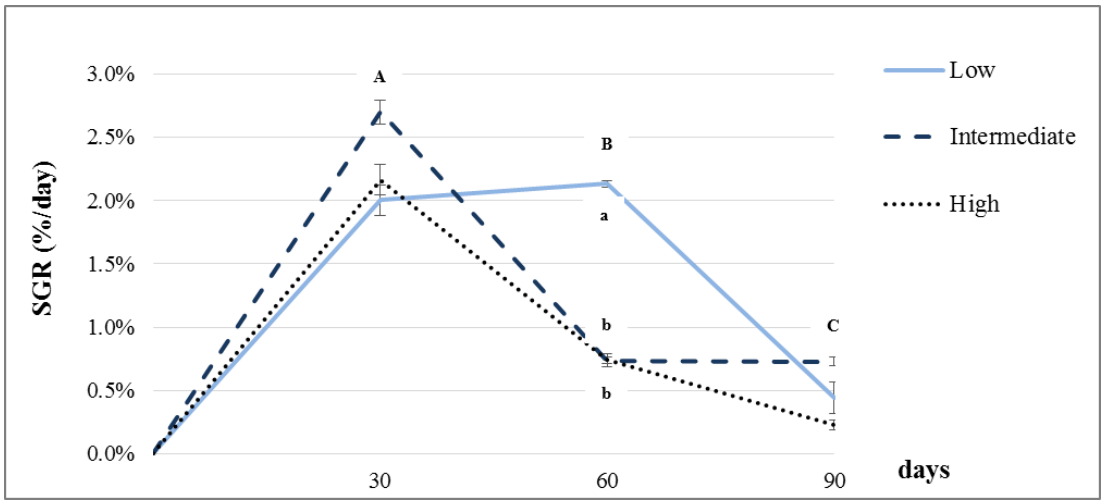

Figure 23: Wet weight Specific Growth Rate (%/day) of *Paracentrotus lividus* at 30, 60 and 90 days, stocked at three densities: Low, Intermediate and High. Capital superscripts indicate significant differences among days; lowercase superscripts indicate significant differences among densities. Values are expressed as mean ± SE (n=3).

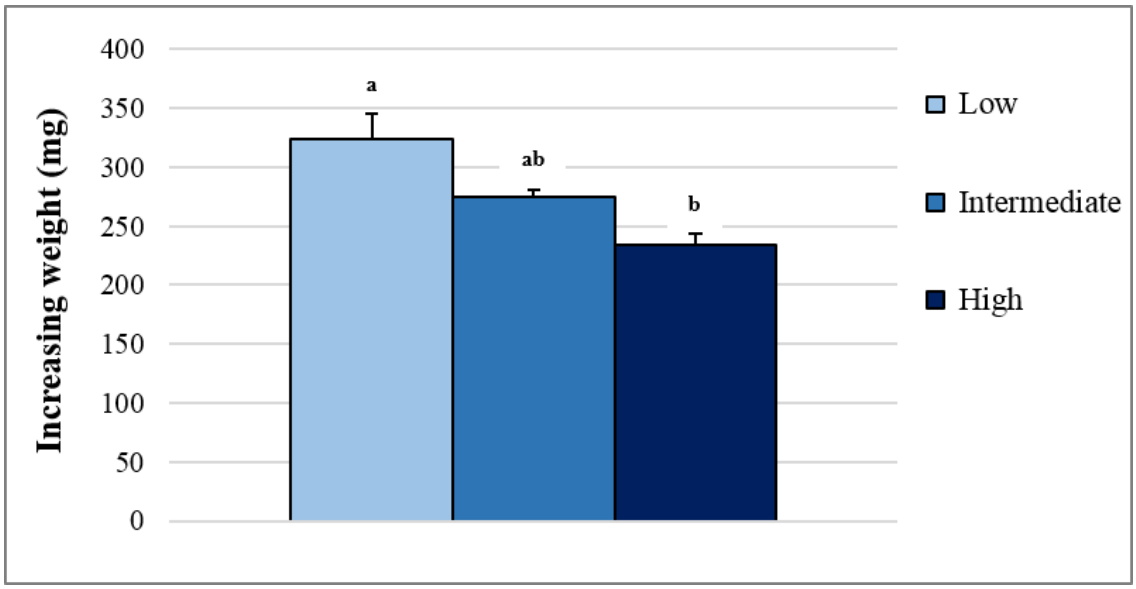

Figure 24: Total wet weight increase (mg) of *Paracentrotus lividus* at 90 days stocked at three densities: Low, Intermediate and High. Lowercase superscripts indicate significant differences among densities. Values are expressed as mean ± SE (n=3).

4.4.2 Feed intake, absorption efficiency and food conversion ratio

Individuals stocked at Low density resulted in a higher amount of wet feed intake in comparison with the other density treatments ($p < 0.001$). Absorption efficiency was influenced by density, resulting in higher absorption at Low than Intermediate and High

treatments ($p < 0.01$). Food conversion ratio resulted in a higher value at High than Intermediate ($p < 0.05$) (Table 7).

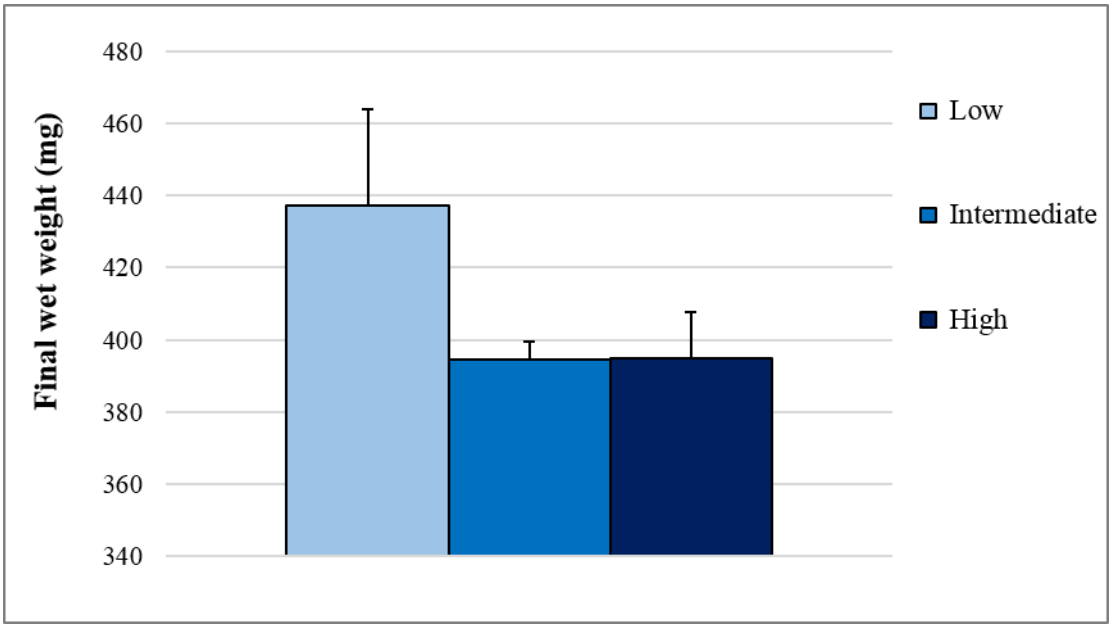

Figure 25: Final wet weight (mg) of *Paracentrotus lividus* at 90 days stocked at three densities: Low, Intermediate and High. Values are expressed as mean ± SE (n=3).

Table 7: Total wet feed intake (g/individual/day), absorption efficiency and food conversion rate (FCR) of *Paracentrotus lividus* stocked at three densities: Low, Intermediate and High. Different letters indicate significant differences among densities. Values are expressed as mean ± SE (n=3).

	Low	*Intermediate*	*High*
Wet feed intake	0.10 ± 0.01 ^{a}	0.08 ± 0.01 ^{b}	0.08 ± 0.01 ^{b}
Absorption efficiency	80.33 ± 2.62 ^{a}	59.99 ± 2.72 ^{b}	66.28 ± 3.03 ^{b}
FCR	4.84 ± 0.18 ^{ab}	4.54 ± 0.11 ^{b}	5.40 ± 0.24 ^{a}

4.5 Discussion

Results of our study indicate that stocking density influences test diameter and wet weight increase of the sea urchin *P. lividus*. The somatic growth of juveniles decreases with increasing density; test diameter and wet weight, indeed, resulted in a higher increase at the lowest density tested, and gradually decreased at Intermediate and High density. Similar pattern was previously observed for the growth of sea cucumber

Apostichopus japonicus (Pei et al., 2012; Dong et al., 2010) and various species of abalone (Lloyd and Bates, 2008; Park et al., 2008) and shellfish (Parsons and Dadswell, 1992).

Pei et al. (2012) wrote that stocking density is an environmental stress factor for the Japanese sea cucumber *A. japonicus*, which could indirectly influence the somatic growth, through conditioning the water quality and the competition for feed. Since all the treatments of our study were maintained in a unique tank, thus in the same seawater, and individuals were fed *ad libitum*, we exclude that these indirect effects caused different somatic growth among treatments. Therefore, we attribute the different diameter and wet weight increase obtained in this study to the stress caused by the competition for space, in agreement with previous studies on invertebrates (Li et al., 2006; Nga et al., 2005). According with these studies, indeed, the crowding stress indirectly causes a feed competition among individuals, because it accelerates the organism energy consumption, which modifies the energy budget and, therefore, influences negatively the growth of subdominant individuals (Pei et al., 2012).

Wet feed intake resulted influenced by density, reflecting in a higher feed intake at Low density than all other treatments. Similar results were obtained by previous studies in unlimited feeding treatments of abalones, which consumed less feed when stocked at high densities (Lloyd and Bates, 2008); on the contrary, this result was not observed for the sea urchin *S. droebachiensis* (Siikavuopio et al., 2007).

It is known that in unlimited feeding conditions, the quantity of feed does not represent a growth limiting factor for the animals (Mgaya and Mercer, 1995), and that the growth rate was higher than in rationed diet conditions (Lloyd and Bates, 2008). With the aim to reduce individual competition for feed and the negative effects of stocking densities on the growth rate, in aquaculture facilities animals are commonly fed *ad libitum* (Mgaya and Mercer, 1995). However, previous studies showed that at low densities corresponded a high growth rate and a low absorption efficiency of sea urchins, thus indicating no crowing-stress and competition for space in this density condition. Indeed, they grew faster at a higher quantity of feed, but absorbed lower than individuals stocked at higher densities (Fernandez and Boudouresque, 2000; Mukai and Nojima, 1985). According to Fernandez and Boudouresque (2000) the transition time of the feed into the gut increases when the feed intake is low, causing a better digestive activity and

an increasing absorption efficiency of the animals. In our study, we could not confirm this correlation due to the difficulty on siphoning and collection of faeces. Being small animals, indeed, the quantity of faeces was very low and we found difficult to collect faeces except than other material (e.g. naturally developed biofilm and other particulate organic matter). Therefore, we are not completely confident about the reliability of data on absorption efficiency and FCR.

This study provides for the first time preliminary information on stocking density in echinoculture of *P. lividus* and highlights its influence on the somatic growth of *P. lividus*. Specifically, it demonstrated that stocking density is one of the key factors for successful urchin culture. Low stocking densities, indeed, improve the somatic growth rate of the animals, while High densities cause low growth rates. However, at Low density conditions individuals needed a higher pro capite amount of feed.

4.6 Acknowledgments

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5 BIOREMEDIATION OF AQUACULTURE WASTEWATER FROM *MUGIL CEPHALUS* (LINNAEUS, 1758) WITH DIFFERENT MICROALGAE SPECIES

In submission

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Keywords:

Phytoremediation, biotreatment, bioreactors, wastewater, algae

Contribution:

The author contributed to design the trial, carried out statistical analysis and wrote the manuscript.

5.1 Abstract

Current aquaculture practices have a detrimental impact on the environment, in particular due to the release of high concentration of nitrogen and phosphorus that can induce eutrophication. This study investigates and compares the capacity of three microalgae species *Tetraselmis suecica*, *Isochrysis galbana* and *Dunaliella tertiolecta*, in the bioremediation of grey mullet *Mugil cephalus* wastewater. The experiment was conducted in batch conditions for 7 days using completely mixed bubble column photobioreactors. After two days, *T. suecica* and *D. tertiolecta* were able to remove more than 90% of Dissolved Inorganic Nitrogen (DIN) and Dissolved Inorganic Phosphorous (DIP), whereas *I. galbana* removed only 32% and 79% of DIN and DIP, respectively. A higher biomass yield resulted for *T. suecica* (0.60 ± 0.03 g/L, mean ± SE). This study confirms the potential to employ *T. suecica* in an Integrated Multi Trophic Aquaculture system for bioremediation of wastewater and identifies *D. tertiolecta* as another valid candidate species. Moreover, these species can growth in unsterilized culture media, and this reduces energy consumption, costs and efforts.

5.2 Introduction

Aquaculture is one of the fastest-growing food producing sectors in the world, providing almost about 50% of all fish for human consumption; within 2030, this share is projected to rise to 62%1. On the other hand, aquaculture represents one of the major contributors to the increasing levels of dissolved and particulate nutrients in the aquatic ecosystems (Lamprianidou et al., 2015). A high nutrient loading into the aquatic environment, in particular nitrogen and phosphorus may cause eutrophication, oxygen depletion and siltation (Burford et al., 2003).

With the aim to reduce the impacts of traditional aquaculture, several Countries around the world are developing Integrated Multi-Trophic Aquaculture (IMTA) systems, which re-use the wastewaters for the growth of micro and macroalgae. Indeed, aquaculture wastewater provides nutrients (ammonia, nitrite, nitrate, dissolved organic nitrogen and phosphate) (Converti et al., 2006; Soletto et al., 2005; Abe et al., 2002) which can be used for the production of microalgae. The uptake of dissolved nutrients by microalgae is considered as the main way to remove nitrogen in aquaculture wastewaters (Attasat et al., 2013; Sirakov et al., 2013).

Previous studies showed that it is possible to remove nutrients from wastewater (fishes and shrimp production plants) employing microalgae and macroalgae as key elements in biological treatments (Gao et al., 2016; Michels et al., 2014; Sirakov and Velichkova, 2014; Bartoli et al., 2005; Borges et al., 2005; Lefebvre et al., 2004; Hussenot et al., 1998; Lefebvre et al., 1996; Hammouda et al., 1995; Shpigel et al., 1993).

This phycoremediation is an eco-friendly method that offers the advantage to be a low-cost way to nutrient removal (Mulbry et al., 2008). In addition, the biomass produced through bioremediation could have multi-purpose uses including fuels, fertilizers, fine chemicals production and feed in aquaculture (Mulbry et al., 2006; Vilchez et al., 1997).

One of the most common microalgae species employed in aquaculture bioremediation wastewater is *Tetraselmis* spp. (Michels et al., 2014; Sirakov and Velichkova, 2014; Borges et al., 2005). A recent study Michels et al. (2014) showed for the first time that it is possible to use *Tetraselmis suecica* for the nutrient assimilation of fishfarm wastewater through its cultivation in controlled photobioreactors.

The aim of this study is to evaluate and compare the capability of *T. suecica*, *Isochrysis galbana* and *Dunaliella tertiolecta*, widely used in aquaculture as feed for rotifers (Mason, 1963), echinoderms (Paredes et al., 2015; De La Uz et al., 2013; Azad et al., 2011; Miller and Emlet, 1999; Zamora and Stotz, 1994), filter feeders (Carboni et al., 2016; Nevejan et al., 2003) and fin fishes (Fabregas et al., 1986), for the removal of dissolved inorganic nutrients (nitrogen and phosphorous) of wastewater aquaculture. We evaluate the biomass yield of these species in controlled bubble column annular photobioreactors, by using untreated mullet wastewater as culture medium. Contrarily to previous studies that sterilized the wastewater before its use for bioremediation to eliminate zooplankton, bacteria and suspended solids (Michels et al., 2014), we avoided the use of expensive pre-treatment procedures as filtration and sterilization, aiming to reduce the costs of seawater treatment and simulate more real operation conditions of a wastewater treatment system.

5.3 Materials and methods

5.3.1 Aquaculture wastewater

Aquaculture wastewater was provided by an experimental fish hatchery located in the International Marine Centre - IMC Foundation (Oristano, Sardinia, Italy). Juveniles of grey mullet *Mugil cephalus* (Linnaeus, 1758) were obtained in laboratory and reared in a recirculating aquaculture system (RAS) consisting of 4 tanks of 2000 L volume. In this system, the tanks were linked in a single biological (trickling filter) and cartridge mechanical filter (10 µm) and supplied with UV lamp (UVPE5, 80 W) and protein skimmer (Panaque). Temperature was maintained at 23 ± 2 °C (mean ± SE) with a chiller (TECO TR60, 0.91 Kw) and natural photoperiod (14/10 L/D) was adopted (Figure 26).

Natural seawater (NSW) at 37.0 ± 1.0 ppt salinity was previously micro-filtered (0.5 µm) and UV lamp sterilized. Juveniles of 0.35 ± 0.43 g body weight (BW) were fed at 3% BW per day with the commercial formulated feed for sea fish supplied by Skretting SpA (PERLA LARVA) composed of 62% crude protein, 11% crude oils and fats, 9% crude ash, 0.8% crude fiber and 1.2% crude phosphorus. Fishes were stocked at an average density of 0.5 g body weight/L.

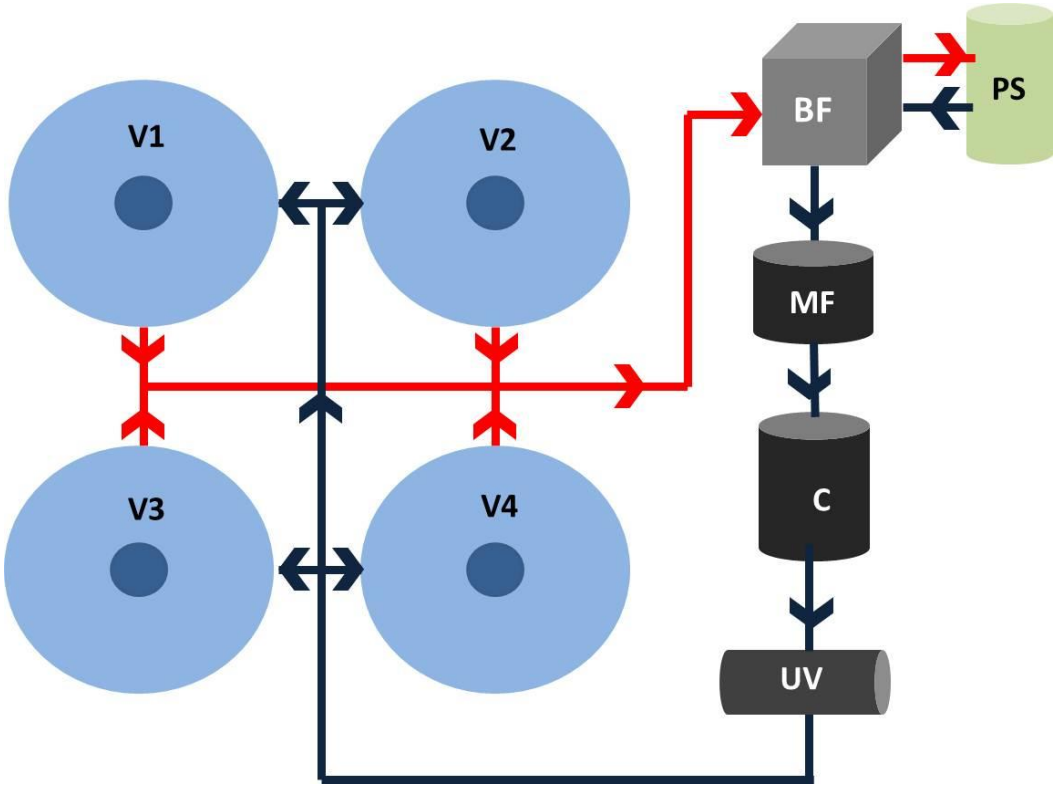

Figure 26: Recirculating aquaculture system (RAS) for rearing of juvenile grey mullets *Mugil cephalus*, consisting of four circular fiberglass tanks with 2000 L volume (V1, V2, V3 and V4). The system was equipped with biological (BF) and mechanical filter (MF), protein skimmer (PS), chiller (C) and UV lamp (UV). Dotted arrow = seawater outlet; continuous arrow = seawater intake.

Tanks were monitored daily for checking mortality; the uneaten food and faeces were siphoned out twice a week for maintaining good water quality. A 30% water exchange was weekly performed, and a part of this 30% was employed as wastewater in our experiment. Wastewater was taken at the inlet of the tank, after UV lamp.

5.3.2 Microalgae culture

The microalgae species were provided by the Agency for Agricultural Research in Sardinia (AGRIS) and sourced from the Culture Collection for Algae and Protozoa (CCAP: Oban, Scotland). Pre-culture inocula were permanently kept in Erlenmeyer flasks in Pyrex glass with total capacity of 2 L, closed with cotton and covered with gauze and aluminum foil. NSW was autoclaved at 121 °C for 30 min and enriched with Guillard F/2 medium (Guillard, 1975; Guillard and Ryther, 1962). Cultures were exposed to a constant illumination (155 μmol/s/m^2) provided by 4 fluorescent lamps

(OSRAM type Natura). Continuous aeration 3 L/min was supplied by peristaltic pump (ECOH Air Pump) and temperature was maintained at 23 °C by air conditioning.

5.3.3 Experimental design

Nutrient uptake and biomass production of *T. suecica*, *I. galbana* and *D. tertiolecta* were evaluated during seven days in batch conditions using two completely mixed bubble column photobioreactors of 6 L; five runs were done for a total of three replicates per treatment.

Lighting system was composed by four neon daylight lamp (four fluorescent lamps type cool daylight, OSRAM Lumilux FQ 24W/865), with light intensity of 100 μmol/s/m^2. This system was monitored with a Programmable Logic Controller (PLC) that it is a device that performs discrete or continuous control logic in process plant or factory environments (Figure 27). These controllers are hardware and software engineered microcomputers, used to provide industrial control operations (Netto et al., 2013). Reactors were equipped with temperature and aeration regulation control system; temperature was maintained at 23 °C, aeration was ensured by a blower at flow rate of 3 L/min. On the contrary, pH was not controlled and resulted at 7.7 ± 0.2. Phytoplankton laboratory-culture methods and photobiorectors operation were adopted according to Saiu et al. (2016).

Microalgae growth was measured as dry weight biomass (DW) (Clasceri et al., 1999). DW was measured once a day in 40 mL of water sample previously filtered through 0.45 μm Whatman fiber-glass. After filtration, filters were washed with 20 mL of deionized water to remove salts and dried in an oven at 105 °C until constant weight, following Saiu et al. (2016). The supernatant liquid fraction obtained after filtration was used for nitrate, nitrite, ammonia and phosphorous analysis. In order to monitor the microalgae nutrient uptake, nutrients were daily analysed by an automatic chemical analyzer μCHEM based on Loop Flow Analysis (Systea, Italy). Microalgae removal efficiencies of Dissolved Inorganic Nitrogen (DIN) and Dissolved Inorganic Phosphorous (DIP) were calculated according to the method used by Michels et al. (2014), as follow:

N removal efficiency (%) = ((DIN influent - DIN effluent) / DIN influent) x 100

P removal efficiency (%) = ((DIP influent - DIP effluent / DIP influent) x 100

DIN values were calculated as the sum of nitrite (NO_2^-), nitrate (NO_3^-) and ammonia (NH_4^+), while DIP corresponded to the total dissolved phosphate (PO_4^{3-}).

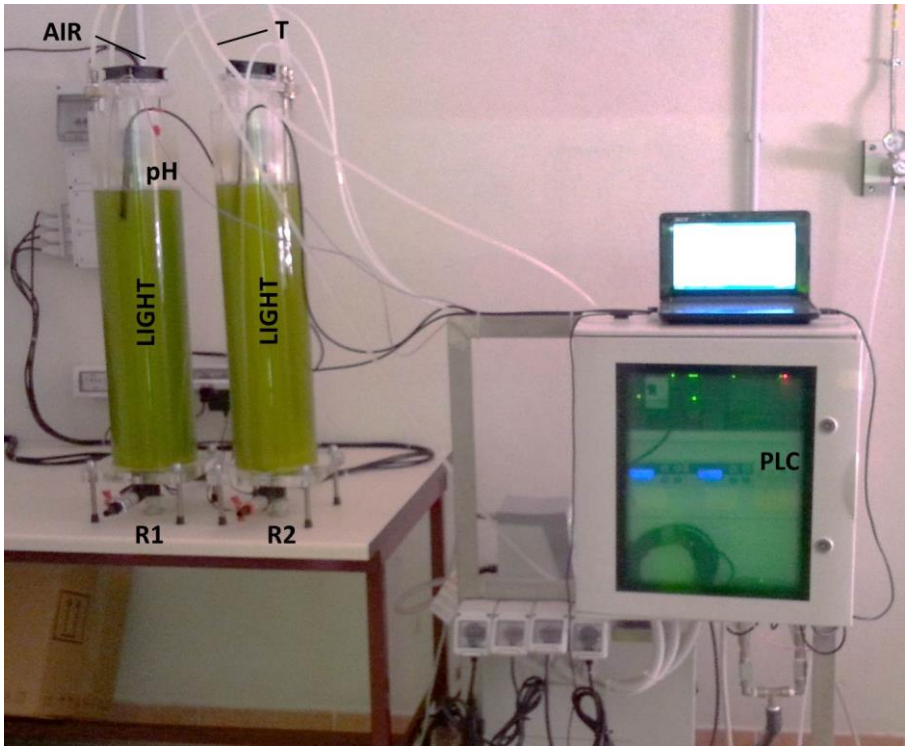

Figure 27: Bubble column annular photobioreactors of 6 L volume (R1 and R2) used for the growth of phytoplankton, supplied with LIGHT, Programmable Logic Controller (PLC), gentle aeration (AIR), probes for temperature (T) and pH (pH).

5.3.4 Statistical analysis

Data were analyzed by Statistica 6.1 StatSoft, Inc. (2004). Differences in the removal efficiences among phytoplankton species were analysed using analysis of variance (ANOVA). Shapiro Wilk's W test was used to verify the normality of the data distribution and Levene's test was used to verify the homogeneity of variances. Biomass was analyzed using repeated-measures ANOVA, with species as independent factor and days as repeated factor. Tukey's honestly-significant difference (HSD) test was used to evaluate all pair-wise treatment comparisons ($p < 0.05$).

5.4 Results

The nutrient concentration of the wastewater was regularly measured before each experiments (Table 8). It was possible to observe that the composition of wastewater was very similar in each experiment, being nitrate the N species with the higher concentration.

Table 8: Nutrients dissolved in the *Mugil cephalus* wastewater. Values are expressed as mean ± SE (n= 3).

	Tetraselmis suecica	*Dunaliella tertiolecta*	*Isochrysis galbana*
NO_3^- -N (mg/L)	4.1 ± 0.4	4.2 ± 0.1	4.2 ± 0.4
NO_2^- -N (mg/L)	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
NH_4^+ -N (mg/L)	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
PO_4^{3-} -P (mg/L)	0.3 ± 0.1	0.6 ± 0.1	0.6 ± 0.1

5.4.1 Nutrients removal efficiency

At the end of the experiment a clearly higher DIN removal efficiency ($p < 0.001$, two-way ANOVA) resulted for *T. suecica* (94.4 ± 1.0%, mean ± SE) and *D. tertiolecta* (95.4 ± 0.3%) in comparison with *I. galbana* (66.0 ± 1.5%). There were not statistical differences between the three species in the removal of DIP at the end of the experiments (Table 9).

Table 9: Influent and effluent DIN and DIP values (mg/L) and removal efficiency (%) of *Tetraselmis suecica, Dunaliella tertiolecta* and *Isochrysis galbana*. Values are expressed as mean ± SE (n= 3). Superscripts indicate significant differences among species.

	Tetraselmis suecica	*Dunaliella tertiolecta*	*Isochrysis galbana*
DIN Influent (mg/L)	4.5 ± 0.5	4.6 ± 0.1	4.6 ± 0.5
DIN Effluent (mg/L)	0.3 ± 0.1	0.2 ± 0.1	1.6 ± 0.1
DIN %	94.4 ± 1.0 [a]	95.4 ± 0.3 [a]	66.0 ± 1.5 [b]
DIP Influent (mg/L)	0.3 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
DIP Effluent (mg/L)	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
DIP %	96.0± 2.5	91.2 ± 2.3	91.9 ± 4.0

T. suecica and *D. tertiolecta* showed a similar pattern of nutrient uptake (Figure 28A, 28C). Both species removed more than 90% of DIN and DIP after 2 and 1 day, respectively. On the contrary, *I. galbana* showed a slower nutrient uptake, lower than 35% and 80% removal for DIN and DIP, respectively, after 2 days (Figure 28B). The

nutrient uptake of DIN showed significant differences ($p < 0.001$) between *I. galbana* and the other two phytoplankton species (Repeated-measures ANOVA).

5.4.2 Biomass yield

Ciliate protozoan *Paramecium* spp. was observed in all cultures through the duration of the experiement, but we did not evaluate the abundance of this species. This was mainly due to lack of the wastewater pre-treatment procedures (i.e. filtration and sterilization). We found a significant difference in biomass yield among the three species (Repeated measures ANOVA, $p < 0.001$). *T. suecica* resulted in a higher DW (0.57 ± 0.02 g/L, mean ± SE) than *I. galbana* (0.12 ± 0.01 g/L) from 3 days up to the end of the experiment, 0.60 ± 0.03 g/L for *T. suecica* and 0.16 ± 0.02 g/L for *I. galbana*. We found no difference between *D. tertiolecta* and the other two species (Figure 29).

5.5 Discussion

In this study, we tested the capability of three microalgae species to remove nutrients dissolved in the wastewater of a hatchery pilot rearing system of *M. cephalus*. We found two out of three species, *T. suecica* and *D. tertiolecta*, able to remove more than 90% of the DIN and DIP after two days of treatment. Differently, the phytoplankton species *I. galbana* employed 7 days to remove 92% of DIN, while DIP were not completely removed at the end of the experiment (66%).

This is the first time that the *D. tertiolecta* was used as aquaculture wastewater species, while previous studies obtained efficient results by using *T. suecica*. Michels et al. (2014) showed that with a biomass concentration of 0.5 g/L, *T. suecica* resulted in a removal efficiency of 49.4% for N and 99.0% for P, after 15 days and using continuously operated tubular photobioreactor. Michels et al. (2014) obtained a higher N removal efficiency (95.7 ± 1.0%) after addition of extra orthophosphate to compensate the insufficient amount of DIP in the wastewater. Culturing *T. suecica* under batch condition, on the contrary, Borges et al. (2005) obtained a maximum P removal of only 52-63% at 8 days, even after nutrient (+N) ratio correction.

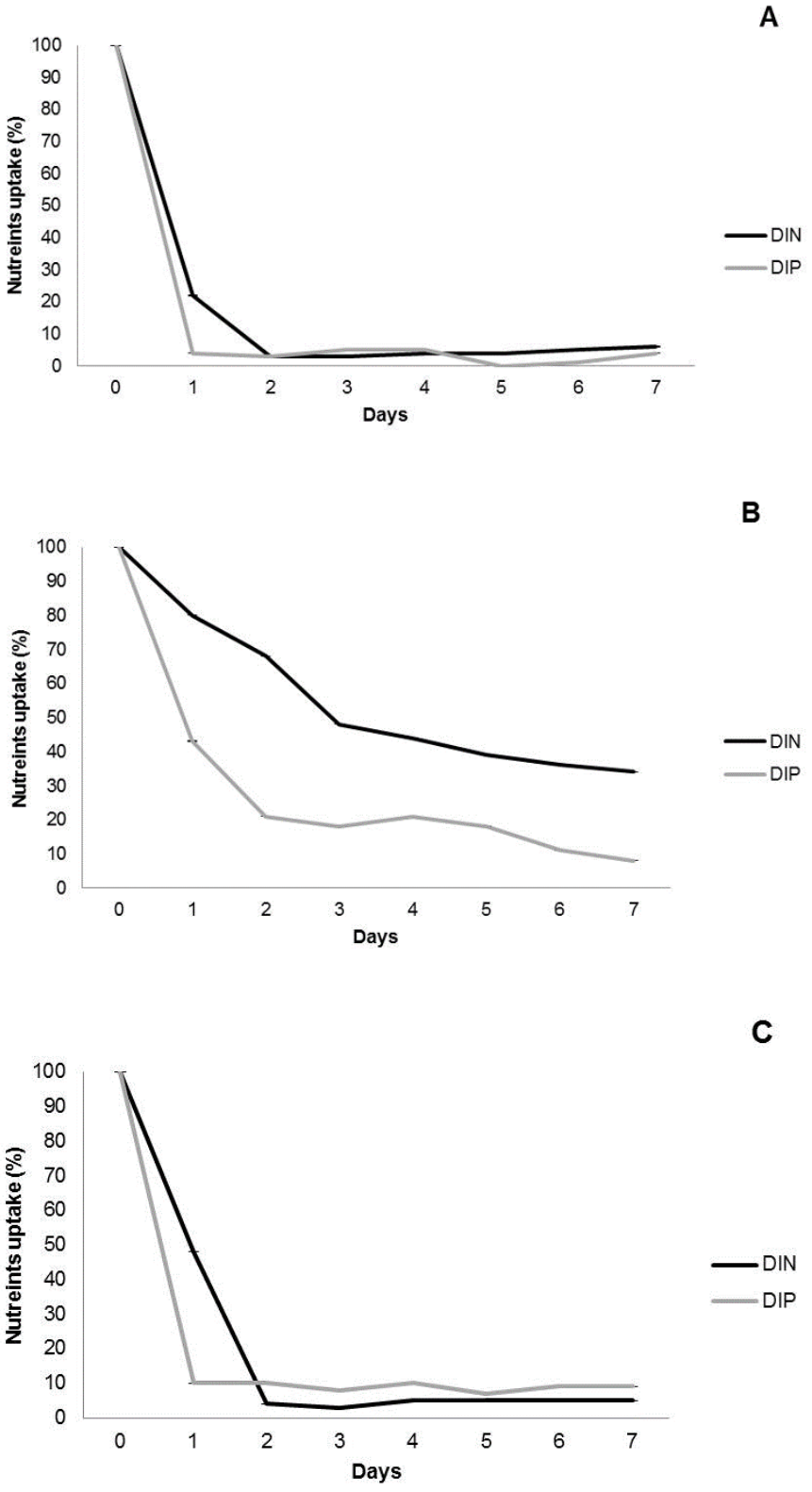

Figure 28: Nutrient uptake (%) of Dissolved Inorganic Nitrogen (DIN) and Dissolved Inorganic Phosphorous (DIP) for *Tetraselmis suecica* (A), *Isochrysis galbana* (B) and *Dunaliella tertiolecta* (C), during 7 days. Values are expressed as mean ± SE (n= 3).

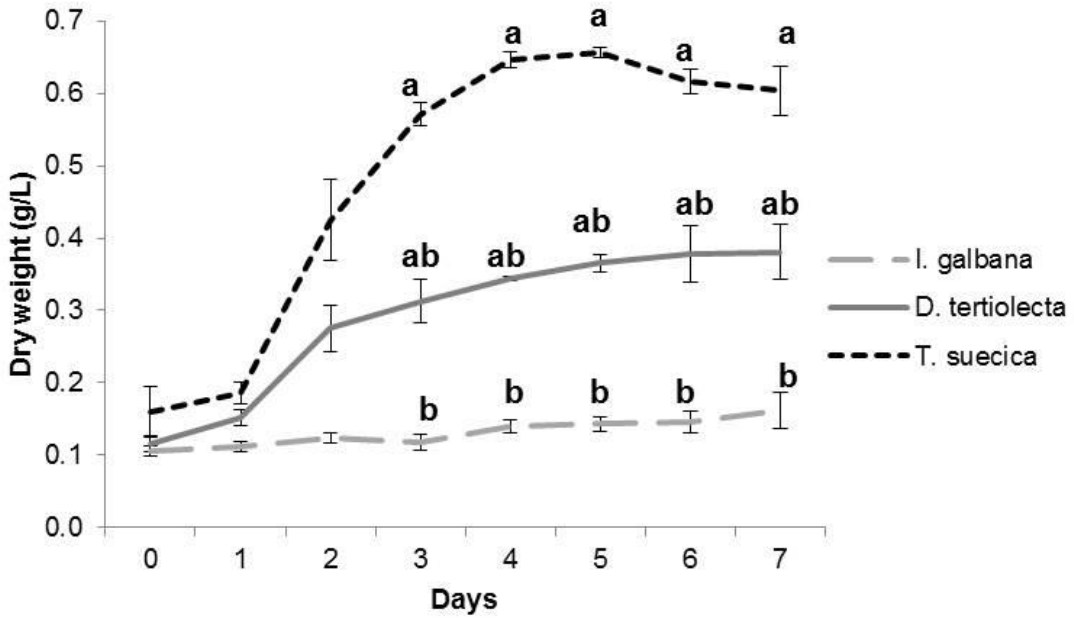

Figure 29: Microalgal growth curves as DW (g/L) of *Tetraselmis suecica*, *Isochrysis galbana* and *Dunaliella tertiolecta*, during 7 days. Values are expressed as mean ± SE (n= 3). Superscripts indicate significant differences among species.

The growth of microalgae is influenced by the culture medium composition and variables such as temperature, light intensity and pH (Molina et al., 1991). Moreover, it was previously observed that other factors are determinant for the growth of phytoplankton, as the N:P ratio. Once microalgae reaches the stationary phase, indeed, Molina et al. (1991) observed that the biomass concentration increases with the N:P ratio up to different levelling-off values, which depends upon temperature, with concentration remaining nearly constant for values beyond this point. At 25 °C, the N:P levelling-off value registered by Molina et al. (1991) for *Tetraselmis* spp. (10) is lower than values registered in the wastewater used for this study, 18 for *D. tertiolecta*, 16.3 for *I. galbana* and 32 for *T. suecica*.

In this study, the highest biomass yield (DW) was obtained with *T. suecica*, 0.6 ± 0.06 g/L, while 0.38 ± 0.06 and 0.16 ± 0.04 g/L was recorded for *D. tertiolecta* and *I. galbana*, respectively, at the end of the experiment. We hypothesize that these differences were due to a diverse species-specific cell size; according to FAO (2004), indeed, *T. suecica* has the largest median cell volume (300 μm^3), followed by *D. tertiolecta* (170 μm^3) and *I. galbana* (40-50 μm^3).

I. galbana is not suitable for the nutrient removal of *M. cephalus* aquaculture wastewater. According with Borges et al. (2005) *I. galbana* resulted in a low biomass yield and removal efficiency of DIN and DIP. We hypothesize that the ciliate *Paramecium* spp. influenced negatively the growth of *I. galbana*, because this organism effectively feeds on other live microorganisms (Wichterman, 1986). *Paramecium* spp. was observed also in the cultures of *T. suecica* and *D. tertiolecta*, but the presence of this protozoan did not seem to affect the growth of these phytoplankton species. *I. galbana* is smaller than the other two species, therefore it could be a more easy prey for the zooplankton. Moreover, it has been previously reported a large spectrum of antimicrobial activity and antibiotic substances of the genus *Tetraselmis* spp. (Austin et al., 1992; Austin and Day, 1990) and *Dunaliella* spp. (Chang et al., 1993), which could limit the negative effects of *Paramecium* spp. on the growth of cultures. When aquaculture wastewater is used as a nutrient source for algae, sterilization may be necessary to minimize the negative effects of bacteria and other organisms on the algae growth (Cai et al., 2013; Stein, 1979). However, sterilization process increases the capital cost of the algae cultivation system, representing a negative point for an efficient phytoplankton bioremediation system at large scale. Microalgae production, indeed, must be a low cost system, easily installable and maintainable (Cai et al., 2013). Avoiding to pre-treat and sterilize the wastewater, as in our experiment, reflects in a reduction of management costs, as manual labour and energy. Moreover, it was demonstrated that microalgae cultures with protozoans such as *Paramecium* spp. represent suitable diets for fish fries (FAO, 1980).

During last decade, research efforts have been focused towards the development of more efficient, higher surface-to-volume ratio photobioreactors for microalgae cultivation (Tredici, 2004; Rodolfi et al., 2008). This is the first study that compared the ability of these three microalgae species in nutrient removal of aquaculture wastewater by using controlled bubble column annular photobioreactors. Gao et al. (2016) recently tested *Chlorella vulgaris* and *Scenedesmus obliquus* cultivated in shrimp *Penaeus vannamei Boone* wastewater, in batch conditions and by using photobioreactors. A better performance in the biomass production was recorded for *C. vulgaris* (7.3 mg/L/day) in comparison with *S. obliquus* (6.2 mg/L/day).

5.6 Conclusions

This study confirmes the potential of *T. suecica* in the assimilation of nutrients dissolved in aquaculture wastewater and in the production of biomass. *D. tertiolecta* also resulted suitable for bioremediation, removing more than 90% of dissolved inorganic nitrogen and phosphorous. Differently from *I. galbana*, *T. suecica* and *D. tertiolecta* are able to grow well in no sterilized culture media contaminated with bacteria and zooplankton (*Paramecium* spp.), reflecting in the potential to reduce manual labour and energy costs for pre-treatment of culture medium in a phytoplankton bioremediation system.

T. suecica and *D. tertiolecta* are valid candidate for the employement in IMTA systems. They can be cultivated for bioremediation of finfish or shrimp wastewater and biomass produced can be re-used as live-feed for hatchery-grown of herbivorous and filter feeders (Alsull and Omar, 2012; Michels et al., 2014). Nevertheless, further studies will be needed to assess the biochemical composition of these phytoplankton species cultivated in aquaculture wastewater and to evaluate their effects as live-feed.

5.7 Acknowledgments

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6 NUTRIENT ASSIMILATION AND GROWTH OF *ULVA* SP. (LINNAEUS, 1753) IN GREY MULLET (*MUGIL CEPHALUS*) WASTEWATER

In preparation for submission

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Keywords:

Nutrients, aquaculture, phytoplankton, treatment.

Contribution:

The author designed and conducted the trial, collected the samples, carried out laboratory and statistical analysis and wrote the manuscript.

6.1 Abstract

This study evaluated the efficiency of the cultivation of *Ulva* sp. in natural environmental conditions, and compared the biomass yield and nutrient removal efficiency for dissolved inorganic nutrients in two culture media: natural estuarine water and grey mullet (*Mugil cephalus*) wastewater. Fresh thalli of *Ulva* sp. were harvested at Mistras lagoon in March and cultivated for 10 days in 5 L transparent cylindrical tanks, with three replicates per treatment. Every two days, we assessed the nutrient removal efficiency of Dissolved Inorganic Nitrogen (DIN) and Dissolved Inorganic Phosphorous (DIP), as well as the growth of *Ulva* sp., biomass yield and specific growth rate. At the end of the experiment, *Ulva* sp. resulted in a higher DIN removal efficiency in estuarine water (95.7 ± 0.3%, mean ± SE) than in wastewater (68.7 ± 1.0%) culture medium ($p < 0.001$). There were no statistical differences between the two water culture media for biomass yield or specific growth rate. This study demonstrates the potential use of this macroalga species as a biofilter for the removal of DIN and DIP as well as for the production of biomass in integrated grey mullet aquaculture systems. In addition, they highlight that the growth of *Ulva* sp. could be enhanced by improving some features of the cultivation system, such as flow rate, exchange rate and water movement.

6.2 Introduction

Algae belonging to the genus *Ulva* are employed in pharmaceutical and chemical applications, as well as for the production of biofuel (van der Wal et al., 2013). Furthermore, *Ulva* spp. are widely consumed by human populations (FAO, 2013) and used in aquaculture as fresh feed for herbivores (Robertson-Andersson, 2003). Indeed, they are among the algae most consumed by sea urchin species (Boudouresque and Verlaque, 2007), and they are widely employed as fresh feed in the stocking and rearing activities of *Paracentrotus lividus* (Shpigel et al., 2005; Paredes et al., 2015).

Due to their versatility and various applications, *Ulva* spp. is widely produced in the world (Boonstra, 2014). Among macroalgae, they are some of the most cultivated, with a production of about 4000 tons during 2010 (Table 3) (Boonstra, 2014). Like for other macroalgae, the cultivation of *Ulva* spp. is a complex practice, because they are often cultivated in natural waters, using only naturally available light, heat, water motion energy and nutrients, so the growing conditions are difficult to control (Boonstra, 2014).

Ulva spp. have a high nitrogen demand (Barr and Rees, 2003). Therefore, they grow well in the presence of sewage, sedimentation and urban pollution (Arevalo et al., 2007; Mangialajo et al., 2007; Eriksson and Johansson, 2005). For this reason, *Ulva* spp. are considered a target macroalgae for the bioremediation of nutrients (nitrogen and phosphorous) from aquaculture wastewater (Copertino et al., 2009; Neori et al., 2003).

It is well demonstrated that *Ulva* spp. effectively uptake nutrients dissolved in aquaculture wastewater derived from fish (Shpigel, 2013; Neori et al., 2004) or shrimp farms (Copertino et al., 2009). This results in a high nutrient-removal efficiency (from 40 up to 90%) and biomass yield (40–100 g of DW/m^2/day) (Neori et al., 2003, 2000, 1998, 1991; Schuenhoff et al., 2003; Martínez-Aragón et al., 2002; Jiménez del Río et al., 1994; Shpigel et al., 1993; Vandermeulen and Gordin, 1990).

The removal efficiency and growth of *Ulva* spp., however, are influenced by many factors. These are the characteristics of the cultivation system and the chemical nutrient balance of the seawater, as well as the temperature, salinity, solar irradiation, currents and waves (Titlyanov and Titlyanova, 2010; Copertino et al., 2009). An optimal growth rate of *Ulva lactuca* is dependent upon ideal water and light conditions (Neori et al., 1996; Vandermeulen and Gordin, 1990). For these reasons, the biomass production and

the nutrient removal efficiency of *Ulva* spp. are highly variable according to location and season (Neori et al., 1998).

The aim of this study is to evaluate and compare the efficiency of *Ulva* sp. in removing dissolved inorganic nutrients (nitrogen and phosphorous) while also comparing the biomass yield of *Ulva* sp. in two culture media, namely estuarine water and aquaculture wastewater, at natural environmental conditions in Sardinia (Italy) during the spring.

6.3 Materials and methods

Fresh thalli of *Ulva* sp. were collected in the nearby Mistras lagoon (39°90'N, 8°46'W) and transported to the laboratory inside a 30 L rectangular tank, containing natural estuarine water which was collected concurrently in the same site. In the laboratory, the thalli were carefully washed with fresh water to remove epiphytes and then cut up with scissors to form disks of a similar size and shape.

The thalli were cultivated in natural estuarine water (EW) and in grey mullet wastewater (WW), with three replicates per treatment. The estuarine water used to transport *Ulva* sp. to the laboratory was filtered (50 µm) to remove epiphytes and then used as a culture medium.

Aquaculture wastewater was provided from an experimental fish hatchery of grey mullet *Mugil cephalus* (Linnaeus, 1758), located at the International Marine Centre-IMC Foundation of Oristano (Sardinia, Italy). Fish were reared in a recirculating aquaculture system (RAS) consisting of four tanks with a volume of 2000 L, containing previously filtered (1 µm) natural seawater (NSW) at 37 ± 1 ppt salinity. The system was provided with a biological and mechanical filter, UV lamp and protein skimmer. A 30% water exchange was done once a week, and 80 L of the water exchanged was employed in the experiment.

Prior to the experiment, EW and WW were transferred to two separate rectangular tanks and 15 disks of *Ulva* sp. were put in each tank. The tanks were supplied with gentle aeration and located outside in natural environmental conditions for 24 h to acclimatize the *Ulva* sp.

The experiment was carried out during spring (March), when *Ulva* sp. grows well, in natural environmental conditions for 10 days. *Ulva* sp. was cultivated in six transparent cylindrical tanks with a volume of 5 L, with three tanks per treatment. The physical and chemical variables of the water (temperature, salinity, pH and dissolved oxygen) were measured every day.

6.3.1 Nutrient uptake and removal efficiency

Every two days, we assessed the nutrient uptake of *Ulva* sp. The assimilation of dissolved nutrients (nitrate, nitrite, ammonia and phosphorous) was analysed by using an automatic chemical analyser µCHEM based on Loop Flow Analysis (Systea, Italy). Efficiencies of fresh thalli in removing Dissolved Inorganic Nitrogen (DIN) and Dissolved Inorganic Phosphorous (DIP) were calculated following the method adopted by Michels et al. (2014).

6.3.2 Biomass yield and Specific Growth Rate

The biomass yield of *Ulva* sp. was evaluated according to the method adopted by Ale et al. (2011). All disks of *Ulva* sp. were scanned and the area was measured prior to inoculation. Before scanning, the disks were transferred to a white rectangular plastic tank containing a small volume of water in order to prevent the seaweed disk from drying. A transparent polycarbonate layer was used to cover the disks and ensure that the entire surface was flat; the disks were photographed with a Canon PowerShot G15 digital camera. Image Processing Analysis in Java (ImageJ 1.49V) was calibrated appropriately for image analysis and measurement. A length of 10 mm was measured using a ruler, the corresponding image was saved and then used to measure the area of each disk in mm^2.

Every two days the growth of the thalli was evaluated. The disk area growth was calculated using the formula: $A_G = [(A_i - A_0) / (A_c \times t)]$. A_G is the disk area growth ($mm^2/m^2/day$); A_i and A_0 are the disk area (mm^2) at the end and the beginning of the sampling interval, respectively; A_c is the area of the culture flask (0.2512 m^2) and t is the time (in days) elapsed between two successive samplings (Ale et al., 2011).

Specific Growth Rate (μ) as a percent increase in disk areal expansion was calculated using the formula: $\mu = 100 \times Ln (A_i/A_0)/t$. A_0 is the initial disk area and A_i is the disk area at time t (DeBoer et al., 1978).

Prior to the experiment, five disks with a known area were weighed, dried in an oven at 105 °C for 4 h and weighed again. The dry weight (DW) of disks was used to convert the disk area into DW, resulting in a conversion factor of 0.15 mg DW/mm^2.

The equivalent DW of the disk was used to calculate the biomass produced as the sum of the DW of all the disks (mg) on the culture flask per culture area of the flask (0.2512 m^2) per unit time (day) expressed in units of mg/m^2/day.

6.3.3 Statistical analysis

Data were analyzed by Statistica 6.1 StatSoft, Inc. (2004). Differences in the removal efficiencies between culture media and nutrients (DIN and DIP) were analyzed using a two-way analysis of variance (ANOVA). Differences in the nutrient uptake of DIN and DIP, biomass yield and SGR were analysed using repeated-measures ANOVA, with water as a factor and days as a repeated factor. Shapiro Wilk's W test was used to verify the normality of the data distribution and Levene's test was used to verify the homogeneity of variances. Tukey's honestly-significant difference (HSD) test was used to evaluate all pair-wise treatment comparisons ($p < 0.05$).

6.4 Results

The amount of inorganic nutrients dissolved in the culture media was measured prior to the experiment, and the results showed different concentrations in estuarine and wastewater (Table 10).

Table 10: Dissolve inorganic nutrient concentration of estuarine water and aquaculture wastewater.

	NO_2^- -N	NO_3^- -N	NH_4^+ -N	PO_4^{3-} -P
Estuarine water	0 mg/L	0.09 mg/L	0.41 mg/L	0.21 mg/L
Aquaculture wastewater	0.68 mg/L	12.09 mg/L	0.71 mg/L	2.27 mg/L

6.4.1 Nutrient uptake and removal efficiency

The assimilation of DIN and DIP by *Ulva* sp. differed between culture media, as well as between days ($p < 0.001$). DIN and DIP percentages gradually decreased from 0 to 10 days for both EW and WW treatments. DIN resulted in a higher nutrient uptake in EW than in WW from 2 to 8 days (Figure 30 and 31).

At the end of the experiment, *Ulva* sp. resulted in a higher DIN removal efficiency in EW (95.7 ± 0.3%, mean ± SE) than in WW (68.7 ± 1.0%) ($p < 0.001$). Moreover, significant differences were also highlighted between nutrients. WW resulted in a higher removal efficiency of DIP (87.1 ± 4.8%) than DIN (68.7 ± 1.0%), while EW showed a lower removal of DIP (83.1 ± 2.1%) than DIN (95.7 ± 0.3%) ($p < 0.001$) (Figure 32).

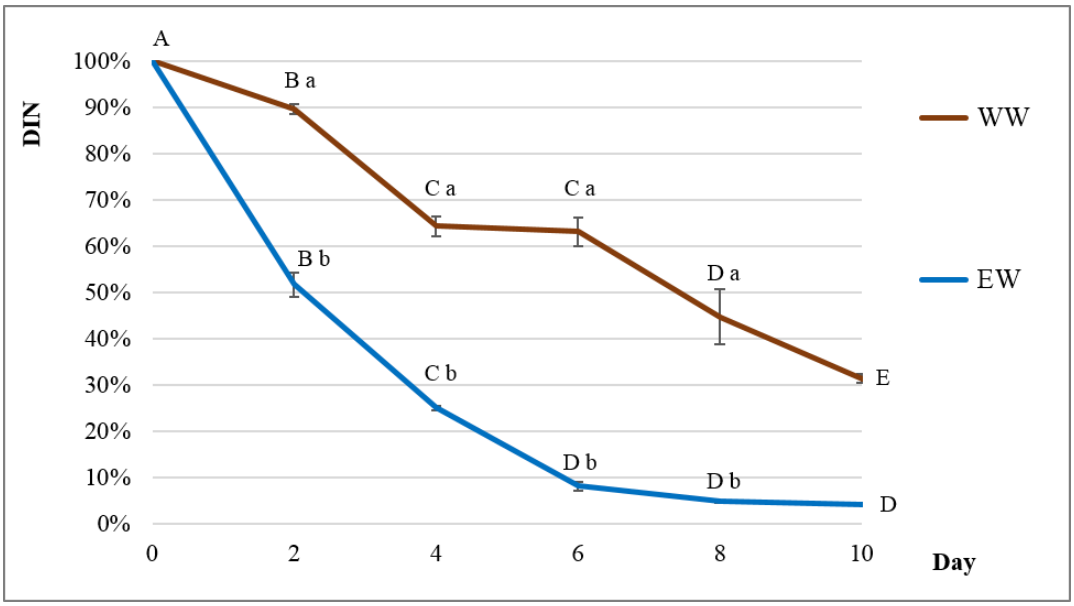

Figure 30: Efficiency assimilation (%) of Dissolved Inorganic Nitrogen (DIN) by *Ulva* sp. cultivated in estuarine water (EW) and aquaculture wastewater (WW). Capital superscripts indicate significant differences among days; lowercase superscripts indicate significant differences between culture media.

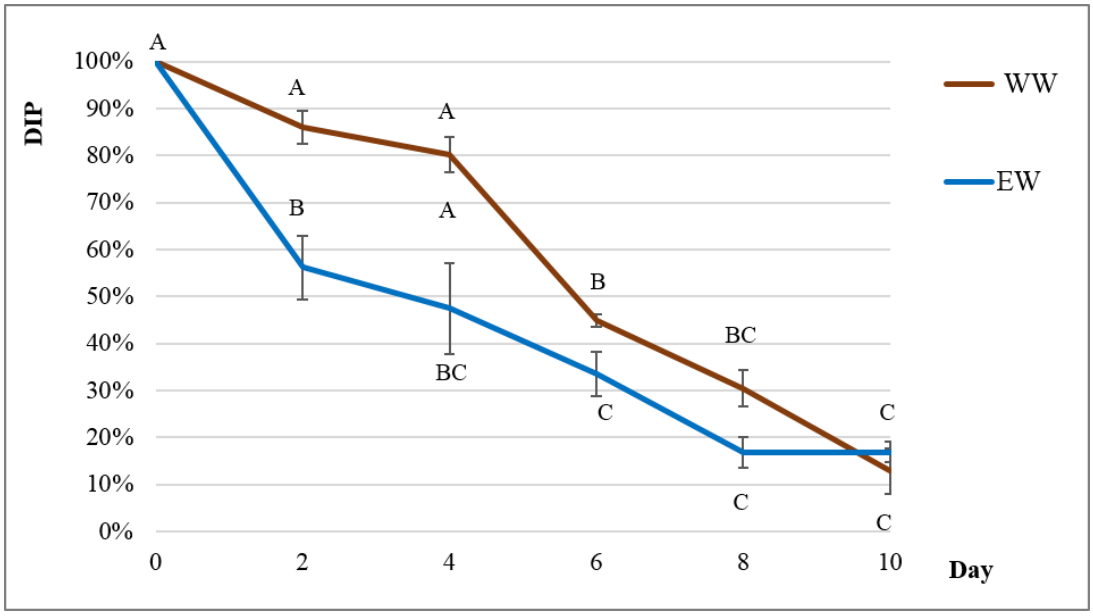

Figure 31: Efficiency assimilation (%) of Dissolved Inorganic Phosphorous (DIP) by *Ulva* sp. cultivated in estuarine water (EW) and aquaculture wastewater (WW). Capital superscripts indicate significant differences among days; lowercase superscripts indicate significant differences between culture media.

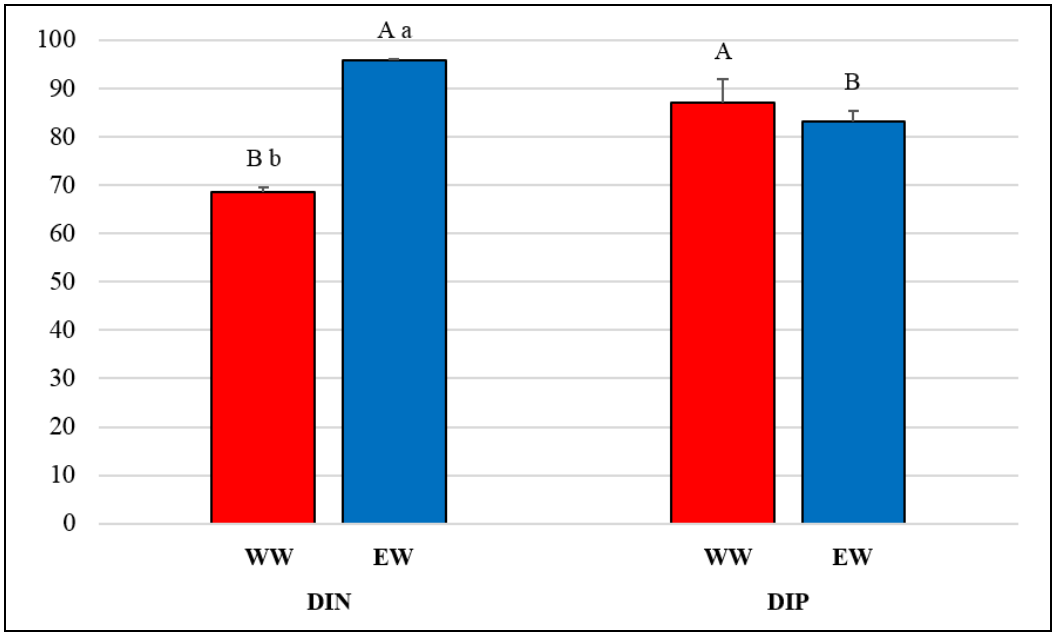

Figure 32: Removal efficiency (%) of Dissolved Inorganic Nitrogen (DIN) and Dissolved Inorganic Phosphorous (DIP) by *Ulva* sp. in aquaculture wastewater (WW) and natural estuarine water (EW). Capital superscripts indicate significant differences between culture medium; lowercase superscripts indicate significant differences between nutrients.

6.4.2 Biomass yield and Specific Growth Rate

Repeated-measures ANOVA showed significant differences in biomass yield of *Ulva* sp for different days of experimentation. It resulted in a higher biomass yield on days 2, 4, 6 and 8 (from 2465 ± 1067 to 4739 ± 700 mg/m^2/day, mean ± SE) in comparison to day 10 (from 652 ± 260 to 873 ± 225 mg/m^2/day) ($p < 0.01$). No significant differences resulted between the two culture media (Figure 33).

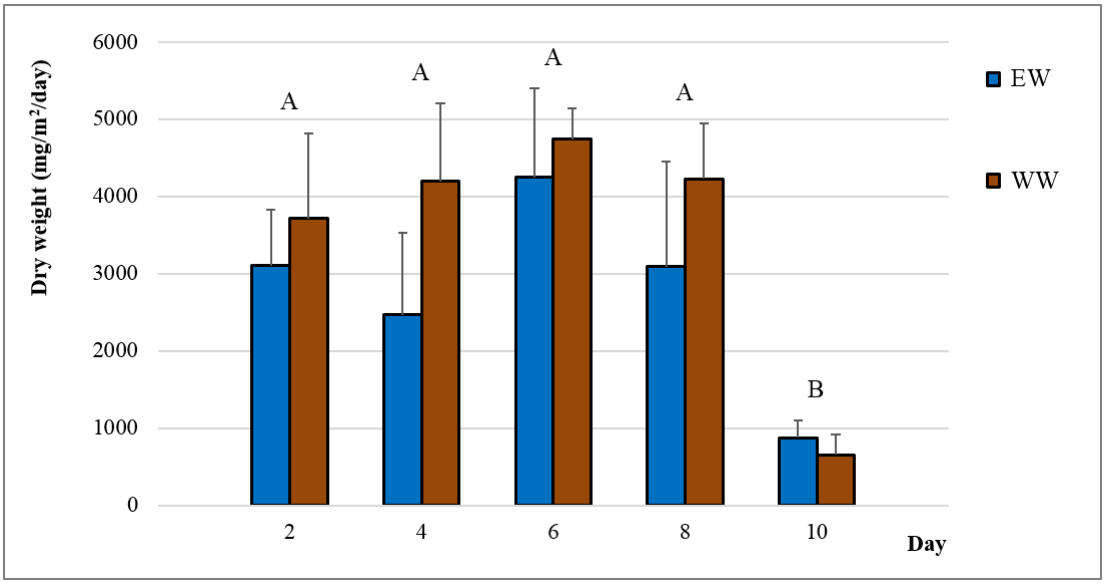

Figure 33: Biomass yield (mg/m^2/day) of *Ulva* sp. during 10-days of cultivation in estuarine water (EW) and aquaculture wastewater (WW). Capital superscripts indicate significant differences among days.

The maximum SGR of *Ulva* sp. was recorded at day 2 for EW (12.1 ± 2.0%/day, mean ± SE) and WW (15.0 ± 4.3%/day). Significant differences were recorded between days, with a higher SGR at day 2 than at day 10 ($p < 0.05$) (Figure 34).

6.5 Discussion

Ulva sp. showed a lower removal efficiency of DIN when cultivated in WW than in EW, while they had a similar pattern in the biomass yield in the two culture media.

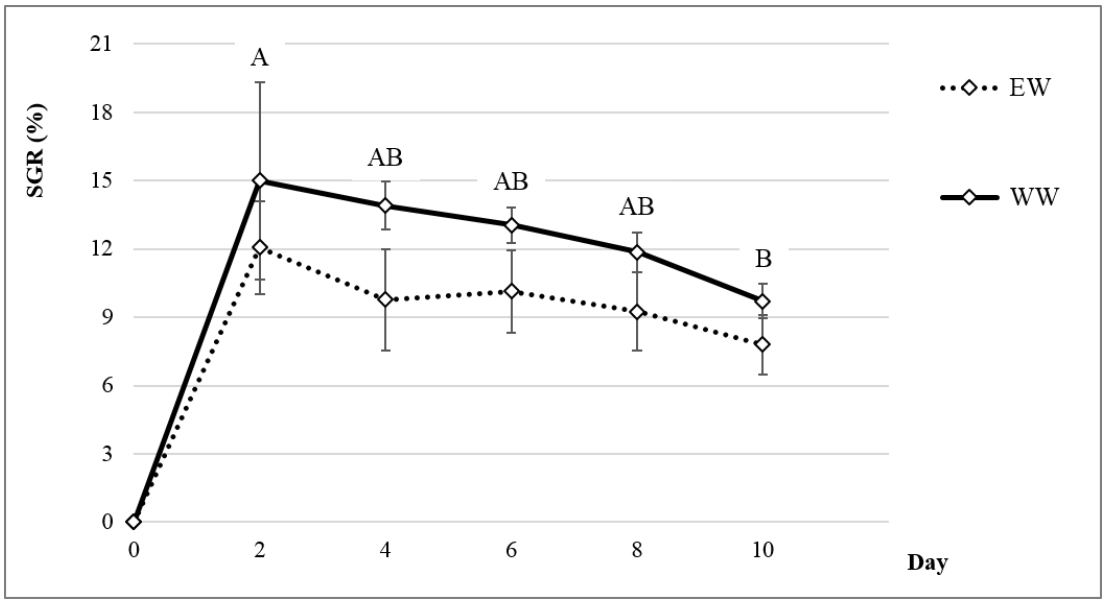

Figure 34: Specific Growth Rate (SGR) of *Ulva* sp. during 10-days of cultivation in estuarine water (EW) and aquaculture wastewater (WW). Capital superscripts indicate significant differences among days.

Our results showed a good removal efficiency of phosphorous (higher than 80%) in both media, and a good efficiency for nitrogen in EW (more than 95%). Although WW resulted in a lower assimilation of DIN (68.7 ± 1%) in comparison with EW, our results are similar to previous studies on integrated systems which reported removal efficiencies from 40 to 90% (Neori et al., 2003, 2000, 1998, 1991; Schuenhoff et al., 2003; Martínez-Aragón et al., 2002; Jiménez del Río et al., 1994; Shpigel et al., 1993; Vandermeulen and Gordin, 1990).

On the contrary, our results for the biomass yield of *Ulva* sp. cultivated in EW and WW (from 0.7 ± 0.3 to 4.7 ± 0.4 g DW/m^2/day) are low in comparison with previous literature data (from 40 to 100 g DW/m^2/day) (Neori et al., 2003, 2000, 1998, 1991; Schuenhoff et al., 2003; Martínez-Aragón et al., 2002; Jiménez del Río et al., 1994; Shpigel et al., 1993; Vandermeulen and Gordin, 1990). It is well known that under farm conditions, the growth and removal efficiency of *Ulva* spp. vary among species, local environmental conditions and characteristics of the cultivation system, due to a variable exposition to nutrient loads, salinity, turbidity, photoperiod and temperature (Copertino et al., 2009).

Previous studies reported a highly seasonal biomass production of *U. lactuca*, with a growth rate predominantly dependent on water temperature and light (Neori et al., 1998, 1996; Vandermeulen and Gordin, 1990). Neori et al. (1998) obtained the highest nitrogen biofiltration efficiency (88%) and fastest growth rate (412 g/m^2/day) of *U. lactuca* in summer at 25 °C. In the same study, these authors registered the lowest biofiltration efficiency (3%) and slowest growth rate (81 g/m^2/day) in winter at 20 °C. Similarly, Copertino et al. (2009) reported the highest growth rate and biofiltering efficiencies of *Ulva clathrata* in mid-summer (about 26.5 °C) and in conditions of low stocking density and nutrient supply. Differently by Neori et al. (1998) and Copertino et al. (2009), we recorded low temperatures during the entire experiment, from 19.5 ± 0.4 °C (mean ± SE) at 3 pm, to 12.1 ± 0.3 °C at 8 am. This could have negatively influenced the growth of *Ulva* sp.

However, we hypothesize that the low biomass yield obtained in this study was caused by the static cultivation system adopted. These results are similar to those reported by Copertino et al. (2009), who observed that the specific uptake rate of *U. clathrata* biomass was influenced by water flow, with the highest values recorded when the algae were cultivated in a continuous flow rather than in a static regime. Water flow velocity is a key factor for seaweed nutrient acquisition (Hurd, 2000) and its effects on the biomass production of *U. lactuca* have been widely demonstrated in other studies (Ale et al., 2011; Msuya and Neori, 2008).

In addition, similarly to Copertino et al. (2009), we observed sporulation events of *Ulva* sp. in both EW and WW culture media. Sporulation appeared as an increase in empty and white/transparent tissues, (the so-called "ghost tissue"), during which *Ulva* spp. stop growing before releasing the internal thallus content as zoids (spores or gametes) (Dan et al., 2002). In *Ulva* species, sporulation could be due to several environmental factors, such as high temperature, increased light and changes in nutrient supply. In our experiment, since we used natural light and temperature, we believe that the sporulation event was caused by the static cultivation system adopted, which determined a change in nutrient supply, as observed by Copertino et al. (2009). This would be consistent with previous studies showing that the biomass yields of *Ulva* spp. can be enhanced by a continuous effluent flow with high exchange rates and high aeration (Lüning and Pang, 2003; Neori et al., 2003; Schuenhoff et al., 2003).

6.6 Conclusions

The results of this study did not show differences in the growth of *Ulva* sp. in natural estuarine water in comparison with their growth in grey mullet wastewater. This demonstrates the potential use of this macroalga species in integrated systems. Due to their high biofiltering efficiencies for DIN and DIP removal, *Ulva* sp. could be used for the treatment of wastewater nutrients. We tested the efficiency of cultivation in spring, when *Ulva* sp. grow well in nature, but the short life cycle of the *Ulva* species can limit the cultivation of biomass production simultaneously with water biofiltration purposes. Instead of the cultivation system adopted here, the growth of *Ulva* sp. could be improved by employing a continuous effluent flow system, characterized by a high exchange rate and water movement. This would improve the acquisition of inorganic nutrients and avoid the release of zoids, which result in biomass disintegration.

7 GENERAL DISCUSSION

In the Mediterranean context, where overfishing causes the depletion of wild stock of the sea urchin population *Paracentrotus lividus*, echinoculture becomes crucial for bridging the gap between supply and demand of this product. It is also important for producing juvenile individuals for reseeding activities. At the same time, it is very important to ensure that environmental well-being is compatible with human well-being in order to make long-term sustainable prosperity a reality for all. To this end, promoting responsible and sustainable aquaculture systems, such as the Integrated Multi Trophic Aquaculture (IMTA), is central to this purpose.

In this thesis project, we evaluated the capability of producing *P. lividus* in an IMTA hatchery system characterized by the cultivation of its feed (micro- and macroalgae) in grey mullet *Mugil cephalus* wastewater (Figure 9). Summarizing, this thesis project consists of two main research lines: the study of the reproduction and rearing of *P. lividus*, and the cultivation of micro- and macroalgae in *M. cephalus* wastewater.

7.1 Reproduction and rearing of *Paracentrotus lividus*

The research done on the rearing of *P. lividus* aimed to increase knowledge and enhance the production of juvenile individuals (~15 mm in test diameter). These individuals, classified as subadults, can then be transferred into other tanks until they reach market size (50 mm) (Grosjean, 2001) or until they are suitably large for enhancing natural populations through reseeding activities (Tegner, 1989).

Nowadays, the echinoculture of *P. lividus* is limited by many factors and bottlenecks. The efficacy of juvenile production has to cope with a high mortality rate during the larval phase (Carboni et al., 2012) and within the first weeks of benthic life (Grosjean et al., 1998), as well as the highly variable and often low metamorphosis rates (Dworjanyn and Pirozzi, 2008; Huggett et al., 2006; Buitrago et al., 2005; Rahim et al., 2004; Grosjean et al., 1998; Gosselin and Jangoux, 1996; Pearce and Scheibling, 1991; Cameron and Hinegardner, 1974). Moreover, the planktonic larval phase is often long (e.g. 30-40 days) and therefore requires a substantial investment of resources (Dworjanyn et al., 2007).

Chapter 2, published in the scientific literature (Aquaculture Research, 2016, 1-11, doi:10.1111/are.12990), focused on the identification of a more suitable larval rearing method that has been largely ignored thus far. Our results demonstrated for the first time that the larval rearing of *P. lividus* does not require continuous seawater and live feed (microalgae) replacements. Conversely, the constant monitoring of phytoplankton consumed by the larvae optimizes the amount of feed necessary and reduces the production of debris and settled materials. This minimizes the need for seawater replacement. Although this method requires more technical labour and expertise, it has the potential to reduce manual labour, water volumes and phytoplankton requirements in a sea urchin hatchery. Moreover, the alternative variable method tested here improves the development and survival of *P. lividus* larvae and enhances the production without the need for physically disruptive operations such as siphoning, sieving and filtration, which can be stressful and could damage the larvae in suspension (Carboni et al., 2014; Russell, 2000; Strathmann, 1978).

In hatcheries, sea urchin larvae are typically induced to settle on a natural biofilm of diatoms (Mos et al., 2011; McBride, 2005; Harris et al., 2003; Shimabukuro, 1991), because this biofilm represents the first feed for newly-metamorphosed juveniles (Kawamura et al., 1983). On the basis of location and season, the biofilm is composed of diverse communities of algae (e.g. diatoms), bacteria and fungi, that often cause a high variability and unpredictability in larval settlement (Daume et al., 2000; Wieczorek and Todd, 1998). For this reason, it becomes important to identify an efficient and reliable alternative as a metamorphosis-inducing factor and first feeding item for *P. lividus* post-larvae (Daume et al., 2000; Wieczorek and Todd, 1998). As showed in Chapter 3 (Aquaculture, 2016, 465: 265-270), this alternative could be the macroalgae *Ulvella lens*, which resulted in a similar larval settlement in comparison with a natural biofilm of diatoms. The juvenile production of *P. lividus*, on the other hand, has been improved by using natural seawater previously "conditioned" by the presence of adult conspecifics.

For about eight days after settlement, metamorphosed individuals have not yet developed a mouth, anus or digestive system, so they are not able to eat (Grosjean, 2001; Gosselin and Jangoux, 1998) and are subject to high mortality rates (Grosjean et al., 1998). In Chapter 3, I demonstrated that the survival of post-metamorphic

individuals could be increased by using suitable diets during larval stage. Although the mixtures of *Isochrysis* affinis *galbana* (T-Iso) + *Dunaliella tertiolecta* and T-Iso + *Chaetoceros gracilis* + *D. tertiolecta* supported the larval development of *P. lividus*, they resulted in a 100% mortality rate at 100 days post-settlement. On the contrary, *D. tertiolecta* alone or mixed with *C. gracilis* resulted in 1.5 ± 1.5% and 3.0 ± 2.0% (mean ± SE) survival at 100 days post-settlement, respectively.

The somatic growth of juveniles (test diameter and the wet body weight) is influenced by stocking density (Chapter 4). As observed for the sea cucumber *Apostichopus japonicus* (Pei et al., 2012; Dong et al., 2010) and various species of abalone (Lloyd and Bates, 2008; Park et al., 2008) and shellfish (Parsons and Dadswell, 1992), the somatic growth of *P. lividus* decreases with increasing density. Due to a competition for space (Li et al., 2006; Nga et al., 2005), when individuals were stocked at a high density (400 individuals/m^2), the increasing diameter (0.9 mm/month) and wet weight (78 mg/month) were lower in comparison to when stocked at an intermediate density (250/m^2): an increase of 1.2 mm/month (diameter) and 92 mg/month (wet weight). The highest somatic growth, 1.5 mm/month (diameter increase) and 108 mg/month (wet weight increase) was recorded at the lowest stocking density tested (100 individuals/m^2). Considering this somatic growth, six-month-old individuals reach 15 mm in test diameter after 5.5, 7 and 9.5 months if stocked at low, intermediate and high densities, respectively.

7.2 Cultivation of micro- and macroalgae

The second research line of this thesis project aimed to assess the efficiency of the cultivation of the microalgae, *Isochrysis galbana* and *D. tertiolecta*, and the macroalga *Ulva* sp. in grey mullet *M. cephalus* wastewater. This was done with the aim of reducing the impacts of traditional aquaculture on the environment and to promote responsible and sustainable IMTA systems.

A previous trial showed that *C. gracilis* did not grow well in *M. cephalus* wastewater, probably due to a low amount of dissolved silicate present, so it was decided not to test this species. On the contrary, the cultivation of *I. galbana* and *D. tertiolecta* was tested in fully-controlled bubble column annular photobioreactors and compared to another

microalga species, *Tetraselmis suecica,* which tested positively for the bioremediation of aquaculture wastewater (Michels et al., 2014; Sirakov and Velichkova, 2014; Borges et al., 2005) (Chapter 5, in preparation for submission).

D. tertiolecta and *T. suecica* were found capable of removing more than 90% of the dissolved inorganic nitrogen (DIN) and phosphorous (DIP) in the wastewater. This indicates that these species are suitable for use in bioremediation, as previously observed for *T. suecica* (Michels et al., 2014; Sirakov and Velichkova, 2014; Borges et al., 2005). Although *D. tertiolecta* resulted in a lower biomass yield in comparison with *T. suecica,* a single photobioreactor was able to produce 94.8 ± 4.32 (mean ± SE) billion cells of *D. tertiolecta* in three days (data not reported in Chapter 5). Considering that one litre of *P. lividus* larval culture (density of 1.5 larvae/mL) needed 13.2 ± 0.1 million cells of *D. tertiolecta* every three days (data not reported in Chapter 3), two photobioreactors working continuously are able to produce live feed for more than 7000 L of *P. lividus* larval culture. On the contrary, *I. galbana* was found unsuitable for the bioremediation of *M. cephalus* wastewater, resulting in a low removal efficiency of nitrogen (66.0 ± 1.5%) and biomass yield at the end of the experiment, probably due to the presence of ciliate *Paramecium* spp.

It has been demonstrated that microalgae cultures with protozoans as *Paramecium* spp. represent suitable diets for fish fries (FAO, 1980), but they could negatively influence the growth of *P. lividus* larvae. Wichterman (1986) reported that *Paramecium* spp. effectively feeds on other live microorganisms (i.e. microalgae), therefore it could compete with larvae for feeding, when they are fed with contaminated microalgae cultures.

Positive results for the assimilation of dissolved nutrients were also obtained with the macroalga *Ulva* sp. (Chapter 6, in preparation for submission). We did not record a total assimilation of DIN (68.7 ± 1.0%) or DIP (87.1 ± 4.8%) at the end of the experiment, but it is probable that *Ulva* sp. needs more than 10 days (the length of our experiment) to assimilate all dissolved nutrients in the grey mullet wastewater. The results indicate that this macroalga species can be used in Sardinia for bioremediation of aquaculture wastewater with success, as observed in other geographic regions, such as Israel (Schuenhoff et al. 2003; Neori et al., 1998, 1991; Shpigel et al., 1993; Vandermeulen and Gordin, 1990), Brazil (Copertino et al., 2009) and Spain (Jiménez del Río et al.,

1994). In this experiment, a biomass yield of about 4.5 g was obtained in ten days. Therefore, we estimate an *Ulva* sp. production of about 45 g and 450 g employing tanks 10x (50 L) and 100x (500 L) larger than the ones we used (5 L). Considering that each juvenile sea urchin needs about 16.5 g of *Ulva* sp. at low density (Chapter 4, feed intake data) to reach 15 mm in test diameter, it is possible that tanks of 50 and 500 L could produce enough live feed for the growth of only 3 and 39 individuals of *P. lividus*, respectively. However, the success of *Ulva* spp. cultivation depends on the improvement of the cultivation method and management protocol, as well as on the season (Copertino et al., 2009; Neori et al., 2004). Hence, our experiment showed that the biomass yield of *Ulva* sp. could be enhanced during the summer months with an average temperature of 25 °C (Copertino et al., 2009; Neori et al., 1998) or by improving the cultivation method, for instance by supplying the tanks with a recirculating system (Ale et al., 2011; Msuya and Neori, 2008).

7.3 Conclusions and further studies

With this thesis project, I demonstrated that it is possible to re-use the wastewater of the grey mullet *M. cephalus* to produce live feed for the larvae (*D. tertiolecta*) and juveniles (*Ulva* sp.) of *P. lividus*. Nevertheless, while photobioreactors allow for the production of phytoplankton for feeding millions of larvae, the cultivation system of *Ulva* sp. is quite poor and needs to be improved to ensure a higher biomass production. On the other hand, the production of juvenile individuals of *P. lividus* in laboratory-controlled conditions is feasible. The adoption of the alternative variable rearing method tested here, coupled with feeding larvae with suitable diets (*D. tertiolecta* alone or mixed with *C. gracilis*), has enabled us to obtain a good larval development and survival at competence. The macroalga *U. lens* represents a valid alternative to the traditional biofilm of diatoms as a metamorphosis-inducing factor. Employing seawater previously "conditioned" by adult conspecifics improves larval settlement. Finally, stocking juveniles at low densities (100 individuals/m^2) and feeding them with the fresh thalli of *Ulva* sp. allows us to obtain individuals with a test diameter of 15 mm in about 13 months.

Nevertheless, the IMTA system tested in this thesis project for the production of sea urchins and feed has only been tested at the laboratory scale. In order to verify the

applicability of these methods to a large-scale production system, further studies would be needed at greater volumes, with a higher number of larvae and juveniles and a higher biomass yield of *Ulva* sp.

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