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EVALUATION OF A MULTITROPHIC BIOFILTRATION SYSTEM WITH NEW ALGAE SPECIES AND THE SEA CUCUMBER *Holothuria sanctori*.

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Trabajo realizado en las instalaciones del Parque Cientifico Tecnologico de Taliarte, el ICCM y el Centro de Biotecnologia marina de Las Palmas de Gran Canaria, España, bajo la dirección del Dr. Ricardo Haroun Tabaue y el Dr. Juan Luis Gomez Pinchetti.

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Hay sol bueno y mar de espuma…
A mi abuela.

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ABREVIATIONS LIST

BCM	Botanica Ciencias del Mar
FAO	Food and Agriculture Organization
GIA	Grupo de Investigacion en Acuicultura
ICCM	Instituto Canario de Ciencias Marinas
IMTA	Integrated Multi-Trophic Aquaculture
NUE	Nitrogen Uptake Efficiency
NUR	Nitrogen Uptake Rate
OM	Organic Matter
P	Production rate in dry weight
PCT	Parque Cientifico Tecnológico de Taliarte
SA	Surface Area
SD	Standar deviation
TAN	Total Ammonia Nitrogen
TOM	Total Organic Matter
ULPGC	Universidad de Las Palmas de Gran Canaria
UV	Ultra violet
μ	Average daily growth in percentage

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ABSTRACT

EVALUATION OF A MULTITROPHIC BIOFILTRATION SYSTEM WITH NEW ALGAE SPECIES AND THE SEA CUCUMBER *Holothuria sanctori*.

By: Luis Felaco **Advisors:** Dr. Ricardo Haroun Tabraue and Juan Luis Gómez Pinchetti

Aquaculture has been increasing rapidly in the past decades and it will continue to increase, this means that the release of nutrients associated with it will also increase; one of the proposed mitigation methods for this is the use of biofiltration systems and organisms. The aim of this study was to evaluate and implement potential new marine species with sediment and water biofiltration capacities of the nutrients from a land-based aquaculture production unit. The organisms associated with the effluents from two inland aquaculture facilities were studied; four species of algae were collected and tested for biofiltration and production under tank conditions *Colpomenia sinuosa*, *Valonia utricularis*, *Ulva rigida* and *Schizymenia dubyi*. *H. sanctori* was collected and placed in tanks with sediments from the aquaculture facility; total nitrogen and organic matter in the sediments were evaluated during a three week period where the effluents from the sea cucumber tanks were connected to *Hydropuntia cornea* tanks, the biofiltration and production of *H. cornea* submitted to the effluents was also tested, finally, a rope culture experiment with *H. cornea*, *Grateloupia imbricata* and *Grateloupia turuturu* was made at the biofilter facility of the PCT (Scientific and Technological Park of Taliarte). *U. rigida* presented the best growth rate, production and nitrogen uptake efficiency, followed by *S. dubyi* which presented the best nitrogen uptake rate. *H. sanctori* was an efficient biofilter of nitrogen in the sediments, returning them to the control conditions; they increased the amount of ammonia available for the growth of *H. cornea* although no significant differences were found in the growth rate, production, physiological state or biofiltration between *H. cornea* cultured with these effluents or directly from the effluents of the sedimentation tank, probably due to the little amount of ammonia present in both. The rope culture experiment showed that it would not be a useful method to stock algae at the biofilter facility from the PCT and a different operational approach increasing the surface for the natural growth of algae should be used.

Keywords: Biofilter, Seaweeds, *Holothuria sanctori*, Sediments, Ecosystemic approach.

1. INTRODUCTION

Aquaculture nutrients release

Aquaculture and specifically, marine aquaculture, has experienced a rapid increase in the past years while wild captured fish have been experiencing stagnation and in some cases decline; this indicates that fish production will increasingly come from aquaculture systems (FAO 2014). However, some current aquaculture practices have potential negative impacts in the environment and, as they increase, the impact on the environment is expected to worsen (Haroun and Makol, 2007).

These negative impacts are primarily due to the release of nutrients, mainly dissolved nitrogen and particulate phosphorus and other compounds that can cause eutrophication into the water column and sediments (Troell *et al.*, 1999; Neori *et al.* 2000; Neori *et al.* 2003; Matos *et al.*, 2006). Thus, intensive culture systems can modify the natural cycling of nutrients, increasing the release of dissolved nutrient and solid excreta and other particulate matter compared to natural environments, these discharges can also alter the sediment biogeochemistry (Honkanen and Helminen 2000, Smaal 1991, Wu*.* 1995, Bouwman *et al*. 2013) and exceed the assimilative capacity for nutrients in the environment, leading to the development of hypoxia (e.g. Honkanen and Helminen 2000) and, due to changes in nutrient quality the promotion of nutrient environments which may favor harmful algal blooms potentially killing or intoxicating the cultured organisms especially in coastal and sheltered areas with little water circulation (Bouwman *et al*. 2013) which may also cause changes in biodiversity.

In finfish aquaculture systems with the addition of exogenous feed, which represent the major nutrient impact from mariculture, although shellfish production represents the majority of total mariculture production by gross tonnage (Bouwman *et al*. 2013), the average efficiency of nutrient retention by the reared organisms is less than 35%. The dissolved part of this excess is mainly nitrogen (N) principally in the form of dissolved ammonia (Porter *et al.*, 1987) while the particulate part is composed mainly by phosphorus (P).

In order to keep aquaculture growth it is necessary to minimize further nutrient effects from the rapidly expanding mariculture sources, with the development of sustainable environmentally sound production and the use of ecological engineering tools (Neori *et al*., 2004, Abreu *et al*. 2011, Bouwman *et al*. 2013)

Currently, most research focuses on culture techniques and effluent treatment methods that reduce the concentration of nutrients in the effluents as well as the volume of them in aquaculture systems, there is a big necessity for this type of research to be broadened and deepen (Phillips *et al*. 2011).

Integrated Multitrophic Aquaculture

In the literature available there have been various proposed techniques for the treatment of the effluents in aquaculture, one of them being the use of biofiltrating organisms to extract the excess nutrients in the water column. One of the main practical approaches in this subject is bacterial dissimilation into gases. Where, bacteria transforms nutrients into gaseous N_2 and CO_2 through a series of oxidation and reduction processes, however, this process is complicated and expensive technology is necessary making bacterial biofiltration suitable only for relatively small land based operations (Neori *et al*. 2004, Crab *et al*. 2007, Martins *et al*. 2010).

The other and more practical approach is the use of extractive organisms which, as the name implies, take out their nourishment from the environment, they, if properly cultured, can turn the excess nutrients into commercial crops and loaded effluents into clean water being this the main concept for Integrated Multitrophic Aquaculture (IMTA) (figure 1). The two economically important marine organisms that fall into this category are bivalve mollusks and macro and microalgae (Neori *et al*., 2004).

Seaweed production and biofiltration is based in the capacity of plants to assimilate nutrients into biomass. plants use solar energy and the nutrients in the effluents (particularly C, N and P) to photosynthesize new biomass (Neori *et al*. 2004); regardless of their importance in biofiltration and Integrated multitrophic aquaculture, there are only few successfully tested seaweed species and information on their economic viability is scarce (Neori *et al*., 2004; Troell *et al*., 2003). The diversity of species trialed in bioremediation systems to date is very limited and the majority of these studies have been focused on temperate aquaculture systems and its organisms (Troell *et al*.

2009). Although several macroalgal species have been shown to be efficient biofilters (Phillips *et al*. 2011), to date, only a handful of seaweeds have been thoroughly investigated for their aquaculture and/or bioremediation potential (Neori *et al*. 2004).

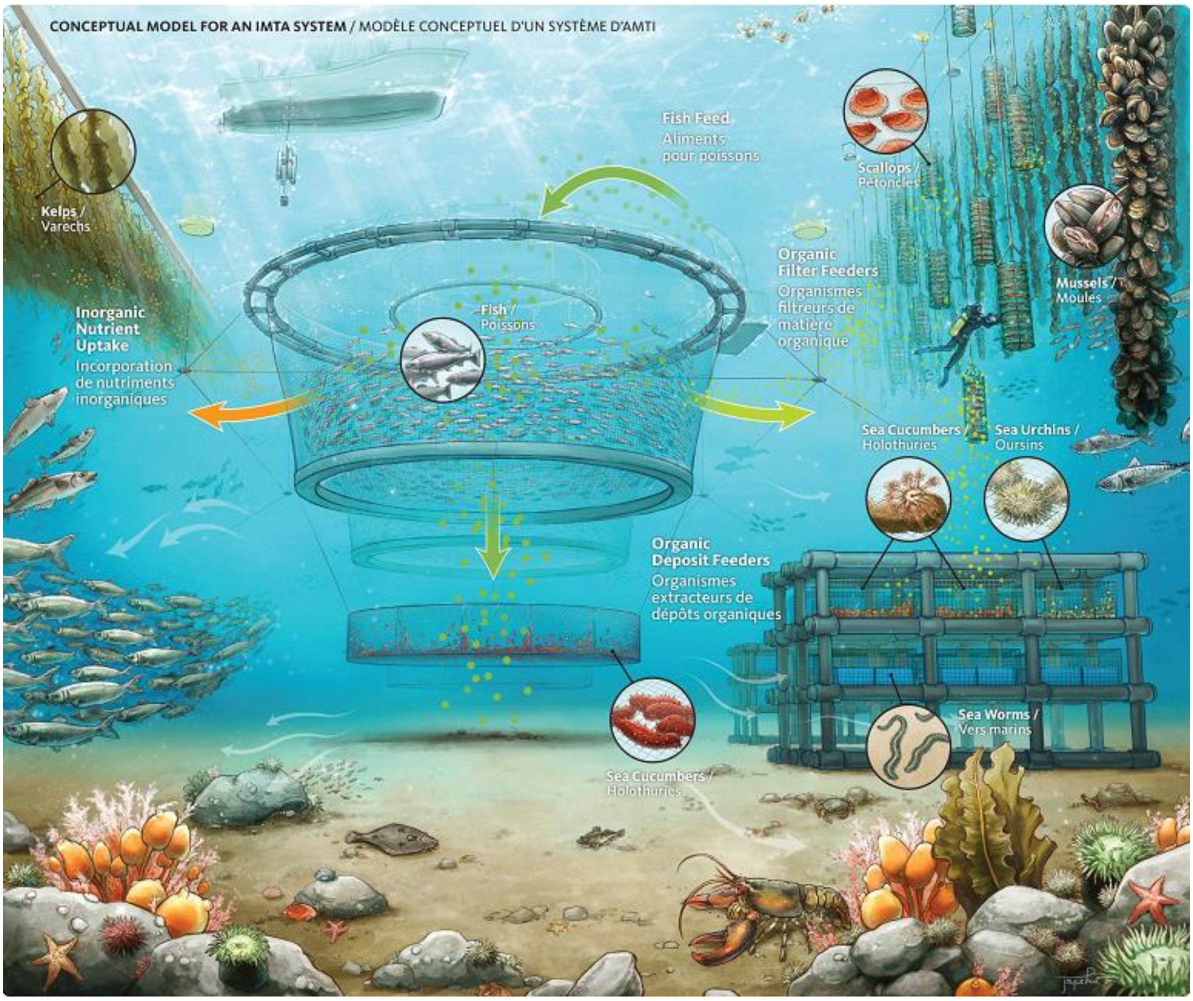

Figure 1: Conceptual model of the ideal IMTA system (from Fisheries and Oceans Canada 2013).

Although some species, for example in the genus Ulva and Enteromorpha, have been identified as ideal candidates for biofiltering fish effluents (Neori *et al*. 2000), since they both fulfill the basic requirements for an efficient biofilter system, none of them have a high market value (Neori *et al*. 2004). To further implement seaweed as biofilter organisms they need to be financially sustainable, to achieve this, various aspects have to be considered. Firstly, the commercial value of the species and its potential market and, secondly, the physiological characteristics of the species and its ability to grow in culture conditions and to resist and remove dissolved nutrients efficiently (Buschmann *et al*. 2001[a]; Chopin *et al*. 2001; Buschmann *et al*. 2008).

The choice of seaweed species for inclusion in an integrated aquaculture system must depend upon meeting a number of basic criteria: high growth rate and tissue nitrogen concentration; ease of cultivation and control of life cycle; resistance to epiphytes and disease-causing organisms; and a match between the ecophysiological characteristics and the growth environment, a biofilter seaweed species must grow very well in high nutrient concentrations, especially ammonium (Neori *et al*. 2004).

Resistance to epiphytes (as well as to small herbivorous animals) is a biological requisite of a biofilter seaweed species. Epiphytes are pest seaweeds and microalgae that use the cultured seaweed or the walls of the pond, tank or rope as substrates and compete for nutrients and light. The growth of these organisms can be prevented, their growth rate can be slowed down or they can sometimes be selectively killed off, for example, one of the most prevalent epiphytes in biofilter systems is precisely the genus *Ulva* their high growth rates, low epiphytism susceptibility, and their worldwide distribution (Msuya and Neori 2002; Muñoz *et al*. 2011) are in fact some of the characteristics that make them efficient biofiltering species.

In addition, given the ecological damage that may result from the introduction of nonnative organisms, it would be preferred if the seaweed is a local species. Beyond these basic criteria, the choice of seaweed will be influenced by the intended application. If, the focus is placed on the value of the biomass produced or if the principal focus is the process of bioremediation, in any case, the optimal system would include a seaweed species that incorporates both value and bioremediation (Neori *et al*. 2004, Schuenhoff *et al*. 2006, Muñoz *et al*. 2011)

The rate of areal total ammonia Nitrogen (TAN) uptake (grams N taken up per square meter per day) is an economically critical feature in seaweed biofilters. Another critical feature of a seaweed biofilter is the N uptake efficiency, the fraction of TAN concentration in the raw effluents removed as they pass through the biofilter. Higher ammonia uptake efficiencies reduce the rate at which the fish/shrimp pond water has to be recirculated. The dependence of TAN uptake rate and of uptake efficiency on TAN load is inversely proportional to each other. A seaweed biofilter has to be N starved to remove TAN with a high efficiency. Unfortunately, N-starved seaweed biofilters perform poorly with respect to the other three important biofiltration parameters—areal TAN uptake rate, yield and protein content. Hence, it is not possible to achieve in one-stage seaweed biofilters that are high in both TAN uptake rates and TAN uptake efficiencies (Cohen and Neori, 1991; Chopin *et al*., 2001; Troell *et al*., 2003).

The main form of seaweed culture for biofiltration is land-based culture in tanks, ponds and ditches; this has been developed in several places following the bottom-aerated pond approach developed by Ryther in the 1970s which consists in the vertical movement, by bottom aeration, of seaweed suspensions in tanks and ponds, and the passage of nutrient-laden water through them. The vertical movement of an optimally stocked seaweed pond allows each algal frond to be exposed to an optimal light dose. The water turbulence generated by the aeration thins the hydro-boundary layer around the frond surface, speeding the inflow to the fronds of nutrients and outflow from the frond of excess oxygen (Neori *et al*. 2004).

Commercial applications

Commercially, seaweeds have been used as food, fertilizers, fodder, pigments, secondary metabolites and phycocolloids such as agar, carrageenan and alginates (Gellenbeck and Chapman 1983). The value of these applications represent a total of US $5.5 – 6 billion a year (McHugh *et al*. 2001; Critchley *et al*. 2006). The primary use for these plants is as a source of food in Asia where *Laminaria japonica*, *Undaria* sp. and *Porphyra* sp. represent the main consumed species.

On the other hand, Industrial utilization is mainly based on the extraction for phycocolloids which are widely used in the food, pharmaceutical and cosmetic industry (Chapman and Chapman 1980). Being Gelidium and Hydropuntia the main genera for extraction of agar (Armisen & Galatas 2000), while *Eucheuma* spp. and *Kappaphycus alvarezii* are a source of carrageenan (Ask *et al*. 2003; Muñoz *et al*. 2004).

As source of fertilizers, some species, mainly brown algae, are commonly utilized as soil conditioners and fertilizer (Blunden. 1991). The large amounts of insoluble carbohydrates in brown seaweeds act as soil conditioners improving aeration and soil structure, especially in clay soils, and have good moisture retention properties. Some studies also show that extracts of brown algae can improve growth rates and reduce pests, consequently increasing crop yields as well as improving the overall quality of the product (Blunden *et al*. 1996; Leach *et al*. 1999).

A more recent approach to seaweed use is as source of fuel and as carbon sinks (Packer. 2009), considering the possible use of large areas of cultivated seaweed, as a tool to mitigate global warming (Muraoka. 2004). CO_2 acquisition by marine macroalgae can represent a considerable sink for anthropogenic CO_2 emissions and harvesting and appropriate use of macroalgal primary production could play a significant role in carbon sequestration and amelioration of greenhouse gas emissions (Chung *et al*. 2011).

At present there are approximately 200 species of seaweeds used worldwide (Zemke - White and Ohno 1999), of which the following species or genera are intensively cultivated: *Laminaria japonica*, *Undaria pinnatifida*, *Porphyra* spp., *Eucheuma* spp., *Kappaphicus alvarezii*, *Hydropuntia* spp., *Monostroma* spp. and *Enteromorpha* spp. (Lüning and Pang 2003).

The inability of traditionally high value genera such as *Hydropuntia*, *Porphyra* and more recently targeted high yield species such as *Asparagopsis* (Dawes *et al*. 1999; Matos *et al*. 2006) to survive the broad environmental fluctuations of tropical pond - based systems provides an impetus for the selection of alternative bioremediation species (Muñoz *et al*. 2011).

Most of the technology for integrated multi - trophic aquaculture (IMTA) strategies already exists, however it has not been properly implemented in a large, marketable, commercial scale (Buschmann *et al*. 2009). Furthermore, seaweed biofiltration of fish farm effluents has not been adopted by the aquaculture industry.

Since most seaweeds are commercially less valuable than cultured marine animals, and considering that one of the most costly component of diets used in aquaculture is protein (Neori *et al*. 1998), One proposed way to increase the economic viability of seaweed biofilters has been to feed the biomass produced to commercially valuable macroalgivores which can convert large quantities of the low-valued seaweed into highly valued commodities (Shpigel and Neori, 1996) such as sea urchins and abalone.

The nutritional value of seaweeds differs according to the species and the family considered. For example, the red seaweeds contain a high level of proteins (Fleurence, 1999; Fujiwara-Arasaki *et al*. 1984) while the green seaweeds, such as *Ulva lactuca*, show a lower protein level and brown algae, such as *L. japonica*, have the lowest protein content. However, seaweed reared in fishpond wastewater effluents regularly increase their protein content and present a significantly higher dietary value compared to algae reared in regular saltwater. This translates to better growth performance as experiments in *Haliotis tuberculata coccinea* (Viera *et al*., 2011) and other commercially important species such as the sea urchins *Psammechinus miliaris* and *Paracentrotus lividus* (Cook and Kelly, 2006) have showed.

These improvements are significant considering that a limiting factor for further expansion of abalone and other macroalgivores aquaculture is the restricted availability of an economically and environmentally sustainable feed, as these cultures frequently require large quantities of wild harvested macroalgae (Viera *et al*., 2005) and that animals fed mixed diets perform significantly better than those fed a single algal diet (Viera *et al*., 2011).

The use of macroalgae as biofilters and as valuable mariculture products is not limited to land based installations, an alternative approach in seaweed pond and ditch culture, using stakes and ropes, has emerged from the technologies used for large-scale coastal seaweed farming of Southeast Asia. This approach, however, has not provided the nutrient removal rates and the areal yields necessary for intensive mariculture, for reasons probably related to insufficient water turbulence, high turbidity and low nutrient concentrations, however, with the increase of interest in offshore aquaculture, they have been shown to work even in harsh offshore conditions (Buck and Buchholz. 2004, Neori *et al*. 2004, Garcia 2012; Leonczek 2013) mostly using long line seaweed aquaculture techniques increasing its potential as integrated multi trophic aquaculture species.

Detritivores in IMTA

Issues such as solid waste management, nutrient recycling and feed conversion enhancement are also more easily and profitably addressed on an industrial scale on land than in open-water fish farms (Neori *et al*., 2004), however, with the expansion of cage aquaculture, there is a need for treating these solid waste directly in open water conditions, that is the reason why other complementary integrated approach for the reduction of the environmental impact on the sea bottom by the net pen sludge has been the culture underneath them of scavengers like sea cucumbers as secondary crops (Ahlgren, 1998) using them as sort of animal biofilter for sediments.

In this way, the integrated multitrophic aquaculture approach is not limited only to the production of macroalgae from the effluents of traditional feed aquaculture, it has also been tested as food source for suspension feeders and detritivores, this gains importance considering that the most significant polluting components of aquaculture effluents is not only ammonia, but also, suspended solids (Tovar *et al*. 2000).

Organic fouling debris made up of fish feces, excess fish food, algae, invertebrates, and other particulate organic matter can accumulate along the sides and floor of the floating pens and on the sea floor below, clogging the nets and restricting water circulation and possibly depleting oxygen in the water and sediments which in turn can stress fish. The feeding habits of some species of sea cucumbers can alleviate fouling debris problems at salmon mariculture facilities this was demonstrated by Ahlgren in 1998 who conducted experiments in which red sea cucumbers *Parastichopus californicus* were allowed to feed inside floating net pens at a salmon rearing facility in Southeast Alaska. These experiments showed that sea cucumbers consumed fouling debris and cleared a significant amount of surface area on the nets.

By doing this, sea cucumbers can take advantage of much of the fouling debris which is derived from protein-rich organic matter such as fish food, both before and after it has passed through a fish gut and even dead fish fry, turning harmful debris into a marketable product (Ahlgren. 1998).

These impacts do not happen only in fish aquaculture, it has been demonstrated that dense assemblages of filter-feeding bivalves enhance the vertical flow of organic matter towards the benthic environment. Palzat *et al.* (2008) gathered evidence that sea cucumbers will utilize, and therefore reduce, the benthic organic deposition from oysters given that when grown in co - culture showing both active selection of organic material from the sediments and digestion/assimilation of these organics in the gut. This suggests the feasibility of developing a commercial-scale co-culture system that would both reduce the amount of organic deposition underneath shellfish farms and produce a secondary cash crop (Paltzat *et al*. 2008).

There have also been trials for the polyculture of shrimp and sea cucumbers (*Holothuria scabra*) but their success has been limited. In several trials, it seemed that shrimp caused the deaths of sea cucumbers, and in some cases it was clear that they did so (Pitt *et al*. 2004). In other trials, survival and growth of sandfish reared with shrimp for 3 weeks were significantly lower than for sandfish reared alone. Confirmed that co-culture is not viable. All sandfish reared in co-culture were dead or moribund after a month (Bell *et al*. 2007).

Some species of sea cucumbers have been reported to be able to eat up to 76 % of Total organic matter (TOM). Zamora and Jeffs in 2011 demonstrated the ability of the sea cucumber *Australostichopus mollis* to use different levels of TOM to generate similar growth rates mainly by changes in their feeding behavior and digestive physiology. The TOM, in the biodeposits sampled beneath mussel farms ranged from 2.8% to 18.2% which were within the range covered in the laboratory feeding experiments (Zamora and Jeffs, 2012). Other diets have been tested by Yuan and collaborators (2006), they reported that a mixed diet containing mostly mussel feces and 25 % powdered algae showed the best standard growth rate.

In the Canary Islands the main representatives of Holothurians are *Holothuria mammata*, *Holothuria arguinensis* and *Holothuria* (Platyperona) *sanctori* Delle Chiaje 1823 the latter species is the most abundant in the archipelago and very conspicuous in the Mediterranean and adjacent eastern Atlantic where it has commercial value (Aydin, 2008). It shows a markedly nocturnal behavior, with larger distances and fastest displacements during the end of the nighttime, remaining hidden in cracks and crevices during daylight and moving at night to feed (Pérez-Ruzafa *et al.* 1984; Pérez-Ruzafa and Marcos 1987) *H. sanctori* is one of the most selective species when it comes to the selection of organic matter from the sediments (Mezali and Soualili. 2013).Its consumption of OM has been shown to increase with OM availability, particularly during formation of the gonads (Navarro *et al*. 2013[a]).

Economic value and uses of sea cucumbers

Sea cucumbers, can be very marketable specifically on their dried form also called beche-de-mer they have been a dietary delicacy and medicine for Asians over many centuries. The collection of sea cucumbers to supply the market has seen a depletion of this resource in the traditional fishing grounds close to Asia and more recently the expansion of this activity to new and more distant fishing grounds. Currently, there are fisheries harvesting sea cucumbers across most of the resource range, including remote parts of the Pacific, the Galapagos Islands, Chile and the Russian Federation. Sea cucumber stocks are under intense fishing pressure in many parts of the world and require effective conservation measures. Sea cucumbers provide an important contribution to economies and livelihoods of coastal communities, being the most economically important fishery and non-finfish export in many countries. A multitude of sea cucumber species are being exploited worldwide, with new species being brought to market as established species become scarcer and more difficult to find. Little is known about the ecology, biology and population status of most commercial species. (Toral-Granda *et al*., 2008)

The majority of sea cucumbers are exported for the beche-de-mer market and few species for the live trade (aquarium) market. In many countries, particularly in the Western Pacific region, some sea cucumbers or their organs are consumed as delicacies or as a protein component to traditional diets (Toral-Granda *et al*. 2008). They have high commercial value coupled with increasing global production and trade. Sea cucumbers have long been used for food and medicine in the communities of Asia and Middle East. Nutritionally, sea cucumbers have an impressive profile of valuable nutrients such as Vitamin A, Vitamin B1 (thiamine), Vitamin B2 (riboflavin), Vitamin B3 (niacin), and minerals, especially calcium, magnesium, iron and zinc. A number of unique biological and pharmacological activities including anti-angiogenic, anticancer, anticoagulant, anti-hypertension, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, antitumor and wound healing have been described for various species (Bordbar *et al*. 2011)

Aquaculture, sea ranching and restocking have been evaluated as possible solutions to wild sea cucumber overexploitation, and some countries have started such ventures (e.g. Australia, China, Kiribati, Philippines, Viet Nam and Madagascar) China is successfully producing an estimate of 10,000 tonnes dry weight of *Apostichopus japonicus* from aquaculture, mainly to supply local demand (Toral-Granda *et al*., 2008).

In the last years, the research group in Aquaculture of the ULPGC has been working on biofiltering processes of the effluents from the aquaculture production unit in the Complementary Module 1 of the Scientific and Technological Park. The main focus has been few seaweed species as nutrient stripers and as source of biomass for abalone production. To our knowledge, there has not been any study where the effluents collected at the sedimentation tanks of in land aquaculture facilities are used to feed detritivores, furthermore, the interaction between sediment feeders, water column and cultured algae has not been thoroughly studied.

This study was started to enhance the potential use of new macroalgae to be incorporated in the biofiltering system as well as to determine the role of sea cucumber as a sediment organic matter consumer. The combination of these two types of nutrient remover organisms (biofilter organisms) has not been evaluated yet and the need to understand the possible synergy between sea cucumber and seaweed aquaculture are the main relevant new factors in this work. In this sense, 3 main research lines have been opened around the concept of integrated aquaculture systems to allow environmental friendly and more profitable land-based aquaculture production. There is a great need to diversify cultivated seaweeds that could be used as biofilters with market viability and it is important to determine the efficiency of filtration of these seaweeds, these are key factors that are addressed in this work.

2. OBJECTIVES

General objective:

The general objective of this study was to evaluate and implement potential new marine species with sediment and water biofiltration capacities of the nutrients from a land-based aquaculture production unit.

Specific objectives:

In order to accomplish the main objective of this study several specific objectives where designed

- Identify the main organisms present in effluents from the in land aquaculture facilities in the Scientific and technologic park of Taliarte (Parque Cientifico Tecnológico de Taliarte, PCT) focusing on the Biofiltration potential of the organisms.
- Evaluate the initial state of the biofiltration facility in the Parque Científico Tecnológico de Taliarte PCT and make the possible and necessary modifications to improve it.
- Determine the biofiltration capacity of the most abundant species of algae present in the effluents as well as their production and resistance to free floating culture systems.
- Assess the viability of adding *Holothuria sanctori* as a way to biofiltrate the sediments from the sedimentation tank at the PCT and increase the nutrients available in the water for the growth of algae in free floating systems.
- Determine the growth and survival of *Holothuria sanctori* fed with sediments from the sedimentation tank of the effluents of the PCT.
- Test the potential use of algal rope culture to cultivate algae with commercial interest in the biofilter installation of the PCT.

3. MATERIALS AND METHODS

3.1. Biofiltration facilities:

This study was made at the integrated culture facilities of the research group in aquaculture (GIA; ULPGC) and the Parque Cientifico Tecnológico de Taliarte (PCT) located in the eastern side of the Island of Gran Canaria, in Taliarte, Telde. The experimental tanks (description of tanks on 3.5) where located inside a greenhouse in the facilities of the complimentary building 1 of the PCT (Figure 2 A); the greenhouse received water through a pump located at an 11 m^3 sedimentation tank that collects the suspended particles of all the effluents from the culture of various species of the facility (Figure 2 B).

On the other hand, at the PCT´s facilities, the biofilter facility located outdoors consists on an elongated tank system with a staircase design that creates cascades of water on each level, the approximated total volume of this biofilter is of 160 m^3 with approximately 30 renovations of its volume per day. The effluents from three fish production facilities enters trough 3 pipes located in the upper side of the biofilter at the center, it has a small sedimentation tank of approximately 1.60 m deep on one of the sides of the first level, from this first level the water falls by gravity to 8 other levels that are made of concrete tanks of approximately 30 cm wide, 40 cm deep by 14 m long. The biofilter ends in a cascade of approximately 2.3 m after which the water flows directly to the ocean (Figure 2 C).

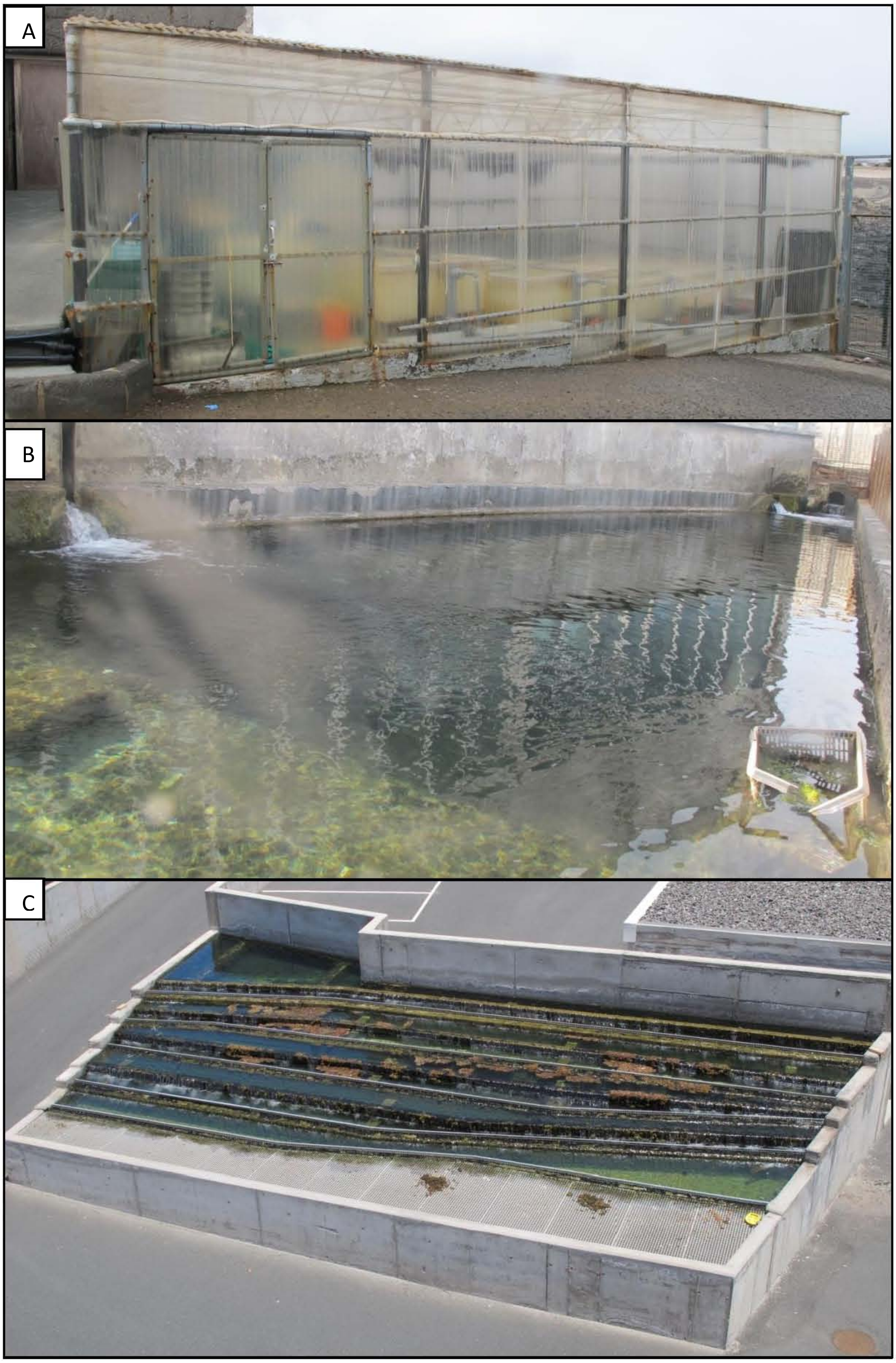

Figure 2: Facilities for the study A) Greenhouse where the culture tanks were located B) Sedimentation tank of the facilities of the complimentary building 1 of the PCT C) Biofilter facility of the PCT.

3.2. Initial state of the biofiltration facilities

The initial state of the biofiltration facility was determined via direct observation of both of the designated biofilter installations (the complimentary building 1 of the PCT and the main building) specially taking into account the sedimentation patterns and the direction of the water flow.

In order to improve the direction of the water flow and the sedimentation of particles (which was too fast and in the center of the biofilter) a series of pipes where connected to the two main exits, redirecting their flow towards the sedimentation tank located on the right side of the entrance of the water to the biofilter. The first set of pipes was connected to the first entrance of the water to the system which was the one with the strongest water flow, this modification enabled the water to enter the biofilter next to the sedimentation tank, the second set of pipes was connected to the second entrance of water located approximately 30 cm to the left of the first entrance and 15 cm deeper, here the water flow was redirected also towards the sedimentation tank, but under the first set of pipes.

Water samples were collected in the left, center and right area at the entrance and the exit of the water through the system to determine total ammonia of the biofiltration facility at the PCT.

3.3. Assessment of associated organisms

A rapid census of the organisms associated with the facility was made by recording every macroscopic organism seen along each level of the biofilter at the PCT and the sedimentation tank of the complimentary building 1 of the PCT facility, pictures and samples were taken for each species with emphasis in algae species, which were collected and placed on the reference collection of the herbarium “Herbario BCM (Botanica Ciencias del Mar)”.

Each species was identified using specialized taxonomic references based on the original descriptions and on a critical analysis of the literature and in the case of algae, stereo and compound microscopes were used to look and describe the morphological and anatomical characters for the proper identification of the species.

3.4. Species selection for culture trials.

The most abundant algae species present in the biofiltration installations were collected and tested in tank conditions (Figure 3). The species of algae tested were *Ulva rigida*, *Codium tomentosum*, *Valonia utricularis,* *Colpomenia sinuosa*, *Dyctiota menstrualis*, *Schizymenia dubyi*, and *Hydropuntia cornea* (Figure 3).

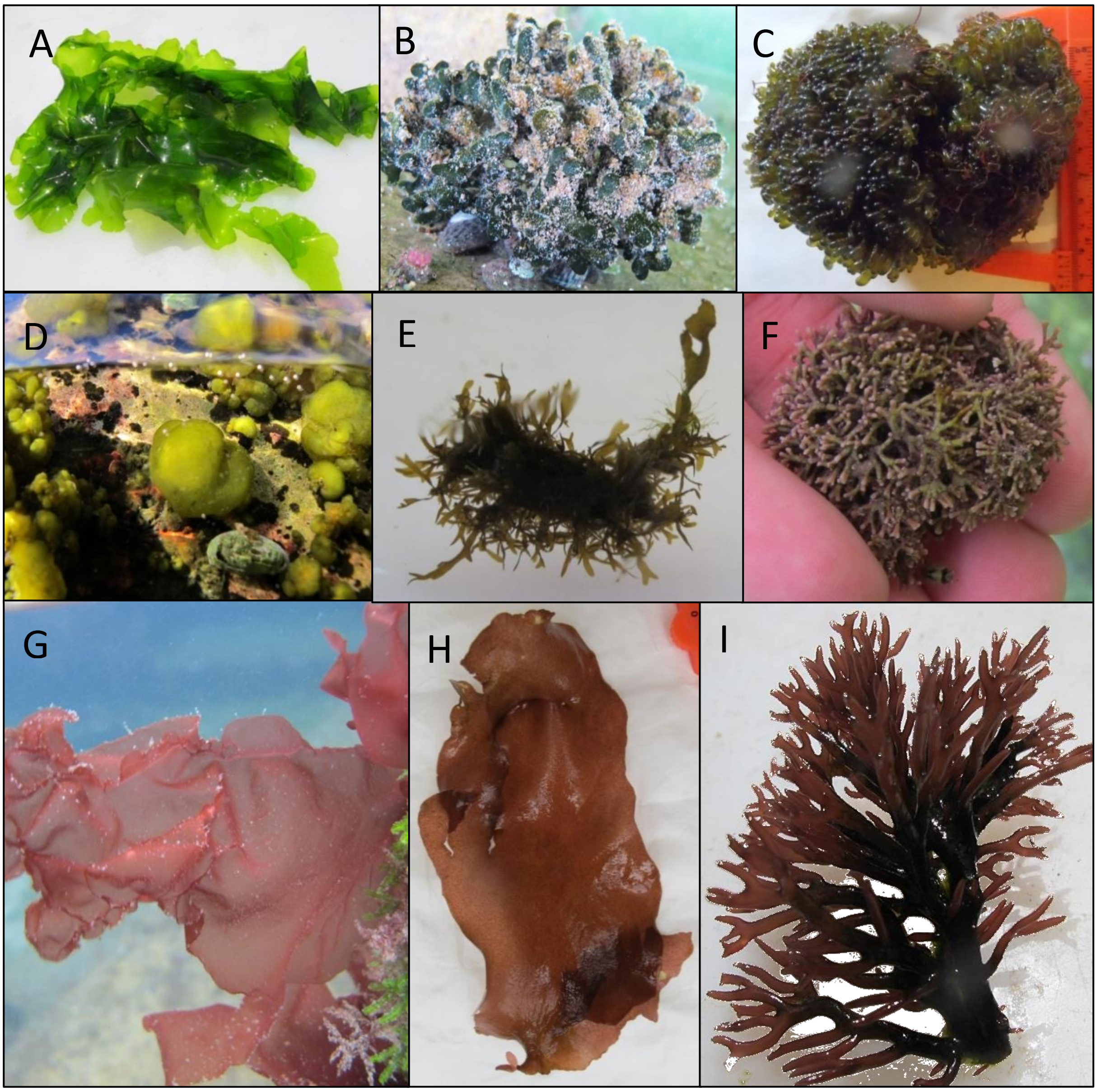

Figure 3: Species of algae tested under tank conditions: A) *Ulva rigida*, B) *Codium tomentosum*, C) *Valonia utricularis*, D) *Colpomenia sinuosa*, E) *Dictyota menstrualis*, F) *Jania rubens*, G and H) *Schizymenia dubyi* and I) *Hydropuntia cornea*.

3.5. Culture care and tank design:

A series of 90 L plastic cylindrical tanks with a surface area of 0.2 m^2 were used to produce each species of algae during winter 2013/2014. Each tank had an adjustable aeration system on the bottom with a plastic tube with holes to distribute air evenly; this system creates enough turbulence to keep the algae suspended on the water column and make them get to the top of the tank periodically to obtain sunlight. Each tank had a drain on the top with a filter of approximately 2mm to prevent algae from falling out of the tank, water renewal in the tanks was set to 10 renovations / day (Figure 4).

In the cases where the amount of algae biomass wasn't enough to create triplicates for a single species, co cultures where made, to select the species with most potential, the resistant species where the ones selected to continue experiments and analyze biofiltration and production separately.

Each triplicate for each species was harvested every week and each algae species was tested during 3 or 4 week period. Algae density was set taking into account recommended densities for similar species (2 g/l for *Ulva rigida*, 5.76 average for *C. sinuosa*, 4.47 average for *V. utricularis* and 2.64 average for *S. dubyi*), the density in each tank was raised or lowered also depending on the production and the amount of epiphytes as a mean to control them.

Tanks were cleaned after each harvest every week by rinsing and scrubbing with freshwater and bleach, after this treatment the tanks were rinsed again with saltwater to eliminate bleach completely and restock them, each week the position of tanks was changed to avoid tank effect.

Algae production and growth rate was determined for each of the tested species each week harvesting the replicates and drying via blot dry using drying paper and pressing on the algae until no more water emerged. After this, each replicate was weighed using a scale to quantify the biomass. The growth rate for each species was calculated using the method described by D´ Elia and Deboer (1978):

$$\mu = \frac{100\,\mathrm{Ln}\left(\frac{P_t}{P_0}\right)}{t} = \%d^{-1}$$

Where: μ = average daily growth in percentage.

P_t = final weight after t days.

P_0= initial weight.

Production rate was also calculated using the DeBoer y Ryther (1977) method.

$$P = \frac{\frac{N_t - N_0}{t} * \frac{PS}{PH}}{A} = gPSm^{-2}d^{-1}$$

Where: P = production rate in dry weight

N_t-N_0= difference in grams of humid weight at t days. Being N_t the weight at t days and N_0 initial weight.

PS / PH= relationship between dry and humid weight (*H. cornea* = 0.12 ; *U. rigida* = 0.21 ; *C. sinuosa*= 0.07 ; *V. utricularis*=0.07 ; *S. dubyi*= 0.17)

A = culture area in square meters (0.2 m^2)

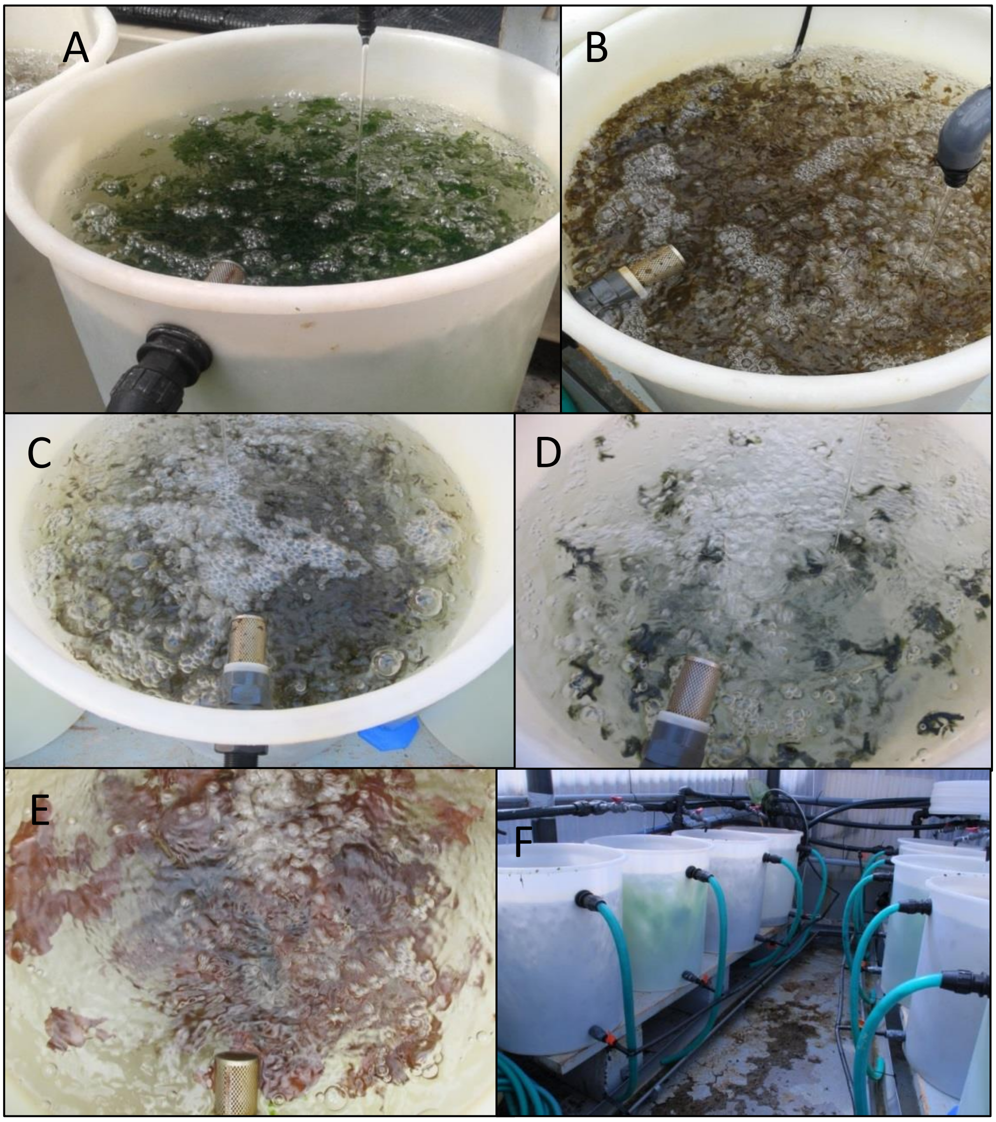

Figure 4: Details of the tanks and some of the cultures tested in them. A) *Ulva rigida*, B) *Colpomenia sinuosa*, C) coculture of *Dyctiota menstrualis*, *Codium tomentosum* and *Valonia utricularis*, D) culture of *Codium tomentosum*, E) *Schyzimenia dubyi*, F) general aspect of the tanks.

All the algae species were selected from either one of the facilities, and tested under the previously described tank conditions, however, only the species with more biomass were used for more through experiments with tank triplicates, this biomass was obtained either by harvest from the installations or by co culture trials when growth was observed, this way, the species *Jania rubens*, *Dictyota menstrualis* and *Codium tomentosum* were eliminated from the study because they did not show enough growth to reach the amount of algae needed to create triplicates (in the case of *J. rubens* and *D. menstrualis*) and because of its slow growth rate in the case of *C. tomentosum* considering also that previous works addressed this algae with little success (Toledo, 1999), but was proven useful to keep cocultures In 90 L tanks at an acceptable density.

3.6. Ammonia biofiltration capacity

Each week, a day after harvesting and sowing (in order to measure biofiltration at the sowed biomass) ammonia biofiltration rates were determined by collecting water samples at the entrance and the exit of each tank, these samples were processed immediately after their collection using the colorimetric method designed by Parsons and collaborators in 1984 using filtered marine water as control, at the facilities of the Marine biotechnology laboratory at the PCT. Nitrogen Uptake Efficiency (NUE) and Nitrogen Uptake Rate (NUR)

$$NUE\ (\%) = CS/CE * 100 = \%$$

Where:

CS and CE are the concentrations of ammonia at the exit and entrance of each tank respectively

$$NUR = Q * (CS - CE) / A = m\ mol\ m^{-2}\ d^{-1}$$

Where:

Q = water flow (L/h),
CE = Ammonia concentration at the entrance of the tank (µM)
Cs = Ammonia concentration at the exit of each tank (µM)
A = Tank surface (0.2 m^2).

3.7. Integrated culture of *Holothuria sanctori* and *Hydropuntia cornea*

The most abundant sea cucumbers species present in the Canary Islands (*Holothuria sanctori*) were collected by free diving at Taliare´s rocky shore at depths between 3 to 6 meters (Figure 5).

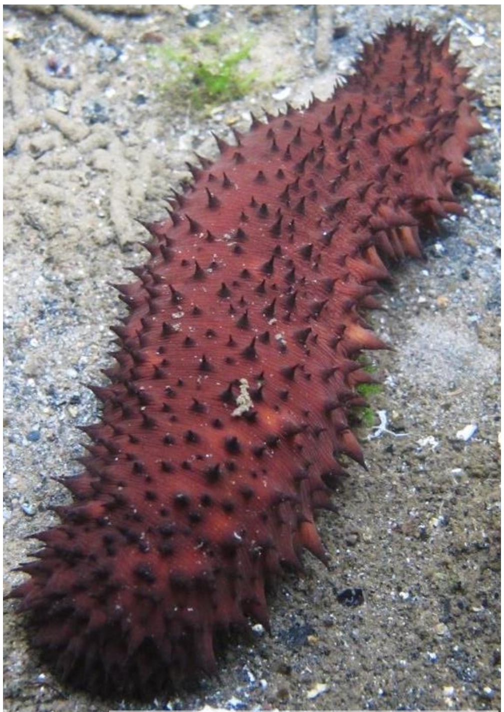
Figure 5: *Holothuria sanctori*.

They were kept in quarantine for 3 weeks at a tank with clean sand and sufficient water flow, after this, Approximately 5 liters of clean sand was placed in the bottom of three 90 liter tanks and a control tank, the air entrance was modified creating an L shape so the air didn't disturb the sediments. Each *Holothuria sanctori* specimen was weighed at the beginning of the experiment by placing each individual in a blotting paper and letting them relax for about 30 seconds until they released the Cuvier tubes and relaxed, and then placing them in a scale, 8 sea cucumbers were placed in each tank, making sure that each had approximately the same sea cucumber biomass (an average of 617 g of total biomass) with approximately the same population structure (Figure 6).

Sea cucumbers were fed by placing approximately 950 ml of wet sediments from the sedimentation tank for the effluents of the aquaculture facility every week during the experiment of integrated culture and every 15 days for the sea cucumber growth experiment. This was done by lowering the tank level, pouring the sediment homogeneously and letting it sink and then refilling the tanks slowly so the sediments would remain undisturbed (Figure 6).

Two of these tanks were connected through each of its drains to a set of three algae tanks using PVC tubes so that the water that entered each algae tank came from the tank with sea cucumbers creating two treatment sets, the experiment lasted 3 weeks, and was made using the algae *Hydropuntia cornea*, each tank was stoked at 4 g/L and harvested each week using the methods described previously. Each week, tree tanks had water from the sea cucumber tanks and the other tree had water directly from the sedimentation tank (Figure 7).

Total ammonia at the entrance and exit of the water was measured each week the day after harvest/ sow of the algae for each tank including the sea cucumber tanks.

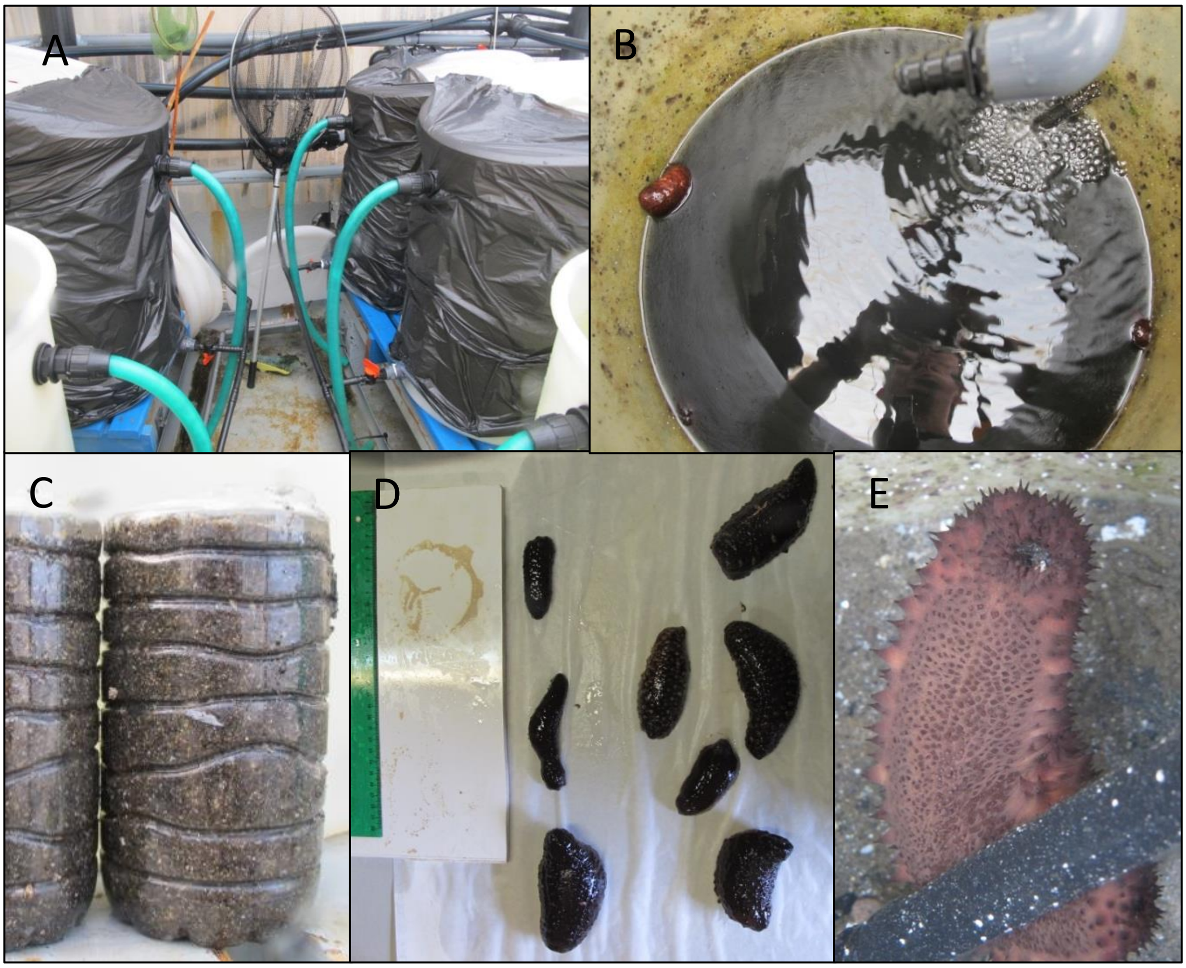

Figure 6: Details of the sea cucumber setup A) the three tanks covered in dark plastic bags to stimulate feeding behavior and avoid growth of unwanted algae B) detail of the sediment adding procedure where the water level is lowered C) detail of the pure sediment added as feed D) relative measures of the sea cucumbers in one tank notice the different sizes F) observed reproductive behavior.

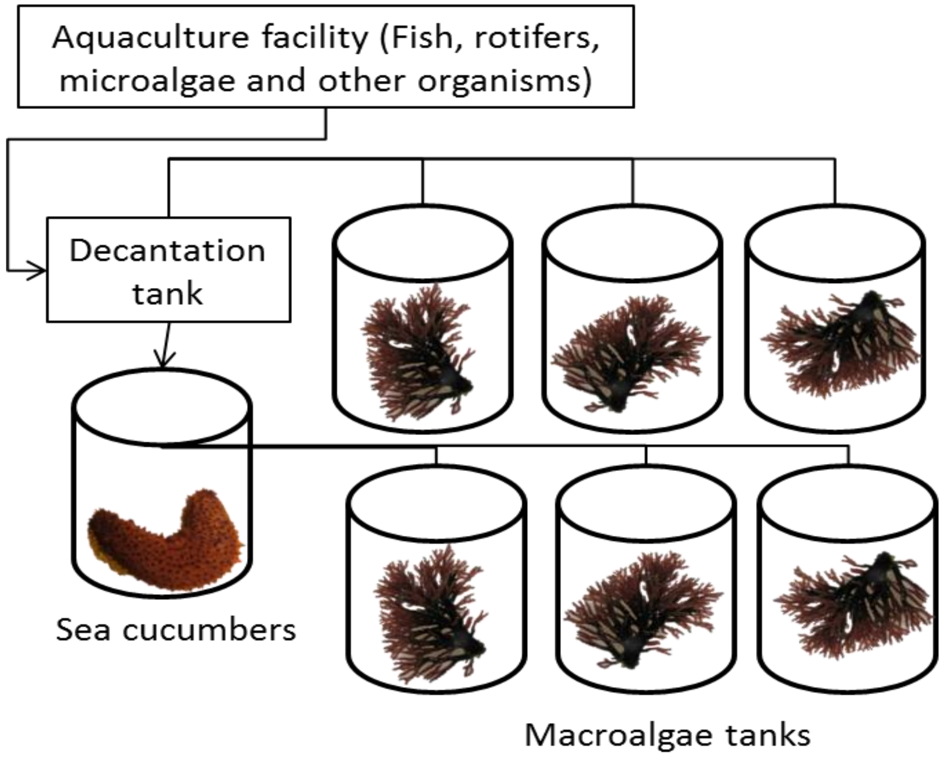

Figure 7: Experimental setting to evaluate the interaction between sea cucumber tanks and algae.

3.7.1. Physiological status of the algae:

The physiological status of *Hydropuntia cornea* submitted to both treatments was verified by measurement of photosynthetic efficiency of chlorophyll (Fv/Fm) by the fluorescence emission using the Hansatech Pocket PEA Chlorophyll fluorimeter.

3.7.2. Sea cucumber sediment biofiltration capacity.

Two surface sediment samples of approximately 100 ml were taken from each tank the day after sediment was poured in and after a week by introducing a urine sample bottle of 100ml at about 1 cm in the sediment at a random place of the tank, and then moving it for about 5 cm in a straight line, each sample was frozen until all the samples of the 3 days trial were collected, then they were dried in a stove at 100 ºC for approximately 24 hours until they reached constant weight, then each sample was homogenized manually for about 20 minutes, each analysis was made in triplicates, the methods used for the analysis are as follows.

Percentage of organic matter was calculated by burning approximately 4 g of sediment sample in a Muffle furnace at 600 ªC during at least 4 hours (AOAC, 2005).

Total nitrogen in the sediments was analyzed using the method described by Kjeldahl (AOAC, 2005), which consists on the digestion of the samples at 420 ªC with concentrated sulfuric acid in presence of a mercuric catalyzer during an hour, after which the sample is distilled with 40% NaOH with boric acid as a receiver substance (using the P Selecta Bloc Digest for the digestion and the P Selecta Kjeldahl distiller pro nitro M for the distillation), in the end, the sample is titrated with HCl 0.1 N and the amount of nitrogen is calculated according to the formula

$$N\ (mg/100g) = \frac{\text{ml of titration} - \text{average pattern titration ml)} \times 0.1\ \text{(molarity of HCl)} \times 14.007}{\text{mg of sample} \times 100}$$

3.8. *Holothuria sanctori* growth

Tree tanks of sea cucumbers were fed every 15 days (except for the period of 3 weeks when the experiment with *H. cornea* took place, when they were fed weekly, also keeping a control tank with no feed) they were weighed at the beginning and then at the end of the 100 days experiment and monitored each week.

3.9. Algae culture in ropes.

Three PVC frames of 35 cm by 1 m were created, three 1 m ropes were placed in each frame and each rope was stocked with either *Grateloupia imbricata, Grateloupia turuturu* or *Hydropuntia cornea* (Figure 5, A, B and C), so that each frame had one species per rope, the algae were put in the ropes by entwining the thallus into the rope (Figure 8 D). Each frame was placed in the same level of the biofilter installation at the PCT at the left, center, and middle section to ensure all possible combinations and to reduce the effect of the location inside the biofilter, they were harvested after 40 days under those conditions.

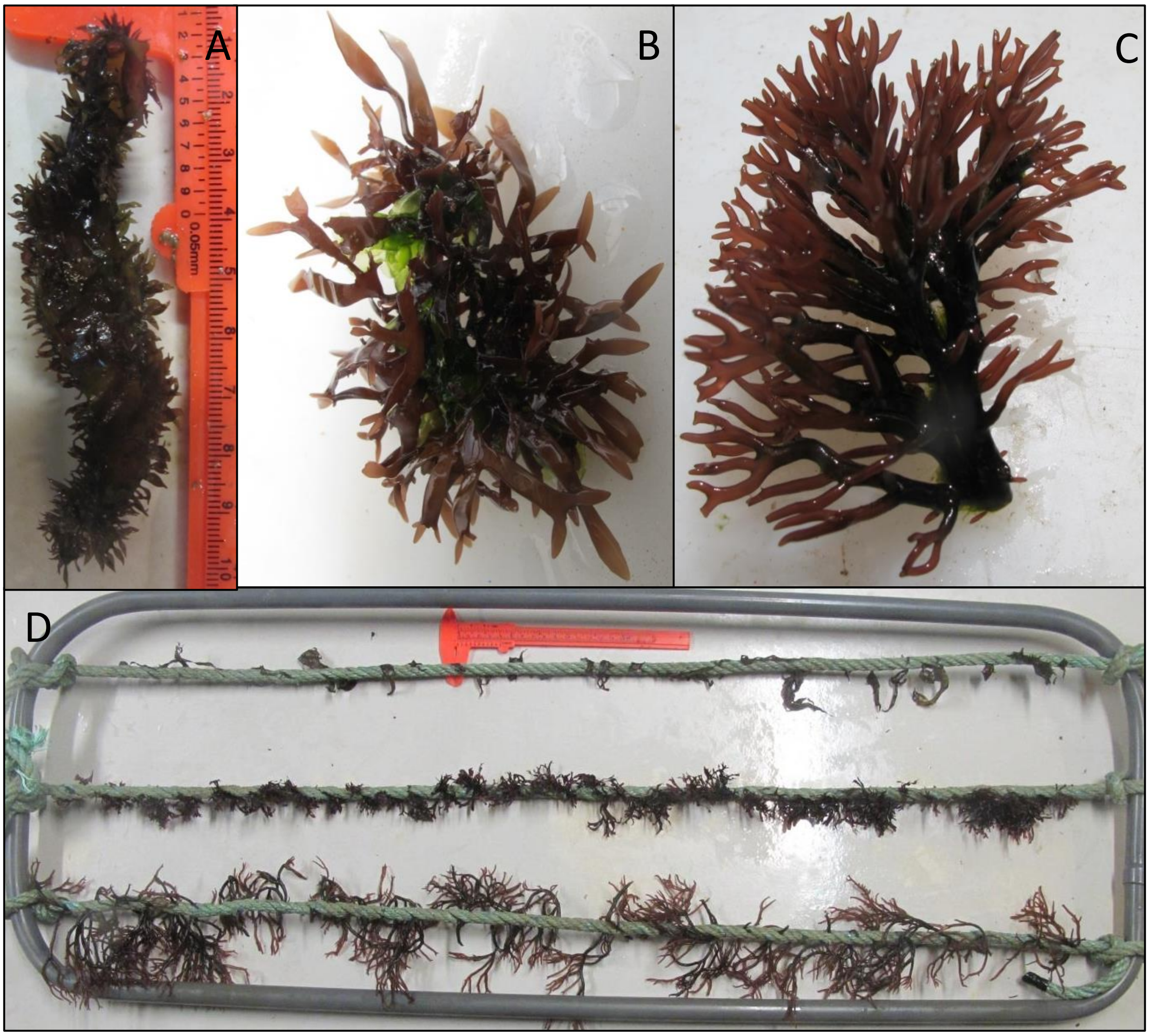

Figure 8: Algae species and setup used for the rope culture experiment A) *Grateloupia turuturu*, B) *G. imbricata*, C) *Hydropuntia cornea*, D) frame with ropes and entwined algae, the first rope have *G. turuturu*, the second *G. imbricata* and the third *H. cornea*.

3.10. Statistical analysis.

Non parametric statistics was used because the data does not comply with the requisites for parametric statistics, the U of Mann Whitney Test and analysis of variance via permutations (PERMANOVA) where used to evaluate if there were significant differences between treatments and species.

4. RESULTS

4.1. Initial state of the effluents and associated organisms.

The temperatures during the study are shown in figure 9, they varied between 24 to 20 ºC. The Total ammonia associated with the biofilter at the main building of the PCTT did not differ at the entrance and exit of the facility (table 1).

The organisms associated with the effluents of both facilities diverged, in general, the sedimentation tank of the complimentary building 1 of the PCT had more species richness, 11 macroalgae (Table 2) and 16 macrofauna species (Table 3) while the biofilter at the PCT only presented 7 species of macroalgae (Table 2) and 14 species of macrofauna (Table 3).

Regarding macroalgae groups, there was a dominance of species of red algae in the effluents from the complimentary building 1 of the PCT with 5 species, followed by representatives of green algae with 4 species, meanwhile, in the biofilter installation at the PCT, Brown algae dominated together with green algae, both with 3 species (Table 2).

The associated fauna presented the same richness in both facilities, with 16 species. The most prevalent and dominant group were the mollusks with 5 species at the installation in the PCT and 7 at the sedimentation tank of the complimentary building 1 of the PCT facility (Table 3), (figure 10).

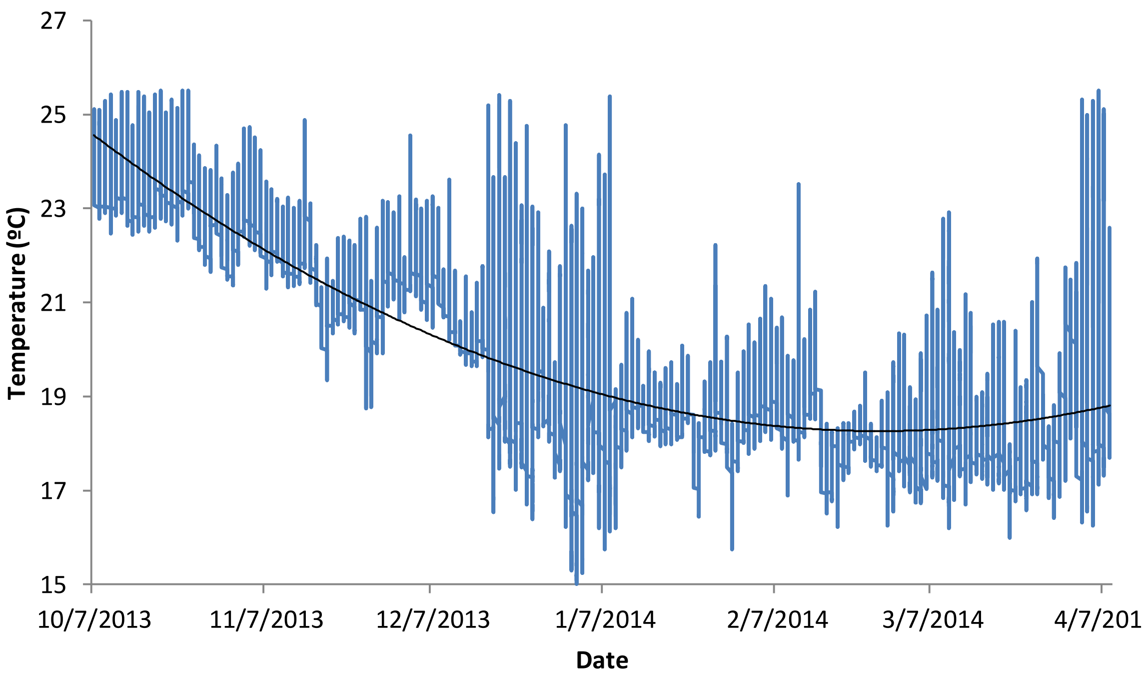

Figure 9: Water temperatures during the study

Table 1: Table showing the ammonia concentrations in µM found at the facilities of the biofilter of the PCT.

	Entrance	**Exit**
Left	8.485	8.345
Middle	9.467	8.626
Right	11.289	10.168
Average	9.747	9.046
Stdev	1.161	0.801

Table 2: Macroalgae species associated to the effluents from both of the aquaculture installations located in Taliarte.

	Species	
	Complimentary building 1	**Biofilter PCT**
Phaeophyta	*Dyctiota menstrualis*	*Dictyota menstrualis*
	Colpomenia sinuosa	*Colpomenia sinuosa*
		Platoma Sp.
Rhodophyta	*Jania rubens*	*Jania rubens*
	Schizymenia dubyi	
	Grateloupia imbricata	
	Grateloupia turuturu	
	Hydropuntia cornea	
Chlorophyta	*Ulva rigida*	*Ulva rigida*
	Valonia utricularis	*Valonia utricularis*
	Codium tomentosum	*Codium tomentosum*
	Caulerpa racemosa	
Total richness	11	7

Table 3: Fauna associated to the effluents from both of the aquaculture installations located in Taliarte.

	Species	
	Complimentary building 1	**Biofilter PCT**
Porifera	*Spongionella pulchella*	
Cnidaria	*Aiptasia mutabilis*	*Aiptasia mutabilis*
Chordata	*Ascidiacea Sp*	*Ascidiacea Sp*
Echinodermata	*Holothuria sanctori*	*Coscinasterias tenuispina*
	Coscinasterias tenuispina	*Ophioderma sp*
	Ophioderma sp	
Annelida	*Polychaeta sp.*	*Eupolymnia nebulosa*
	Sipunculus nudus	*Sipunculus nudus*
		Myxicola infundibulum
Molusca	*Aplysia dactylomela*	*Aplysia dactylomela*
	Patella aspera	*Patella aspera*
	Cerithium vulgatum	*Cerithium vulgatum*
	Osilinus atratus	*Osilinus atratus*
	Siphonaria pectinata	*Siphonaria pectinata*
	Haliotis tuberculata coccinea	
	Aplysia depilans	
Arthropoda		*Pachygrapsus marmoratus*
Vertebrata	*Sparus aurata*	*Sparus aurata*
		Seriola rivoliana
		Dicentrarchus labrax
Total richness	16	16

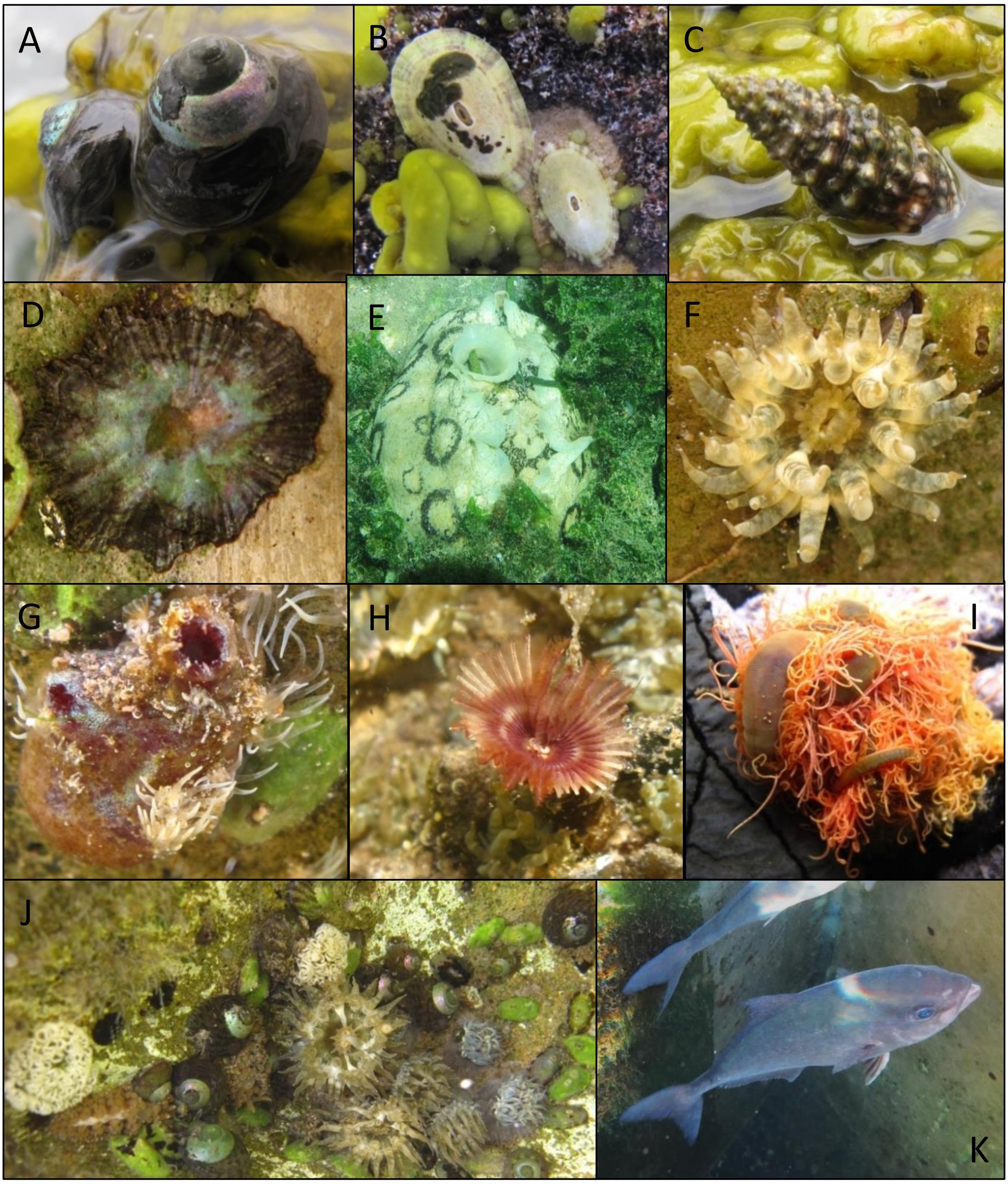

Figure 10: Fauna associated with the effluents at the PCT and complimentary building 1 of the PCT sedimentation tank. A) *Osilinus atratus,* B) *Siphonaria pectinata*, C) *Cerithium vulgatum*, D) *Patella aspera*, E) *Aplysia dactilomela*, F) *Aiptasia mutabilis*, G) Tunicates, H) *Myxicola infundibulum*, I) *Eupolymnia nebulosa*, J) example of an aggregation of various organisms K) Example of escaped fish in this case, *Seriola dumerilii*.

4.2. Algae growth rate and production.

The experiments were carried out between the 15/10/2013 and the 07/11/2013 for *Colpomenia sinuosa* and *Ulva rigida* and for *Schizymenia dubyi* and *Valonia utricularis* between the 27/01/2014 and the 18/02/2014.

Every species of algae evaluated showed a tendency to decrease during each experimentation period, the species that showed the higher specific growth rate was *Ulva rigida* (11.03 %d^{-1}) followed by *Schizymenia dubyi* with 1.58 %d^{-1} and *Valonia utricularis* with 1.31 %d^{-1} and, *Colpomenia sinuosa* decreased during the whole experimentation period with an average of –6.09 %d^{-1} (Figure 11).

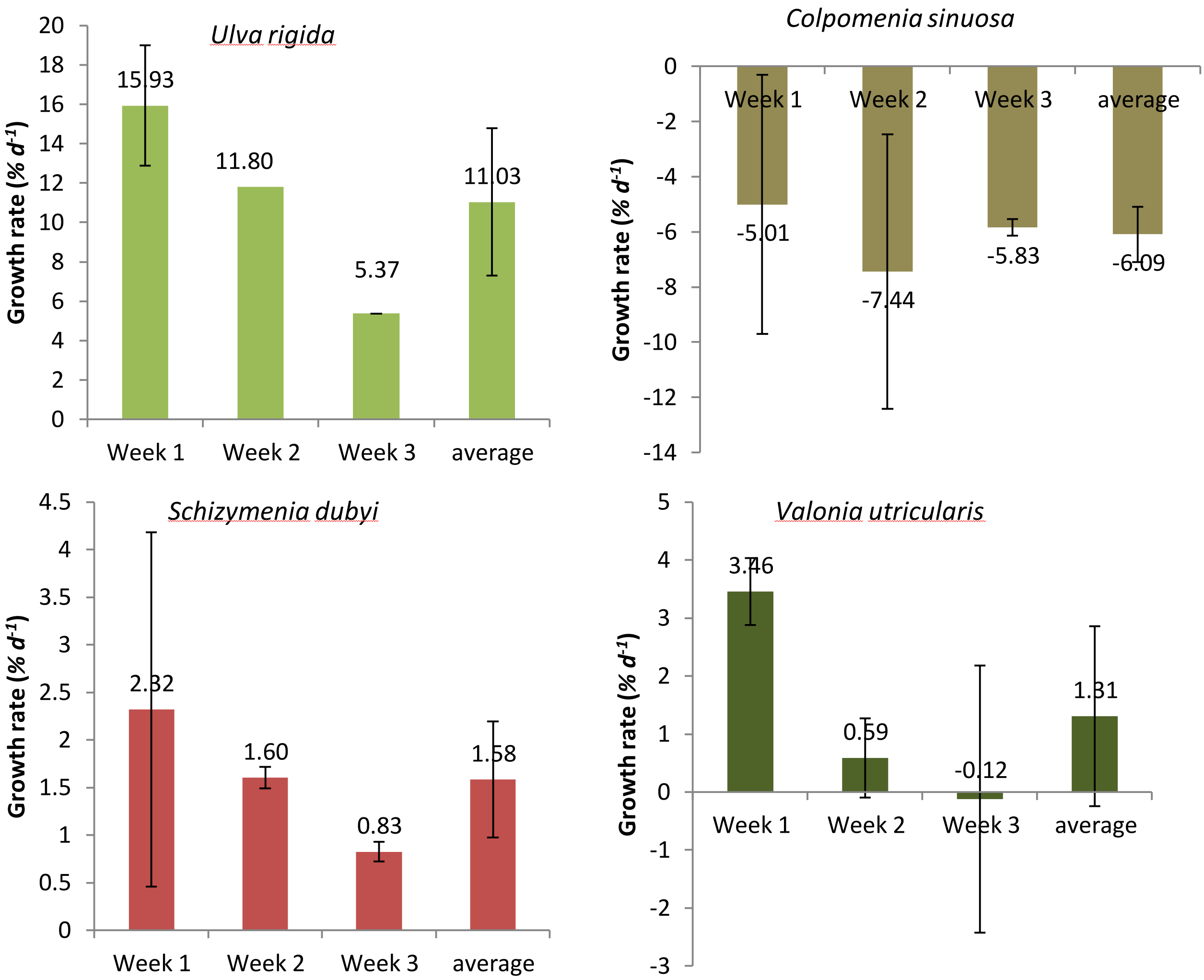

Figure 11: Growth rate presented by the 4 species of algae evaluated during three weeks, standard deviation and average are presented.

Algae production presented a similar tendency as the one showed by the growth rate, with the highest production shown by *U. rigida* followed by *S. dubyi* and *V. utricularis* (figure 12)

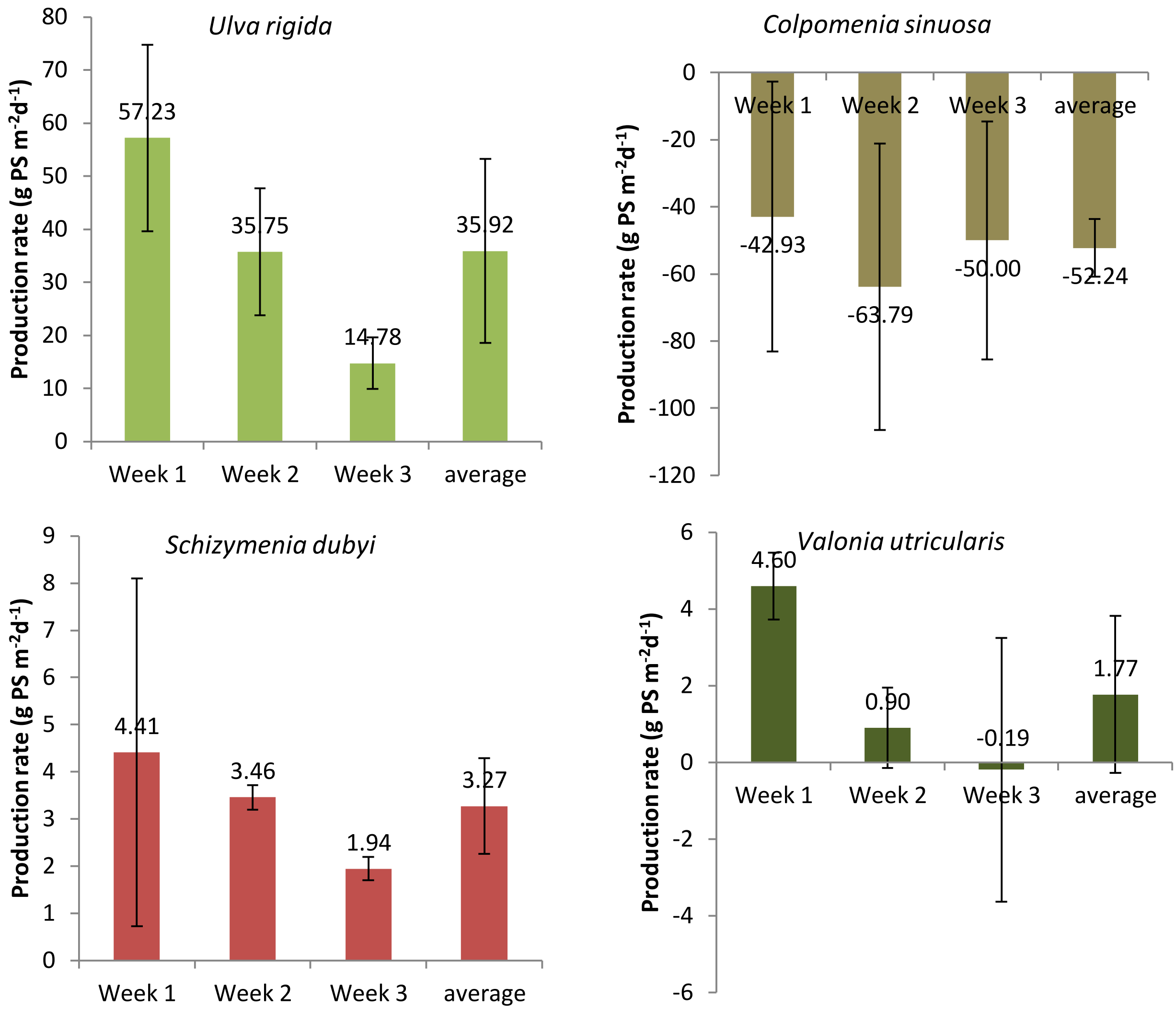

Figure 12: Production rate presented by the 4 species of algae evaluated during three weeks, standard deviation and average are presented.

4.3. Algae biofiltration:

Tables are presented to summarize the algae biofiltration values of Nitrogen uptake rate and Nitrogen uptake efficiency, the Nitrogen uptake efficiency was the highest for *Ulva rigida* with 90% however, the highest nitrogen uptake ratio was shown by *Schizymenia dubyi* with 0.94 mmol NH_4^+ $m^{-2}d^{-1}$, on the other hand, the lowest of these parameters was shown by *Valonia utricularis*. Each value presented is an average of the triplicates for each week (tables 4 to 8).

Table 4: Weekly results summarizing the average amount of ammonia at the entrance of the tanks, the NUE, the NUR, together with the growth and production rate for *Ulva rigida* during the experimentation period with a density of 2g/L and a renovation rate of 10 Vol/day.

	Ulva rígida				
	ENTRANCE NH_4^+	**NUE**	**NUR**	**GROWTH RATE**	**PRODUCTION RATE**
WEEK	µM	%	mmol NH_4^+ $m^{-2}d^{-1}$	% d^{-1}	g PS $m^{-2}d^{-1}$
1	3.501	99.999	0.656	15.930	57.225
2	10.380	70.570	1.373	11.800	35.75
3	0.941	99.999	0.176	5.368	14.775
AVERAGE	**4.941**	**90.190**	**0.735**	**11.032**	**35.916**
SD	3.985	13.873	0.492	4.345	17.330

Table 5: Weekly results summarizing the average amount of ammonia at the entrance of the tanks, the NUE, the NUR, together with the growth and production rate for *Colpomenia sinuosa* during the experimentation period with an average density of 5.76g/L and a renovation rate of 10 Vol/day.

	Colpomenia sinuosa				
	ENTRANCE NH_4^+	**NUE**	**NUR**	**GROWTH RATE**	**PRODUCTION RATE**
WEEK	µM	%	mmol NH_4^+ $m^{-2}d^{-1}$	% d^{-1}	g PS $m^{-2}d^{-1}$
1	4.434	87.931	0.731	-3.359	-42.928
2	7.571	74.842	1.062	-4.714	-63.785
3	0.747	99.999	0.140	-4.787	-50.000
AVERAGE	**4.251**	**87.591**	**0.644**	**-4.287**	**-52.238**
SD	2.789	10.273	0.381	0.656	8.660

Table 6: Weekly results summarizing the average amount of ammonia at the entrance of the tanks, the NUE, the NUR, together with the growth and production rate for *Schizimenia dubyi* during the experimentation period with an average density of 2,64g/L and a renovation rate of 10 Vol/day.

	Schizymenia dubyi				
	ENTRANCE NH_4^+	**NUE**	**NUR**	**GROWTH RATE**	**PRODUCTION RATE**
WEEK	µM	%	mmol NH_4^+ $m^{-2}d^{-1}$	% d^{-1}	g PS $m^{-2}d^{-1}$
1	4.153	78.343	0.610	2.320	4.412
2	9.119	71.315	1.219	1.603	3.460
3	7.442	72.054	1.005	0.825	1.942
AVERAGE	**6.905**	**73.904**	**0.945**	**1.582**	**3.272**
SD	2.062	3.153	0.252	0.610	1.016

Table 7: Weekly results summarizing the average amount of ammonia at the entrance of the tanks, the NUE, the NUR, together with the growth and production rate for *Valonia utricularis* during the experimentation period with an average density of 4.47g/L and a renovation rate of 10 Vol/day.

	Valonia utricularis				
	ENTRANCE NH_4^+	**NUE**	**NUR**	**GROWTH RATE**	**PRODUCTION RATE**
WEEK	µM	%	mmol NH_4^+ $m^{-2}d^{-1}$	% d^{-1}	g PS $m^{-2}d^{-1}$
1	1.825	76.811	0.263	3.457	4.600
2	9.155	64.899	1.114	0.589	0.900
3	5.014	49.855	0.468	-0.123	-0.191
AVERAGE	**5.332**	**63.855**	**0.615**	**1.307**	**1.769**
SD	3.001	11.029	0.362	1.547	2.050

Table 8: Statistical analysis for the average of each parameter for each species.

	U. rigida	*C. sinuosa*	*S. dubyi*	*V. utricularis*
Nitrogen uptake Efficiency[1]	90.19 ±13.87[a]	87.59 ± 10.27[a]	73.90 ± 3.15[b]	63.85 ± 11.02[a]
Nitrogen uptake rate [2]	0.73± 0.49[a]	0.64 ± 0.38[a]	0.94 ± 0.25[a]	0.61 ± 0.36[a]
Growth rate (%) [3]	11.03 ± 4.34[a]	-4.28 ± 0.65[c]	1.58 ± 0.61[b]	1.30 ± 1.54[b]
Production rate (P) [4]	35.91 ± 17.33[a]	-52.23 ± 8.66[c]	3.27 ± 1.01[b]	1.76 ± 2.05[b]

Values in the same row but with different letters are significantly different ($P<0.05$). [1]NUE (*%*). [2]NUR (mmol NH_4^+ $m^{-2}d^{-1}$). [3]µ (*% d^{-1}*). [4]P (g PS $m^{-2}d^{-1}$).

4.3.1. Biofiltration in relation to biomass:

To be able to do a gross comparison of the biofiltration ability of each species of algae a biofiltration relative to the biomass of the algae in the tanks was developed, where the NUR was corrected according to the biomass of algae present in each tank. The results of this index are presented in mmol NH_4^+ $m^{-2}d^{-1}Kg^{-1}$ and show that *Ulva rigida* has the highest biofiltration with 7.63 maximum and an average of 4.05, followed by *Schizymenia dubyi* with 3.93 however no significant differences were found between these two species ($P= 0.90$) but they differ significantly with the rest ($P<0.05$) being *Colpomenia sinuosa* the lowest one with 1.26, this species did not differ significantly with *Valonia utricularis* ($P=0.47$) (Figure 13).

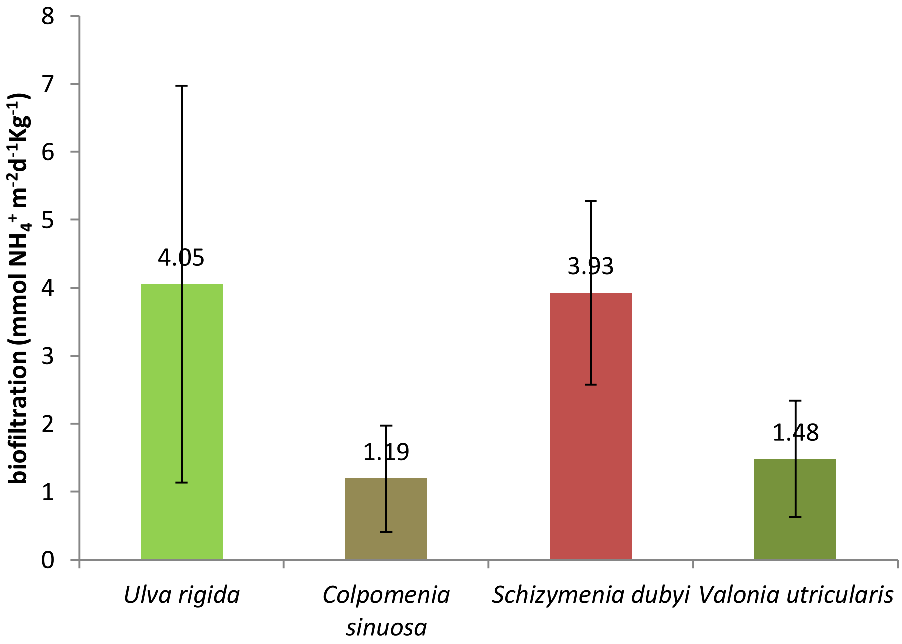

Figure 13: Biofiltration in relation to biomass comparing the four algae species.

4.4. Integrated culture of *Holothuria sanctori* and *Hydropuntia cornea*

4.4.1. Growth and production rates:

The growth rate in $\%d^{-1}$ and the production rate presented in g of fresh weight $m^{-2}d^{-1}$ are presented in the following graphs, for both of the parameters, the tanks submitted to the water coming from the effluents from the sea cucumber tanks presented a tendency to perform better and have a higher growth and production rate, with the exception of the first week where the tanks with the effluents from the sea cucumbers had a slightly lower performance (figures 14 and 15) however, no significant differences were found in the growth rate (U=22 P=0.11) or the production (U=22, P=0.11).

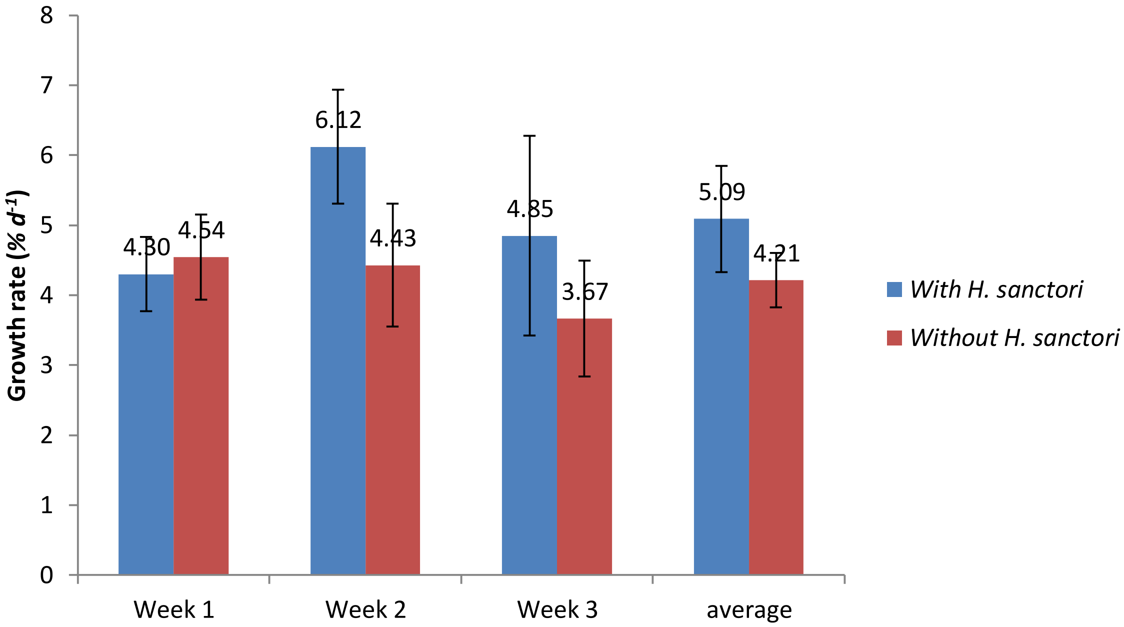

Figure 14: Specific growth rate of *Hydropuntia cornea* submitted to the two treatments showing the standard deviation.

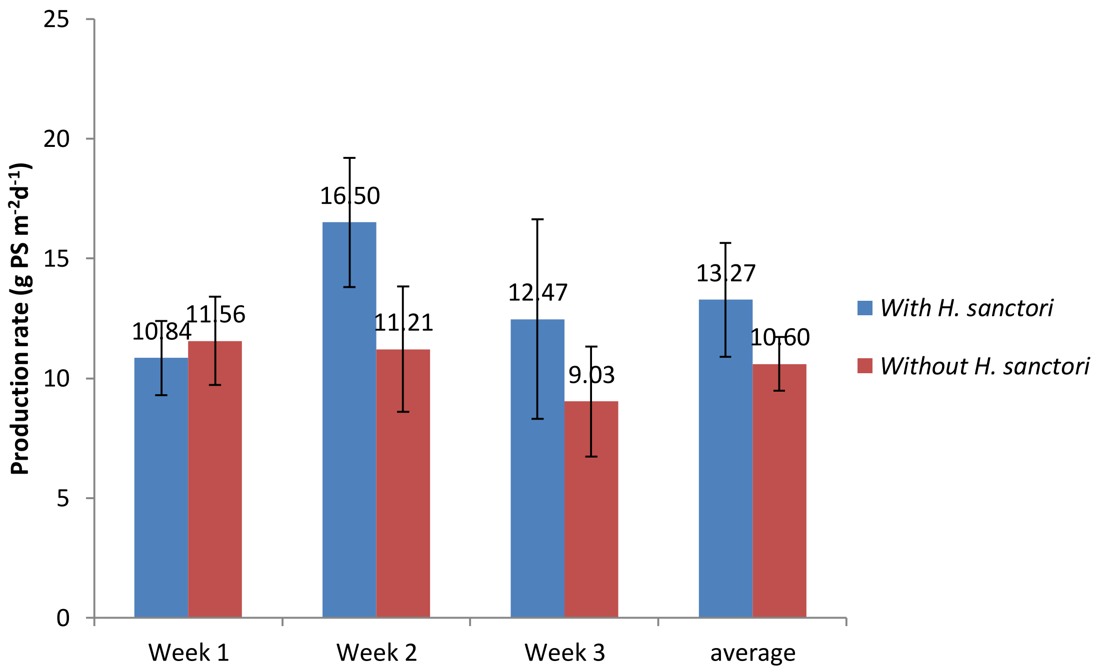

Figure 15: Production rate of *Hydropuntia cornea* submitted to the two treatments.

4.4.2. Biofiltration, Ammonia and physiological state:

Tables are presented to summarize the algae biofiltration values of Nitrogen uptake rate and Nitrogen uptake efficiency of *Hydropuntia cornea* submitted to both of the treatments; the tables show slightly higher nitrogen uptake efficiency for *H. cornea* when submitted to the effluents from the sea cucumber tanks (39.67 %) (Table 9) when compared to the *H. cornea* cultured without the effluents from the sea cucumbers (36.52 %) (Table 10), the same tendency occurs with the nitrogen uptake ratio, with 0.735 mmol NH_4^+ $m^{-2}d^{-1}$ with the effluents from sea cucumbers and 0.282 without them, the NUE and NUR do not differ significantly between treatments (P>0.05).

As for the physiological state of the algae, no significant differences were found between the treatments (P>0.05) (Figure 16).

On the other hand, figure 17 shows the average amount of ammonia present at the entrance and the exit of the sea cucumber tanks, the figure shows higher concentrations of Ammonia for the effluents of the tanks with sea cucumbers than the regular effluents from the sedimentation tank this difference is significant (U=4, P=0.0014).

Table 9: Weekly results summarizing the average amount of ammonia at the entrance of the tanks, the NUE, the NUR, together with the growth and production rates for *Hydropuntia cornea* cultured with the effluents from *Holothuria sanctori* tanks during the experimentation period with a density of 4g/L and a renovation rate of 10 Vol/day.

	Hydropuntia cornea + Holothuria sanctori				
	ENTRANCE NH_4^+	**NUE**	**NUR**	**GROWTH RATE**	**PRODUCTION RATE**
WEEK	µM	%	mmol NH_4^+ $m^{-2}d^{-1}$	% d^{-1}	g PS $m^{-2}d^{-1}$
1	5.799	44.574	0.656	4.301	10.842
2	5.494	29.054	1.373	6.120	16.502
3	6.571	45.381	0.176	4.848	12.468
AVERAGE	**5.955**	**39.670**	**0.735**	**5.090**	**13.271**
SD	0.453	7.513	0.492	0.761	2.379

Table 10: Weekly results summarizing the average amount of ammonia at the entrance of the tanks, the NUE, the NUR, together with the growth and production rates for *Hydropuntia cornea* cultured without the effluents from *Holothuria sanctori* tanks during the experimentation period with a density of 4g/L and a renovation rate of 10 Vol/day.

	***Hydropuntia cornea* without sea cucumbers**				
	ENTRANCE NH_4^+	**NUE**	**NUR**	**GROWTH RATE**	**PRODUCTION RATE**
WEEK	µM	%	mmol NH_4^+ $m^{-2}d^{-1}$	% d^{-1}	g PS $m^{-2}d^{-1}$
1	5.051	43.771	0.414	4.544	11.557
2	3.477	48.940	0.319	4.428	11.214
3	3.642	16.859	0.115	3.665	9.0257
AVERAGE	**4.056**	**36.523**	**0.283**	**4.213**	**10.599**
SD	0.706	14.063	0.125	0.390	1.1212

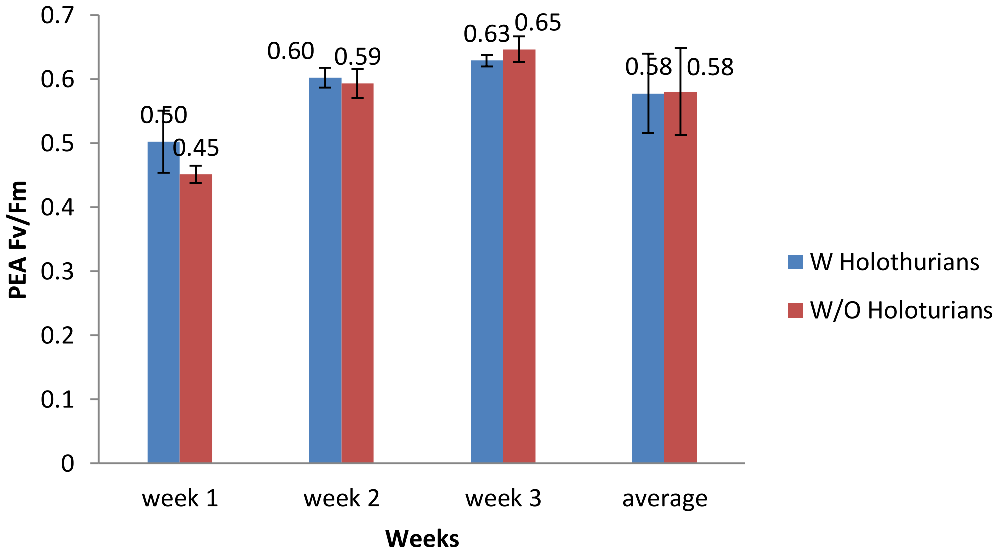

Figure 16: Physiological state in Fv/Fm of *Hydropuntia cornea* submitted to both treatments (with water from the effluents of sea cucumber or directly from the sedimentation tank) during the period of study.

Table 11: Statistical analysis for each parameter evaluated for *H. cornea* with or without the effluents from the sea cucumber tanks.

	With *H. sanctori*	**Without *H. sanctori***
Nitrogen uptake Efficiency[1]	39.67 ±7.51[a]	36.52 ± 14.06[a]
Nitrogen uptake rate [2]	0.73± 0.49[a]	0.28 ± 0.12[a]
Growth rate (%) [3]	5.09 ± 0.76[a]	4.21 ± 0.38[a]
Production rate (P) [4]	13.27 ± 2.37[a]	10.59 ± 2.37[a]
Fv/Fm	0.58 ± 0.062[a]	0.58 ± 0.068[a]

Values in the same row but with different letters are significantly different ($P<0.05$). [1]NUE (*%)*. [2]NUR (mmol NH_4^+ $m^{-2}d^{-1}$). [3]μ (*% d^{-1}*). [4]P (g PS $m^{-2}d^{-1}$).

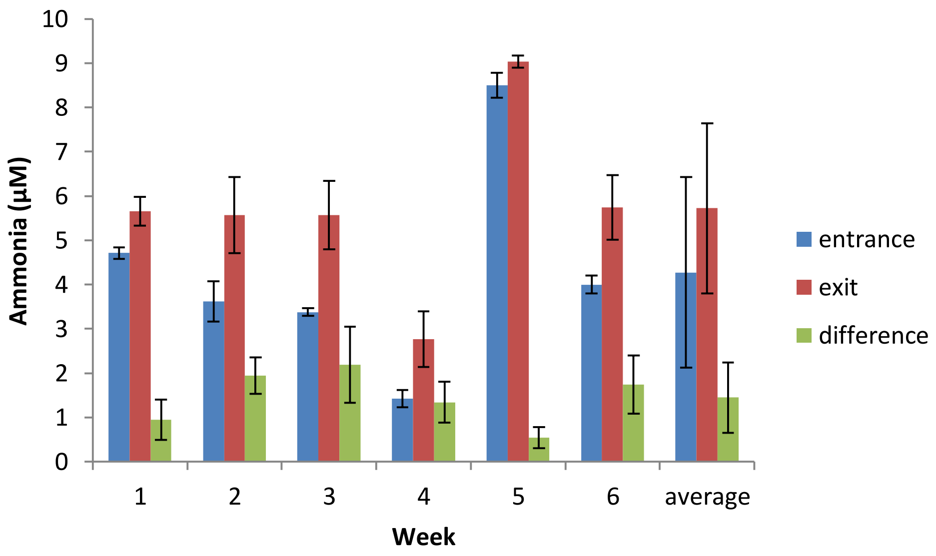

Figure 17: Average amount of ammonia at the entrance and exit for each week measured at the entrance and exit of the sea cucumber tanks and its difference.

4.4.3. Sediment filtration by *Holothuria sanctori*:

The amounts of organic matter and total nitrogen in the sediments are presented in the next figures, the results show an increase for both of the parameters at the beginning of each week, when the sediments from the sedimentation tank were added, and then, after a week of activity by the sea cucumbers, the quantities of both parameters showed a decrease, however, this decrease is not significant for Organic matter (U=25, P=0.18) but in the case of total nitrogen in the sediments the difference is significant (U= 3, P=0.001) and its amount is reduced to the control levels at the beginning of the experiment and does not differ significantly from them (U=38, P=0.85) (figures 18 and 19).

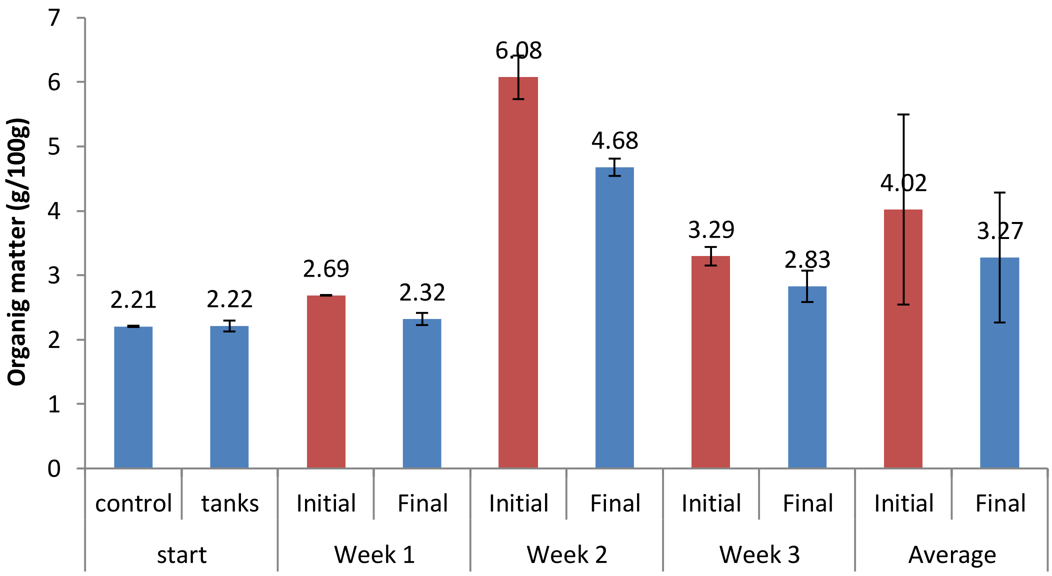

Figure 18: Amount of organic matter (g/100g) present at the beginning and the end of each week during the study.

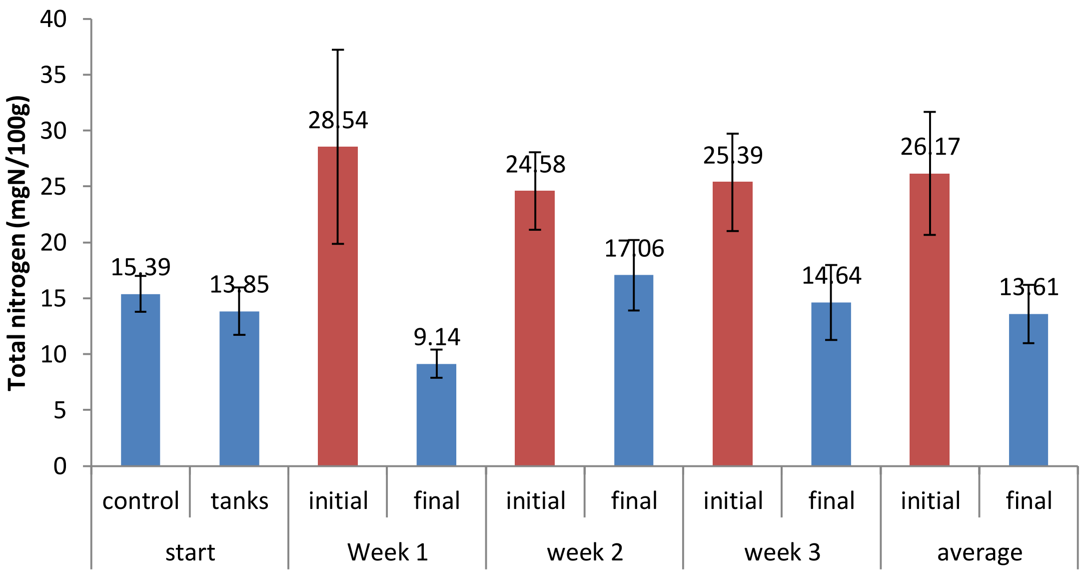

Figure 19: Total nitrogen (mgN/100g) present in the sediments at the beginning and the end of each week of treatment.

4.4.4. Sea cucumber growth and survival:

Regarding sea cucumber growth, after a period of 100 days of feeding with sediments from the sedimentation tank every 15 days in average, the average weight showed a tendency to decrease in all of the tanks including the control however, the differences are not significant (U=2, P=0.11) (Figure 20). Survival in each treatment tank was 100 % while in the control tank only 84.615 % (Table 12)

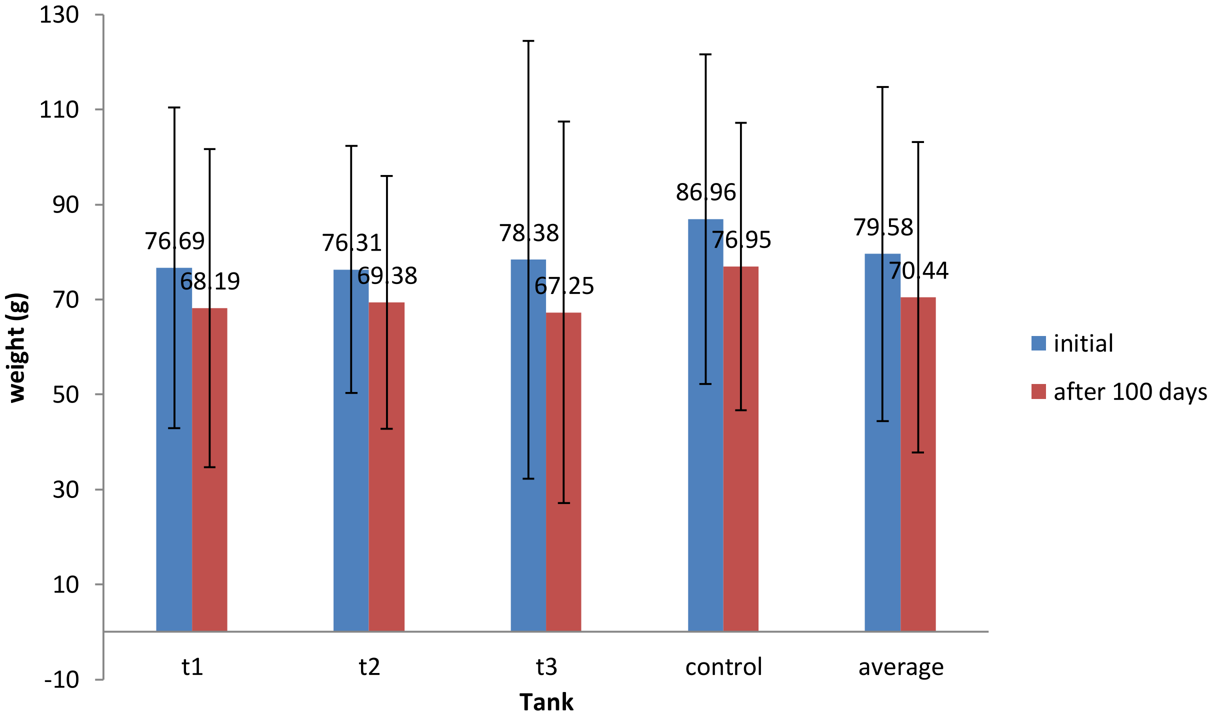

Figure 20: Shows the difference in the weight of *Holothuria sanctori* for each tank after 100 days of feeding with sediment from the sedimentation tank.

Table 12: Survival of *Holothuria sanctori* in the experimentation tanks and the control tank.

Tank	Survival (%)
1	100
2	100
3	100
control	84.6
Average	96.15

4.5. Rope culture experiment

Regarding the experiment of rope culture in the biofilter installation at the PCT, after 40 days of placing the ropes in the biofilter the tendency showed a marked decrease for *Hydropuntia cornea*, a slight increase for *Grateloupia turuturu* and some decrease for *G. imbricata* (figures 21, 22 and 23).

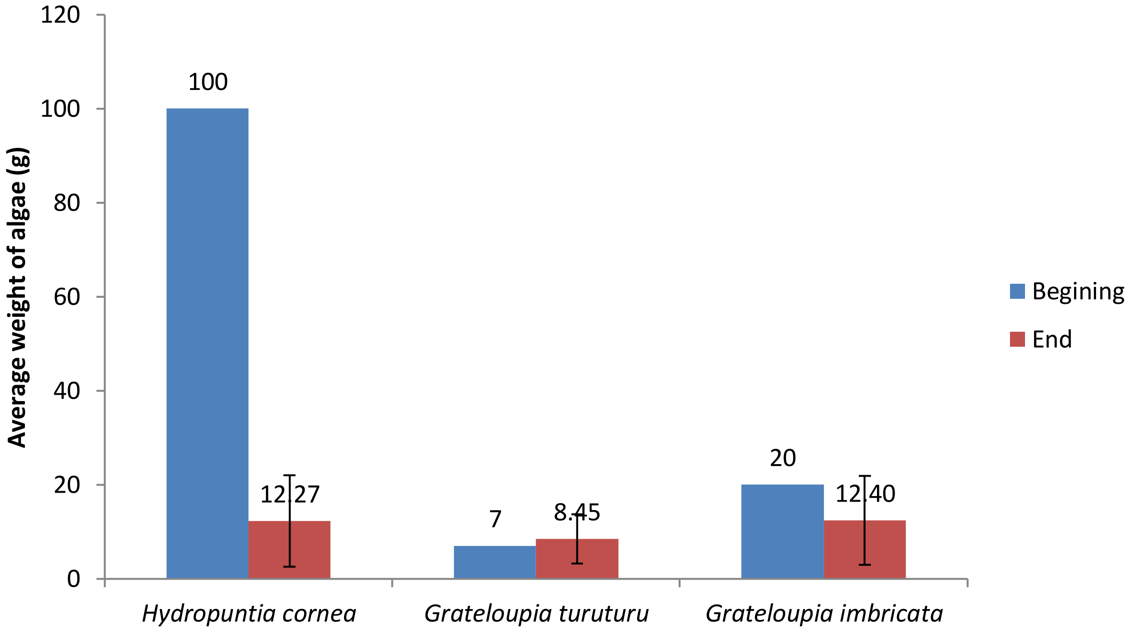

Figure 21: Average weight of *Hydropuntia cornea*, *Grateloupia turuturu* and *G. imbricata* at the beginning of the rope experiment and after 40 days.

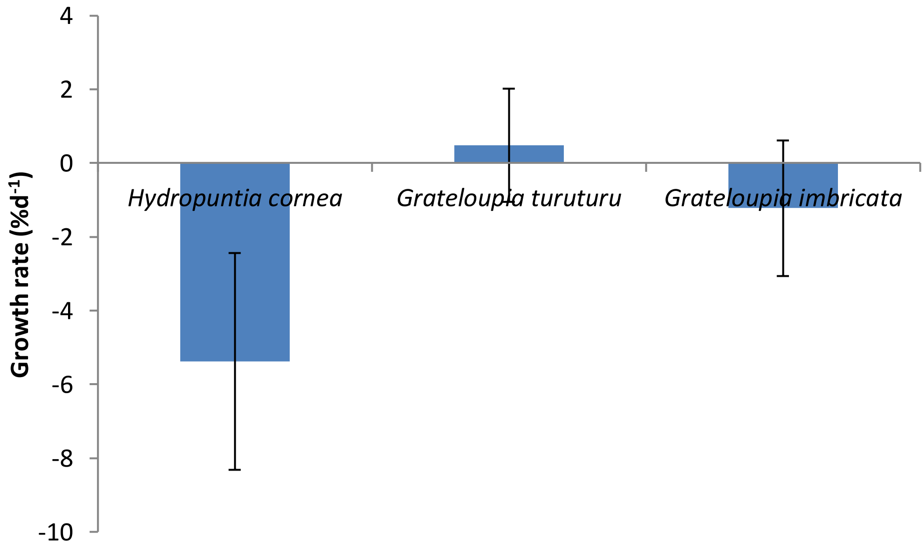

Figure 22: Average growth rate of *Hydropuntia cornea*, *Grateloupia turuturu* and *G. imbricata* at the beginning of the rope experiment and after 40 days.

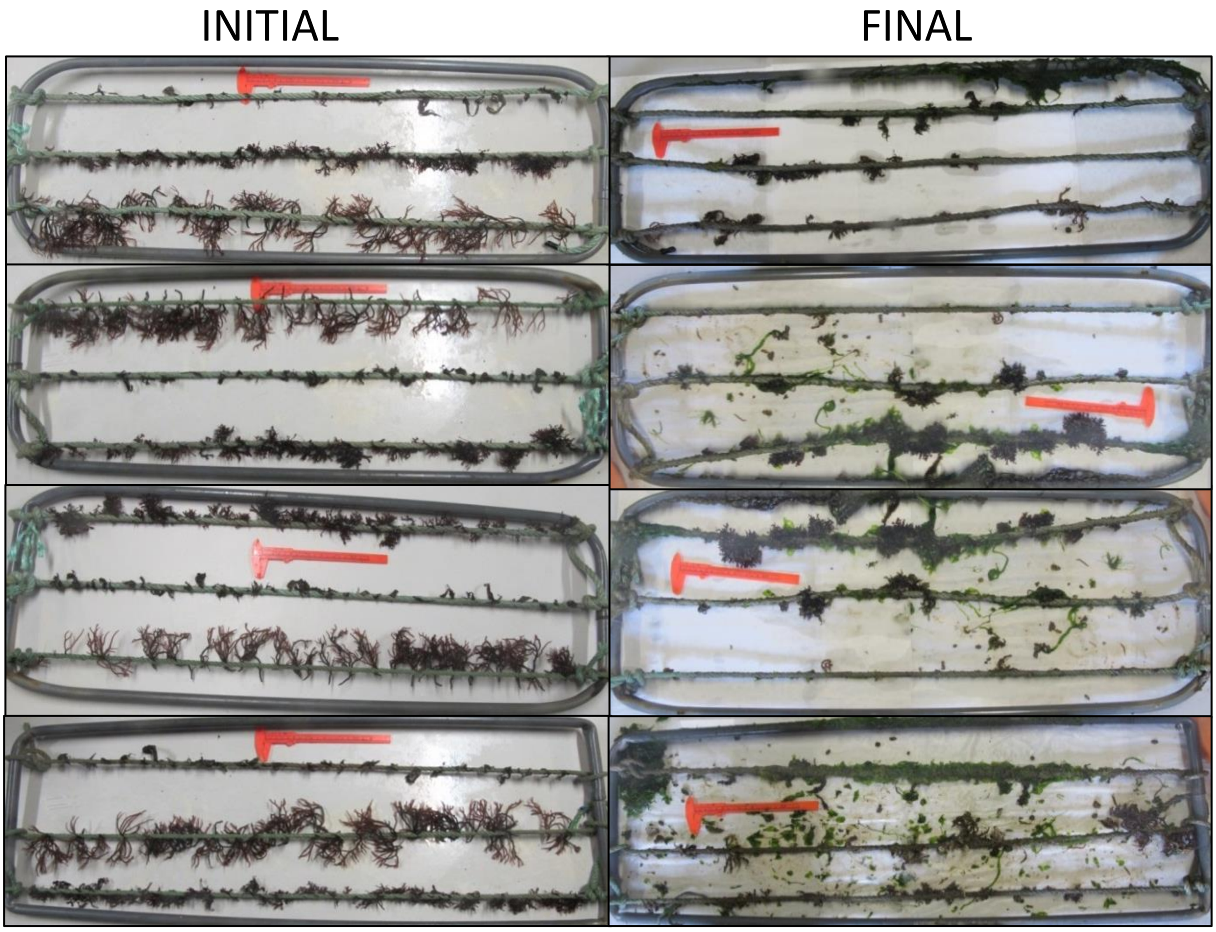

Figure 23: initial and final state after 40 days of the ropes stocked with *Gracillaria cornea*, *Grateloupia imbricata* and *Grateloupia turuturu* at the biofilter of the PCT.

5. DISCUSSION

5.1. Initial state of the effluents and associated organisms.

There was a difference in the main algae groups present at the facilities of the complimentary building 1 of the PCT where green and red algae dominated and the facilities of the PCT where brown algae dominated with occasional outburst of green algae, this is possibly due to the differences in the design of both installations, the complimentary building 1 of the PCT one is design as a large sedimentation tank, while the biofilter at the PCT was originally conceived with the aim of using it as a biofilter and also as an architectonic asset, this means that the water flow is considerably larger at the PCT and also that the surface available for algae that typically grow attached is larger, apart from this, the facilities at the complimentary building 1 of the PCT are older than the PCT ones so there has been more time there for the development of a more diverse community.

The fauna assemblage at both of the facilities was dominated in apparent abundance by suspensivores such as *Aiptasia mutabilis*, and less abundant, but equally common, sponges and tunicates which indicate that the amount of nutritional particles in suspension is high at both areas.

A very important faunistic component due to its diversity and abundance at both facilities were the gastropods and the fact that most of them were macroalguivores (with the exception of *Cerithium vulgatum*) creates important changes in possible management of these installations as algae biofilters because they are expected to eat the algae that was meant to be used as biofilters and could suppose a big problem for production, however, some of these naturally growing organisms are consumed and could have potential for aquaculture, *Patella aspera* and *Osilinus atratus* are examples of this.

The presence of some species can be indicators that some practices in the aquaculture facilities could be improved, for example, in natural conditions it would be extremely rare to find *Haliotis tuberculata coccinea* at the effluents, more importantly, the presence of *Dicentrarchus labrax*, *Sparus aurata* and *Seriola rivoliana* at the effluents mean that further measures should be taken to prevent escapes.

The modifications made to the direction of the flow of the effluents at the PCT is expected to change in some ways the fauna and flora composition in the biofilter, it would create a better disposal of the sediments thus making them easier to manage either by adding organisms able to use them as feed or by taking them out of the biofilter to be disposed elsewhere.

Some selections had to be made of all the algae and animals found at both facilities for further research, these organisms were selected based on various criteria, for example, in the case of *Schizymenia dubyi* the main interest was due to its morphology, secondly because it is a representative of red algae which has been cited as one of the preferred groups for abalone to eat in the Canary Island in wild conditions (Espino and Herrera, 2002 cited in Viera *et al*., 2005), and thirdly because of its abundance at the sedimentation tank of the complimentary building 1 of the PCT and for it being a species that has not been cited for the Canary Islands (Haroun *et al*. 2002), on the other hand, for *Colpomenia sinuosa* the main deciding factor was its abundance at the PCT, *Ulva rigida* was abundant and a proven biofilter organism, and *Valonia utricularis* presented relatively good growth.

For the filtration of sediments, the sea cucumber *Holothuria sanctori* was the only species present that fit the criteria of having potential as a sediment feeder and as a marketable organism, and even though some polychaete worms were abundant, the broad distribution and abundance of *H. sanctori* (Navarro *et al*. 2013), its potential commercial value (Aydin. 2008) and its easy handling were the determinant factors for the selection of this species

5.2. Algae growth and biofiltration

The growth rates differed between the species as expected, being *Ulva rigida* the species with the biggest growth rate and production, followed by *Schizymenia dubyi* this concurs with various references that mention that the genus Ulva is generally a very good biofilter (Neori. 2004) and that one of the most important characteristics to consider a species for biofiltration is the surface area, which increases the area available for the uptake of nutrients

Growth rate of an algae is largely defined by morphology (Littler and Littler, 1980); generally speaking, the higher the ratio of surface area to volume (SA/Vol), the faster the specific growth rate. Phytoplankton have higher SA/Vol than seaweeds, similarly, the thin sheet morphology has a higher growth rate than does the fleshy one that have a growth rate typically less than 10% day^{-1} (Marinho-Soriano *et al*., 2002; Nagler *et al*., 2003; Neori. 2004) this explains the fact that *S. dubyi* and *U. rigida* had a better growth rate than *C. sinuosa* or *V. utricularis*. For these reasons, further research should be done on the Schizymenia genus and specifically in *S. dubyi* since it represents a possibly viable new biofiltration species.

Such large standard deviations indicate that the experimental setting could be improved, not necessarily an instability in the algae cultures, the greenhouse set for these experiments had different conditions of light for each tank and the building next to it casted a shadow upon one of the sets of tanks at some hours, to avoid these problems, each week tanks were rotated, however, further measures should be taken to ensure more replicable results.

These problems could be solved increasing the amount of replicates while changing the disposition of the tanks ensuring a more even distribution of sunlight and using light sensors during the study at each tank if possible to include light and temperature as a covariate in the statistical analysis.

The variability in the cultured species could also be explained by the variability of the nutrient content in the effluents which change according to the reared organisms at the facilities, the hour of their feeding, the day of the week (no feeding on Sundays) and other factors such as the cleaning of the facilities with formalin and chlorine.

The growth rate and production of *Colpomenia sinuosa* was negative, probably because it could not adapt to the free floating conditions; the temperature and photoperiod are not expected to have an important role in these results because during the whole experiment it was growing naturally at the biofilter of the PCT, under tank conditions it usually changed color from green to brownish and then fragmented and started to get epiphytes

Specifically in one of the weeks during the culture of *S. dubyi* and *Valonia utricularis* a broad cleaning with chlorine and formalin was done in one of the areas established for larval rearing, these chemicals got to the sedimentation tank and could have gotten to the algae culture tanks in high amounts.

Biofiltration expressed by NUR and NUE also presented the expected results with *U. rigida* and *S. dubyi* performing good at both parameters, *Colpomenia sinuosa* also presented a very high nitrogen uptake efficiency even though it did not perform well under the culture conditions, this could mean that the epiphytes that grew on the Colpomenia are the real responsible for the biofiltration or that some bacterial biofiltration could be occurring.

To further improve these results and the overall health of the algae cultures some options for preventing and combat epiphytes can be applied such as filtered and even UV-treated inflow water which has been cited to greatly reduce the potential for epiphyte contamination of a system, also, the control over environmental conditions in the tanks may be used strategically to influence competition between maricultured seaweeds and epiphytes, using nutrients pulses and a high stocking density have also been successfully proven anti epiphytes techniques (Friedlander *et al*., 1987, 1991; Neori. 2004).

The proposed calculation of NUR/ Kg indicates that, apart from Ulva (that may be greatly underestimated due to the little amount of ammonia present during the weeks of study for this species), the species with more potential for biofiltration and production is *S.dubyi*. however, this calculation has some limitations because it depends on the amount of ammonia at the entrance and the exit of the biofilter which varied each week and varies even according to the hour of the day; to really evaluate the potential of different species at the same time the amount of ammonia should be controlled and the stocking density of each species should be the same, as well as the amount of light and its physiological state.

Another important parameter to be controlled is light, during the weeks of study, it varied due to cloudiness and logically, It is important to mention that the maximal biofiltration of any nutrient occurs, in daytime, yet some TAN is also taken up at night, particularly when the TAN load to the algae is low (Cohen and Neori, 1991; Schuenhoff *et al*., 2003) such as the conditions of the complimentary building 1 of the PCT.

5.3. Integrated culture of *Holothuria sanctori* and *Hydropuntia cornea*

The effluents from the sea cucumber tanks presented a significantly larger concentration of total ammonia this means that sea cucumbers have the ability of releasing nutrients from the sediment into the water column, the amount of ammonia from these effluents was also less variable than the effluents from the sedimentation tank which would make it easier to estimate the production of the whole system.

The growth and production rate of *H. cornea* had a tendency to increase when sediments and sea cucumbers were added to the setup and, even though this difference was not significative, it is expected, and the magnitude of the effect may increase with increased amounts of nutrients being released by the sea cucumbers from the sediments, the large variability between tanks can be explained by differences in light because of the orientation of the tank sets at the greenhouse.

As for the NUE and the NUR the results had no significant differences, this means that both parameters depend more on the species of algae than on the amount of ammonia being added and thus, being the same species these parameters do not change.

On the other hand, the Fv/Fm indicates that the treatment is not interfering with the physiological state of the algae and they seem to be well adapted for both cases.

The results of organic matter at the beginning of each week of experimentation where very similar to those encountered by Lucia Molina in 2000 directly under the aquaculture cages located at Melenara bay, in Gran Canaria.

Organic matter presented high variations between weeks and no big differences at the beginning and the end of each week; this could be associated with the error of the method for estimating this parameter, which may vary if the sample was not properly dried to a constant weight due to the humidity in it or if different scales where used to weigh it.

For total nitrogen in the sediments there was a marked difference between the beginning and the end of each week, especially at the first week of the experiment the difference between both dates was larger, probably because of an increased feeding activity by *H. sanctori* due to previous starvation during its time in quarantine.

The total reduction of nitrogen in the sediments does not differ significantly from the control or the beginning of each tank before the experiment took place, which was clean sand this means that the sea cucumbers where efficient in filtering the nitrogen from the sediments.

The similarity of the values for organic matter and nitrogen with the ones encountered below cages in Melenara where Molina in 2000 reported values of around 4 to 10 % for organic matter directly under the aquaculture cages, and between 10 – 18 mgN/100g for nitrogen under the same conditions, even though there were seasonal differences for both parameters obtaining the highest concentrations in warm months (June- August) and the lowest in winter, she reports that the effects of this seasonality directly under the cages are minimal and the concentrations remain almost constant. This suggests that sea cucumbers may be effectively used as natural filtration systems under aquaculture cages reducing greatly the potential impact and creating a secondary crop.

The differences between the organic matter and the nitrogen may be explained by the ability of various species of sea cucumbers to actively select patches of sediment with high nutrient value, they may be selecting the more palatable parts of the sediment, for example, they would not eat pieces of wood but these would be counted in the organic matter method (Navarro *et al*. 2012).

There are not significant differences for the growth of *H. sanctori* at the beginning and after 100 days, the high variability in each tank is due to the fact that sea cucumbers of different sizes were used, thus, even though each tank had roughly the same biomass of sea cucumbers, each individual may have different growth rate.

This may even be underestimated considering that the amount of nitrogen and organic matter in the pure sediments was higher than the amount measured at the beginning of each week, this could happen because of a large overnight feeding activity by *H. sanctori* and the fact that the samples taken had a depth of about 1 cm into the sediments and the pure sediment accumulated at the surface as well as the feeding activity of the sea cucumbers.

The growth rate of these animals is very slow so 100 days are not enough to truly evaluate this parameter, however, the weight did not increase but rather showed a tendency to decrease, this could be due to low nutritional value of the sediments because insufficient amount of sediments for the density of sea cucumbers, so more research should be done to optimize the amount of sediments and the nutrients that the sediments should have to be able to feed a given density of *H. sanctori*.

Also, at the end of the experiment some sea cucumbers were observed to exhibit reproductive behavior, even though the dates do not coincide with the reported reproductive period for this species which is once a year during the warm months (Navarro *et al*. 2012) an abnormal spawning could have occurred with its associated gonadal and weight reduction, which has been cited before by Paltzat *et al*. 2008 where overall growth was affected by the absence of visceral organs and the cessation of feeding activity who also reported a growth of 42.9g in 12 months with growth rates of 0.061 to 0.158 g d^{-1} this substantial weight loss has been also mentioned by Chuanxing *et al*. in 2009 during the aestivation phase as well as decreased growth rates were during the winter phase. Further research should be performed to evaluate this and determine the best way to ensure growth for *H. sanctori*.

The efficiency with which deposit feeders assimilate organic matter in the sediments is related to its quality or composition of the sediment (Yingst. 1976, Yingst and Aller 1982; Hauksson. 1979) and may also be influenced by seasonal differences in the quantity of organic matter in the sediments (Hauksson, 1979) these are parameters to consider if a culture of *H. sanctori* is going to be tested under cages at the Canary Islands, since as Molina cited in 2000 the organic matter and total nitrogen under the cages is variable.

However, in sedimentation tanks from inland aquaculture facilities total organic matter should be more constant and larger since they are in a close environment, it has been cited that as the total organic matter level in the feed increases, the selection of organic particles decreases and most importantly overall nutrient absorption increases, which is likely to lead to improved growth rates (Zamora and Jeffs. 2011). Zamora and Jeffs In 2012 also mention that as the total organic matter content increases in the food from 4% to 20%, the ingestion rate of sea cucumbers decreases and both the apparent assimilation and food conversion efficiencies increased. There was no significant growth of sea cucumbers in the 1% total organic matter feed treatment, while sea cucumbers in the remaining treatments had similar final wet weights with a combined mean daily specific growth rate of 0.6% d^{-1} (Zamora and Jeffs. 2012) this means that a constant and higher than 4% total organic matter in sediments should be sufficient to guarantee enough growth rates for sea cucumbers, however, this has to be tested with *H. sanctori* since it was tested with *Australostichopus mollis.*

The results of this work agree with the results observed by Zheng *et al*. in 2009 who suggested that non-artificial-feeding sea cucumber culture ponds could not only yield valuable seafood products, but also effectively remove nutrients from the aquaculture systems and consequently alleviate nutrient loadings of the nearby coasts, this also translates to the sediments from in land aquaculture facilities as this work demonstrates.

5.4. Algae rope culture at the biofiltration facility

The results observed for the culture of algae in ropes showed that it is not viable to use this system for the conditions of the biofilter at the PCT, it takes too much time and effort to stock the ropes with the algae and the growth, when it occurred, was too low.

Hydropuntia cornea specially suffered more the conditions of the rope culture, probably due to it being too brittle and easy to break, even though other species of the same genus have been successfully implemented in rope cultures by entwining or tying, as an example of this is the work by Redmond *et al* in 2014 at the New England Seaweed culture handbook where *Hydropuntia tikvahiae* is mentioned as an ideal species for rope cultures, this is probably due to its flexibility compared to *Hydropuntia cornea*.

The selection of *Grateloupia turuturu* and *G. imbricata* responded to their interest as possible species for rope culture close to aquaculture cages since they are regularly observed close to these environments and both could have interesting properties as abalone feeds.

The decrease of *H. cornea* and *G. imbricata* and the very low growth of *G. turuturu* may also be explained by other factors such as the high epiphytism observed at the frames and ropes and also the presence of various species of gastropods which got to the frames and ropes and were seen feeding on the algae (Annex 8).

The elimination of the epiphytes and gastropods would be very difficult and also, the amount of time and effort it takes to stock the ropes and place them properly makes this approach very unpractical, however, the epiphytes (mostly *Colpomenia sinuosa*) can also be used as biofilters in this setup if surface for their natural attachment and growth is increased, creating this way a biofilter installation with an Eco-systemic approach although it would not be monospecific, the production algae could be easily manageable this way and could also be used for soil nutrition or even as feeds after careful cleansing. More experiments to achieve this are already being done and a test of this is shown on annex 9.

6. CONCLUSIONS

- Different organisms with biofiltration capabilities were identified in the environments created by the facilities destined for the effluents, all of the algae species identified are potentially capable of biofiltration given the right conditions.
- Various identified animals have potential biofiltration capacities, most of them as suspension feeders such as *Aiptasia mutabilis*, sponges and tunicates, and *Holothuria sanctori* for the sediments.
- *Ulva rigida* had overall the best biofiltration efficiency and nitrogen uptake rate followed by *Schizymenia dubyi*, on the other hand, *Valonia utricularis* presented an acceptable growth and production but the nitrogen uptake rate and nitrogen uptake efficiency were not enough for it to be considered an important biofiltering species; *Colpomenia sinuosa* did not resist the free floating conditions which makes this species not suitable for typical biofiltration and production systems but further research should be made as a fixed species at land facilities such as the PCT.
- *Holothuria sanctori* was proven to be a viable organism to filter efficiently the nitrogen and organic matter in the sediments, furthermore, this reduction in the amount of nutrients in the sediments also meant the increase of nutrients available for algae uptake and production, however, more experiments should be made to define the optimal amount of sediments that the sea cucumbers are able to filtrate and their rate of filtration.
- Sea cucumbers were able to survive living in nutrient rich water and also being fed sediments from in land aquaculture facilities but they did not show growth.
- The cultivation of algae in ropes at the facilities of the PCT was proven not to be feasible a different operational approach is recommended for these facilities.

7. RECOMMENDATIONS

- More modifications should be made to the biofiltration facility at the PCT, one of them being adding a level tube at the exit of the facility; increasing drastically the volume of the biofilter and adding more area available for biofiltration.
- The seasonal variations of the flora and fauna associated to the effluents of aquaculture should be evaluated.
- Proximal analysis of the algae tested should be done, especially for *Schizymenia dubyi* to further address its potential and evaluate its palatability as as abalone feed.
- Further testing should be done to reduce epiphytes in the tanks, for example, implementating of UV filters or automatic nitrogen pulses.
- The possible use of dried macro or microalgae produced directly from the effluents of the aquaculture facility, added to the sediments to increase its nutritional value for sea cucumbers should be evaluated.
- Based upon the results we consider that the best way to improve the efficiency of the biofilter at the PCT is using an ecosystemic approach increasing the surface available for the algae to grow naturally with structures as the one presented in annex 9 and adding sea cucumbers to the sedimentation tank in both facilities.
- Further studies should be addressed to determine the optimal density of sea cucumbers being fed sediments from the aquaculture facilities.
- Sporulation and growth of *S. dubyi* to be stocked in ropes for long line culture should be studied as well as the use of *H. sanctori* directly under the aquaculture cages as biofilters and secondary crop.
- It would be ideal to evaluate the changes of the nutrients in sediment and water continuously during the biofiltration periods and also establish a correlation between the nitrogen in the sediments and the water column to be able to predict the precise effect of the sea cucumbers.

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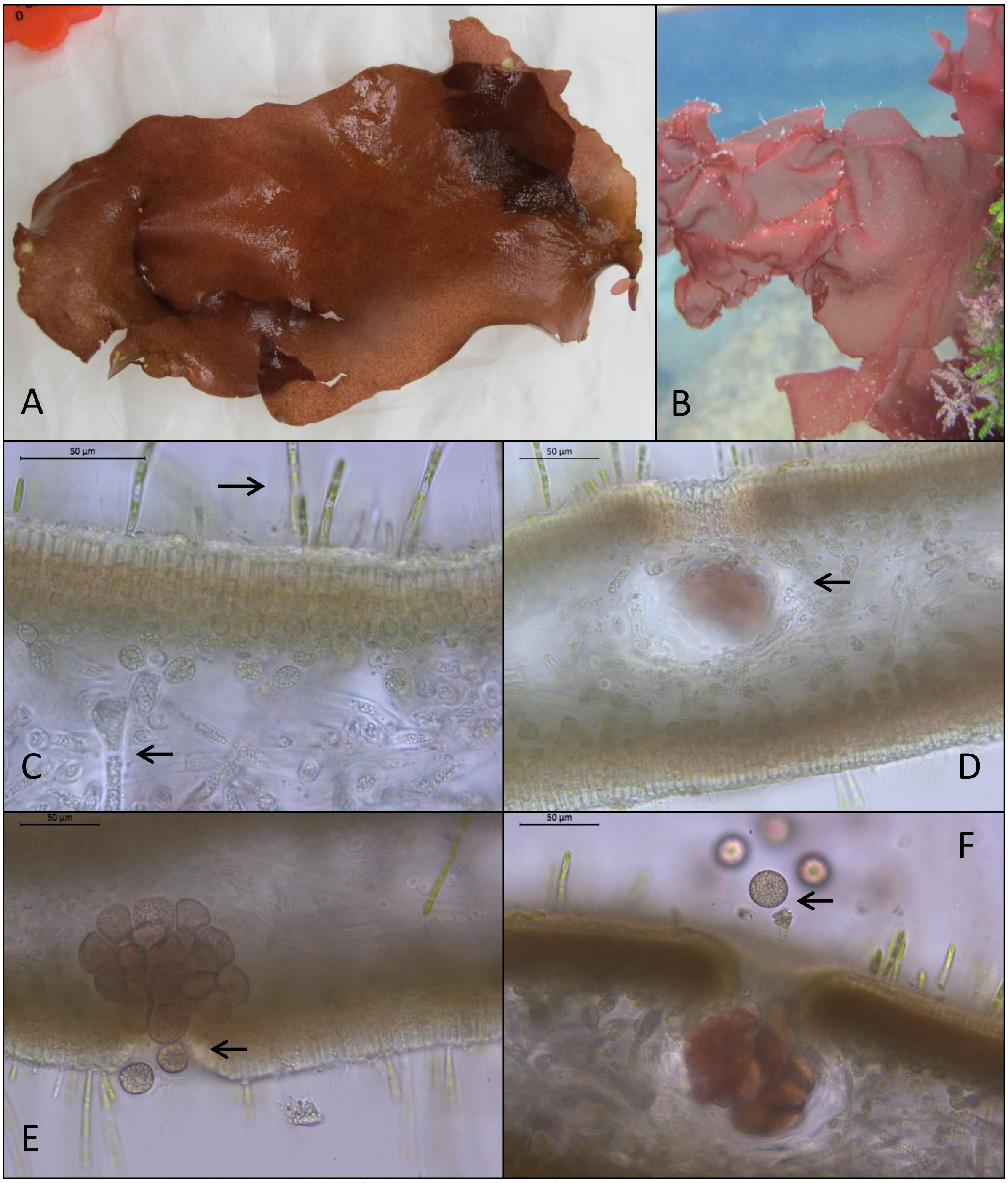

Annex 1: Example of the identification process of *Schizymenia dubyi* using its macroscopic and microscopic morphology. A) morphology of the thallus with the very small stipe B) Morphology of thallus in natural conditions, C) Cross section of the blade showing epiphytes (right arrow) and medular filaments (left arrow), D) Cross section of the blade showing early development of the gonimoblast (left arrow), E) cross section showing the ostiole in a well-developed gonimoblast and the expulsion of spores (left arrow), F) detail of the spores (left arrow).

Annex 2: Proposed mechanism to increase the surface available for algae growth at the biofilter of the PCT A) initial state, B) condition after a month viewed from the surface and C) condition after a month filled with *Colpomenia sinuosa* at the continuously submerged side.