Nutrients and organic matter reduction by sea cucumber in marine multitrophic aquaculture system

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Doctorando

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Dedicado

a la gente de

España y Iran

Dedicated to the kind and hospitable people of Spain y Iran

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General Abstract

Wastewater from traditional aquaculture contains high concentrations of mineral nutrients and organic compounds that generate environmental problems as eutrophication, oxygen depletion and water transparency reduction in the recipient ecosystems. Therefore, a sustainable aquaculture is the global challenge for scientists, policy makers and food producers. The integrated multitrophic aquaculture (IMTA) emerges as a new procedure that can alleviate these handicaps of the traditional aquaculture. This procedure includes the use of species trophically complementary, where the excretion and fecal wastes derived from the primary species are nutritional resources of the secondary species named as "extractive species".

Sea cucumbers are frequently selected as extractive species due to capacity to uptake particulate matter and detritus. However, their effects on mineral nutrients and dissolved organic components have been scantly explored. In this PhD thesis, we determined the effects of the presence of two species of holothurians (*Holothuria tubulosa* and *Holothuria forskali*) co-cultured with *Anemonia sulcata* as primary species on water transparency and quality. We determined the effects of these sea cucumbers on mineral nutrients, particulate organic components and optical properties of dissolved organic matter (DOM) including chromophoric and fluorescence components. Finally, we used fluorescence spectroscopy (excitation-emission matrixes (EEMs) and PARAFAC analysis) to assess the actual influence of holothurians on humic-like compounds (biologically more refractory) and amino acid-like compounds (biologically more labile). To reach these objectives we used two approaches: time-series monitoring in two big tanks with and without holothurians and short-term experiments in smaller tanks with all the other sources of variability controlled to corroborate the observations obtained in the time series.

In the time-series of two big-volume tanks, we monitored during more than one year the concentration of mineral nutrients (ammonium, nitrite, nitrate, and total phosphorus), total organic carbon (TOC), particulate organic matter (POM), transparent exopolymer particles (TEP), chlorophyll-a, bacterial abundance, chromophoric dissolved organic matter (CDOM) including absorption coefficients and spectral slopes and fluorescence

dissolved organic matter (FDOM). The short-term experiments consisted of manipulate the presence of holothurians. Each experiment was carried out in seven tanks that contained individuals of A. sulcata. At the initial time, in three of the tanks we also included individuals of H. tubulosa. These three tanks are the replicates of the +holothurians treatment. The other four tanks only contained A. sulcata and represent the replicates of the –holothurians treatment.

We found that the concentration of ammonium, nitrate, TOC, POM, TEP, and bacterial abundance in the effluent waters from the tank with holothurians was significantly lower than the effluent waters of the tank without holothurians. The consumption of transparent exopolymeric particles by holothurians was a result particularly remarkable and novel. The experiments confirmed the time-series results with reductions statistically significant in nitrates, bacteria and TEP concentration in the treatments with holothurians. This TEP and bacteria consumption by holothurians is of great relevance in the maintenance of the tank hygiene and the control of potentially pathogenic bacterial outbreaks that can be associated to biofilm generation by exopolymeric particles.

In relation to DOM optical characterization, in the time-series we observed that absorption coefficients (a_{325}) and spectral slopes ($S_{275-295}$) were significantly lower in the effluent of the +holothurian tank than in the effluent of the -holothurian tank. This reduction in the absorption of the dissolved organic matter appears to be mediated by the POM consumption by holothurians. The experiments confirmed the results observed in the time-series. The a_{325} and $S_{275-295}$ values were significantly lower in the treatment with holothurians than in the treatment without holothurians indicating a reduction in the concentration of chromophoric organic compounds, particularly of compounds with lower molecular weight. This reduction in the concentration of particulate and chromophoric organic matter can affect positively to water transparency by reducing light scattering and absorption.

EEMs and PARAFAC analysis provided a six components model with four humic-like components (C1, C2, C3 and C4) and two amino acid-like components (C5 and C6). We observed than holothurians were able to reduce significantly both humic-like and amino acid-like components, likely due

to their great efficiency removing particulate organic matter. In the short-term experiments we confirmed that holothurian were able to reduce two of the humic-like components (C2 and C4) and the two amino acid-like components (C5, tryptophan-like and C6, tyrosine-like). We suggest that symbiotic bacteria in the tentacle epidermis and below the cuticle in holothurians could directly assimilate these amino acids and explain the remarkable significant reduction of the two components that we observed in both the time-series and particularly the short-term experiments.

Resumen General

Las aguas residuales procedentes de la acuicultura tradicional contienen elevadas concentraciones de nutrientes minerales y compuestos orgánicos que generan problemas de eutrofización, disminución de oxígeno y reducción de la transparencia del agua en los ecosistemas receptores. Por lo tanto, una acuicultura sostenible es un desafío global para los científicos, los políticos y los productores de alimentos. La acuicultura multitrófica integrada (IMTA) surge como un nuevo procedimiento que puede aliviar estos hándicaps de la acuicultura tradicional. Este procedimiento incluye el uso de especies complementarias tróficamente, dónde los desperdicios de excreción y fecales derivados de la especie primaria son recursos nutritivos para especies secundarias denominadas "especies extractivas".

Los pepinos de mar son seleccionados frecuentemente como especies extractivas debido a su capacidad para ingerir materia particulada y detritus. Sin embargo, sus efectos sobre los nutrientes minerales y los componentes disueltos orgánicos han sido escasamente explorados. En esta tesis doctoral, nosotros determinamos los efectos de la presencia de dos especies de holoturias (*Holothuria tubulosa* y *Holothuria forskali*), co-cultivadas con *Anemonia sulcata* como especie principal, sobre la transparencia y calidad del agua. Nosotros determinamos los efectos de los pepinos de mar sobre la concentración de nutrientes minerales, componentes orgánicos particulados y propiedades ópticas de la materia orgánica disuelta (DOM) incluyendo los componentes cromofóricos y fluorescentes. Por último, usamos espectroscopía de fluorescencia (matrices de emisión-excitación (EEMs) y análisis PARAFAC) para valorar la influencia real de las holoturias sobre los compuestos similares a los ácidos húmicos (biológicamente más refractarios) y los compuestos similares a aminoácidos (biológicamente más lábiles). Para alcanzar estos objetivos hemos usados dos aproximaciones: el seguimiento en una serie temporal de dos tanques de gran volumen en el que uno contenía holoturias y el otro no y experimentos cortos con tanques más pequeños pero con más réplicas y con todas las otras fuentes de variación controladas para corroborar las observaciones de las series temporales.

En las series temporales en los dos tanques de gran volumen, hicimos un seguimiento durante más de un año de la concentración de nutrientes minerales

(amonio, nitrito, nitrato, fósforo total), carbono orgánico total (TOC), materia orgánica particulada (POM), partículas exopoliméricas transparentes (TEP), clorofila-a, abundancia bacteriana, materia orgánica disuelta cromofórica (CDOM) incluyendo coeficientes de absorción y pendientes espectrales y materia orgánica disuelta fluorescente (FDOM). Los experimentos cortos consistieron en manipular la presencia de holoturias. Cada experimento se realizó en siete tanques con individuos de A. sulcata. A tiempo inicial, en tres de los tanques se incluyeron individuos de H. tubulosa. Estos tres tanques son las réplicas del tratamiento +holoturias. Los otros cuatro tanques sólo contenían A. sulcata y representan las réplicas del tratamiento –holoturias.

Encontramos que la concentración de amonio. Nitrato, TOC, POM, TEP y abundancia de bacterias en las aguas efluentes del tanque con holoturias fue significativamente menor que en las aguas efluentes del tanque sin holoturias. El consumo de partículas exopoliméricas transparentes por las holoturias fue un resultado particularmente remarcable y novedoso. Los experimentos confirmaron los resultados de las series temporales con reducciones estadísticamente significativas para el nitrato, bacterias y concentración de TEP en los tratamientos con holoturias. Esta consumo de TEP y bacterias por las holoturias puede ser de gran relevancia en el mantenimiento de la higiene en los tanques y el control de brotes de bacterias potencialmente patógenas que se asocian a la generación de biopelículas por TEP.

En relación a la caracterización de la materia orgánica disuelta, en las series temporales observamos que los coeficientes de absorción (a_{325}) y las pendientes espectrales ($_{S275\text{-}295}$) fueron menores en los efluentes del tanque +holoturias que en el tanque –holoturias. Esta reducción en la absorción de la materia orgánica disuelta parece que estuvo mediada por el consumo de POM por las holoturias. Los experimentos confirmaron los resultados de las series temporales. Los valores de a_{325} y $S_{275\text{-}295}$ fueron significativamente menores en el tratamiento con holoturias que en el tratamiento sin holoturias indicando una reducción en la concentración de compuestos orgánicos cromofóricos, particularmente de compuestos con bajo peso molecular. Esta reducción en la concentración de materia orgánica particulada y cromofórica puede afectar positivamente a la transparencia del agua

reduciendo la dispersión y absorción de la luz.

El análisis de EEMs y PARAFAC dio un modelo de seis componentes con cuatro similares a ácidos húmicos (C1, C2, C3 and C4) y dos similares a aminoácidos (C5 y C6). Observamos que las holoturias fueron capaces de reducir significativamente tanto compuestos similares a húmicos como a aminoácidos, probablemente debido a su gran eficiencia retirando materia orgánica particulada. En los experimentos cortos confirmamos que las holoturias redujeron dos compuestos húmicos (C2 y C4) y dos como amino ácidos (C5, similar al triptófano y C6 similar a la tirosina). Especulamos que la presencia de bacterias simbióticas en la epidermis de los tentáculos y por debajo de la cutícula en las holoturias podrían asimilar estos aminoácidos directamente y explicar con ello esta reducción tan remarcable de estos dos componentes que observamos tanto en la serie temporal como en los experimentos cortos.

Glossary of abbreviations

Aquaculture

IMTA	Integrated Multitrophic Aquaculture
FAO	Food and Agriculture Organization of United Nation
APHA	American Public Health Association
AWW	Aquaculture waste Water

Nutrients and Exopolymer

DIN	Dissolved inorganic nutrients
TN	Total nitrogen
SRP	Soluble reactive phosphorous
TOC	Total organic carbon
TP	Total phosphorous
DOM	Dissolved organic matter
POC	Particulate organic carbon
POM	Particulate organic matter
TDS	Total dissolved solids
TEP	Transparent exopolymer particles
XG	Xanthan gum

Prokaryotes

DMSO	Dimethyl sulfoxide
SSC	Bivariate plots of Side scatter
FL1	Green fluorescence
FL2	Orange fluorescence
FL3	Red fluorescence

CDOM

CDOM	Chromophoric dissolved organic matter
a_t	total spectral absorption coefficient
a_w	absorption coefficient of pure water
a_{ph}	absorption coefficient of phytoplankton pigments (in algae cells)
a_{nap}	absorption coefficient of non-algal particulate material
a_g	absorption coefficient of chromophoric dissolved organic matter
a_s	absorption coefficient of inorganic salts dissolved in seawater
a_{325}	absorption coefficient at 325 nm

$S_{275\text{-}295}$ Spectral slope over the wavelength range 275–295 nm
$S_{350\text{-}400}$ Spectral slope over the wavelength range 350–400 nm
SR Ratio of spectral slopes over the ranges 275–295 nm and 350–400 nm
UV Ultraviolet

FDOM
FDOM Fluorescent dissolved organic matter
EEM Excitation Emission Matrix
Peak A/C Humic-like FDOM peak at Ex/Em < 270-370/470 nm
Peak M Humic-like FDOM peak at Ex/Em 320/400 nm
Peak T Tryptophan-like FDOM peak at Ex/Em 290/340 nm
Peak B Tyrosine-like FDOM peak at Ex/Em 270/310 nm
PARAFAC parallel factor analysis
RU Raman Unit

Chapter 1

Introduction

IMTA for water protection

Introduction

Traditional aquaculture

During the last several decades, human activities such as overfishing, pollutants addition, and climate change have greatly affected species diversity in the marine ecosystems (Halpern *et a*l., 2008; Purcell *et al*., 2013). The exponential growth of the human population has increased the global demand of fish and seafood (FAO, 2016). Consequently, the human consumption of seafood is increasing, but extractive fisheries are more and more limited. In fact, aquaculture meets more than 40% of human demand for fish and seafood (Bostock *et al*., 2010). In the last FAO report (2016) the aquaculture production, for the first time, overtakes fisheries as fish and seafood provider. Therefore, a responsible aquaculture is a global challenge for both aquatic scientists and food producers (Diana *et al*., 2013).

Traditional aquaculture produces waste-water, which usually contains high quantities of organic and inorganic nutrients, antibiotic, uneaten food pellets, etc. (Black, 2001; Read & Fernandes, 2003; Klinger & Naylor, 2012). This aquaculture waste-water (AWW) affects the marine environment depends on the aquaculture production system (extensive vs. intensive/semi-intensive), aquaculture system (tank (inshore), pond, cage (out-shore)), and the cultured species, but also on the carrying capacity of the recipient waters. AWW discharges from aquaculture facilities can cause coastal eutrophication and/or sediment anoxia (Crab *et al*., 2007). Ecosystem health and, consequently, biodiversity are influenced by nutrient concentrations and their ratios. This is largely because the concentrations of limiting nutrients (typically N in marine systems) frequently control primary productivity. AWW can be an important source of nitrogen for marine waters, stimulating primary producers and increasing the risk of algal blooms or red tides (Ajin *et al*., 2016). Consequently, eutrophication and oxygen depletion (with consequent release of ammonia and sulfide from sediments) modify physicochemical properties of water (Kalantzi & Karakassis, 2006) and might be toxic for some fishes and coral reefs. In extreme cases, the decomposition of organic material will cause "dead zones' (Dodds, 2006)". These "Dead zones" have been a common re-occurring problem in the half past century. Therefore, the promotion of techniques for nutrient removal or limited nutrient enrichment will help to optimize ecosystem health. Indeed, inorganic and organic extractive aquaculture, independently or as components of integrated multitrophic

aquaculture (IMTA), can be relevant in the assimilation of these nutrients before they reach the environment. In addition, fecal waste and uneaten foods make up a small percentage of particulate organic matter that settles down in the bottom of the tanks or below the cages on marine sediments affecting bacterial activity and sediment properties and can contribute to detritus-feeder species.

Particulate organic matter (fecal pellets, detritus, and uneaten food) as a component of AWW can settle in the bottom affecting the structure of sediments and bacterial activity. Consequently, an oxygen depletion and other harmful byproducts such as ammonia, sulfides can affect macrobenthic community. Many studies have analyzed AWW effects on community structure in shore and out-shore marine waters. The AWW inputs can change species richness (Weston, 1990; Frouin, 2000), abundance, biomass and diversity of macrobenthos (Apostolaki *et al.*, 2007; Kutti *et al.*, 2007; Nickell *et al.*, 2003; Yokoyama, 2002; Boyd & Massaut, 1999).

AWW also affects species. Inorganic nutrients as, for instance, nitrite can be toxic and act as contaminant factor through the passive transport into the gills. When nitrite convenes with the hemoglobin produces methemoglobin. This form of hemoglobin cannot uptake oxygen resulting in hypoxia symptom for many aquatic species, especially fishes. Therefore, in the case of nitrite contamination, fish death in natural environment can happen (Lewis & Morris, 1986; Tilley *et al.*, 2002). Nitrite is generally less toxic in seawater than in freshwater. It is converted into nitrate by bacterial activity (*Nitrobacter sp.*). Nitrate is toxic for marine aquatic organisms only at high concentrations (Camargo *et al.*, 2005; Hickey, 2009). Some inorganic nutrients, such as ammonium, are produced during the remineralization of organic matter (Chen *et al.*, 1993). Ammonium is highly toxic for fishes. In aquatic systems, normally there is equilibrium between various forms of ammonium (ionized and unionized). Both forms depend on water alkalinity and the unionized form (NH_3) is more toxic for the fishes. NH_3 concentrations higher than 0.05 mg L^{-1} damage the gills and higher than 2.0 mg L^{-1} are lethal for many fishes.

In the particular case of inshore installations, wastewater effluents from aquaculture tanks usually are treated before being returned to the aquatic ecosystems. Several procedures to treat aquaculture wastewaters are in practice. To decide which treatment is more appropriate several factors

should be considered such as land and water availability, wastewater local regulation and operational expenses. Wastewater treatments such as Fenton's oxidation (Lee & Shoda, 2008), sequencing batch reactor (Fontenot *et al*., 2007), up-flow anaerobic sludge bed or integrated anaerobic/aerobic biological treatments (Bortone, 2009) have been used but all imply high economical costs and can eventually generate toxic by-products and membrane fouling in comparison with alternative treatments with a more biological approach.

In general, biological treatments are more acceptable by fish producers and policy makers. For instance, Da *et al*. (2015) proposed the reuse of wastewater from Striped Catfish farms in rice crops. Other authors proposed the recovering of phosphorous from wastewaters using the gastropod shell (Oladoja *et al*., 2015) or aquatic plants (Buhmann & Papenbrock, 2013 and Zhang *et al*., 2014b). Diana *et al*. (2013) recommended IMTA and poly-culture as responsible procedures that can decrease inorganic and organic nutrient loads in the effluents using "extractive species" that also can reduce the costs of wastewater treatments.

Poly-culture and Integrated Multitrophic aquaculture (IMTA)

Poly-culture and IMTA, unlike monospecific aquaculture, use trophically complementary species where the excretion, fecal and food wastes from the primary (fattening) species are nutritional resources for the extractive species (Chopin *et al*., 2012). Therefore, these aquaculture systems include the primary species (e.g., a commercial fish species), the organic extractive species that filter or ingest the suspended or settled down organic matter and detritus (e.g., mussels, oysters, sea cucumbers), and species that assimilate inorganic nutrients (e.g., seaweeds) reducing the loads of inorganic nutrients and organic matter in the effluents (Fig.1.1). Therefore, it is desirable that the future expansion of aquaculture develops these co-culture procedures to remove or, at least, reduce these organic and inorganic loads in the effluents and, simultaneously, provide an extra co-cultured species with additional economical and ecological value making aquaculture more sustainable (Diana *et al*., 2013). The IMTA concept is extremely flexible. It can be applied in open-water cages or land-based systems as tanks or aquaponic installations, both in marine and freshwater ecosystems.

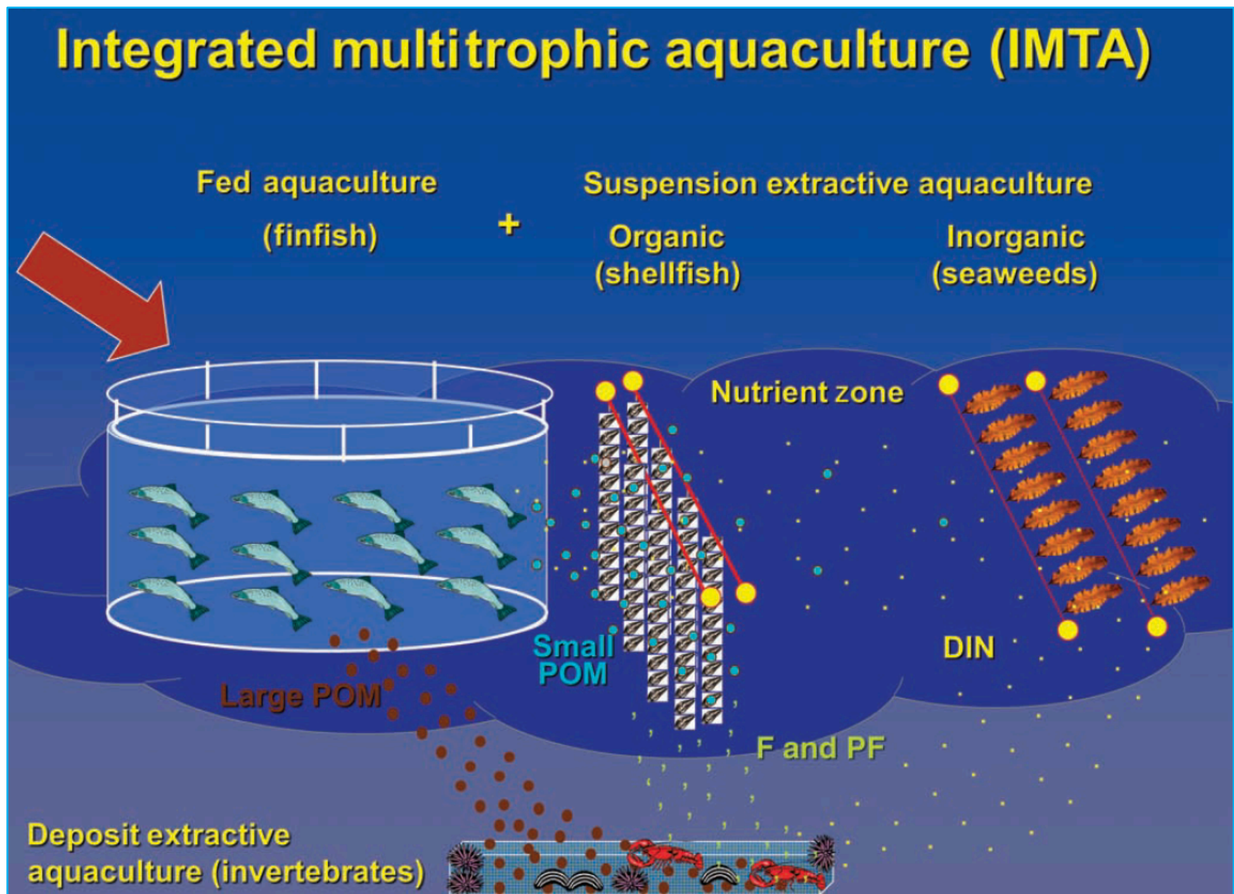


Fig. 1.1. Conceptual diagram of an integrated multi-trophic aquaculture (IMTA) system. POM means particulate organic matter, DIN means dissolved inorganic nutrients, and F and PF mean feces and pseudofeces, respectively. Figure taken from Chopin *et al.* (2012).

Mono-specific aquaculture produces wastewater that usually contains high concentrations of organic matter as well as inorganic nutrients, antibiotics and uneaten food pellets (Read & Fernandes, 2003; Klinger & Naylor, 2012). Therefore, as we have mentioned previously, at the ecosystem level, on the one hand the effluents with mineral nutrients associated can produce eutrophication (Ajin *et al.*, 2016; Ruiz-Zarzuela *et al.*, 2009). On the other hand, the effluents also include dissolved and particulate organic matter; which can reduce water transparency due to an increase in light backscattering and absorption by organic compounds with chromophoric groups (Ibarra *et al.*, 2012; Del Bel Belluz *et al.*, 2016). Therefore, a sustainable aquaculture with low environmental impact should to consider reductions in mineral nutrients and organic matter in its effluents. Indeed, poly-cultures and IMTA can alleviate to some degree the handicaps of traditional aquaculture.

Diana *et al*. (2013) have suggested that the integrated multitrophic aquaculture (IMTA) or multispecies co-cultures are responsible procedures; which can decrease inorganic and organic nutrient loads in the effluents, reducing the costs of effluent treatments. On the other hand, co-cultured, extractive species might be an additional source of food or, alternatively, biotechnological resources (Bhatnagar & Kim, 2010; Valliappan *et al*., 2014; Leon-Palmero *et al.,* 2018).

More recently, poly-cultures, IMTA and aquaculture in general have been proposed not only as procedures to obtain food resources, but also for restoration of natural marine and freshwater ecosystems (Froehlich *et al.,* 2017). Aquaculture production can diversify the cultured species and this diversification can also help to maintain species that have been overexploited and are declining such us sea cucumbers in some regions as Asia (Purcell *et al.,* 2013), but also in the Mediterranean region (González-Wangüemert *et al.,* 2018). This diversification will depend, to some extent, on local demands, culture price, food history, availability, etc.). The benefits of diversification are not only limited to human consumption but also, through economical aspects related to marine products with pharmaceutical or cosmetic potentials. However, more research is needed to provide evidence of new species with diverse economical and ecological interests.

Poly-culture and IMTA in European Union regulation

For many years in the European Union, several rules and monitoring program for coastal water contamination from aquaculture sources have been promoted headed to the named "Blue Growth" (COM, 2013). It seems, more than ever, important to design and apply production methods with low cost operation, streamlined, sustainable and with less wastewater effect on natural environment, accommodating with international-regional rules and economic-effective benefits such as polyculture and IMTA. Eight directives are related to the management of the environmental impacts of aquaculture. One of the most important includes Environmental Impact Assessment Directive, Strategic Environmental Assessment Directive and Water Framework Directive (Codling *et al*., 1995; Read & Fernandes, 2003). In addition, these directives are not the only regulation for AWW, but all aquaculture activity has to be organized under other International Conventions (Read & Fernandes, 2003).

Components of Particulate Organic Matter (POM)

POM extractive species can uptake bacteria, phytoplankton cells, detritus, fecal pellets, and transparent exopolymer particles. **Bacteria** are prokaryotes in the range of 1 μm in size and consist of species that are both autotrophic and heterotrophic (Fig.1.2.). Usually, bacteria abundance ranges from 10^5 to 10^7 cells ml^{-1} in seawater, with higher concentrations in the surface ocean (Emerson & Hedges, 2008). The different species of cyanobacteria Synechococcus and Prochlorococcus dominate the **Pico-phytoplankton** in most oceanic regions Partensky *et al*., (1999). Their importance was discovered in the 1970s and 1980s with the development of epifluorescence microscopy.

Transparent exopolymer particles (TEP) are polysaccharide-enriched and organic gel-like particles with a sticky nature generated by aquatic microorganisms (Passow, 2002). TEP origin is complex, involving microorganisms of several trophic levels and physiological conditions. TEP in marine systems are predominantly formed by colloidal precursors (fibrils 1-3 nm diameter) (Fig.1.2). when pieces of mucus detach from phytoplankton cell surfaces(Fig.1.3). Polysaccharides precursors are mainly formed during the exponential growth phase of phytoplankton, the sloppy feeding by grazers, bacterial growth, and viral infections (Passow, 2002; Ortega-Retuerta *et al*., 2010; Vardi *et al*., 2012).

TEP and their dissolved precursors has a major role as a micro-habitat for bacteria providing physical structure and forming biofilms (Bar-Zeev *et al.,* 2012). Providing surface for bacteria colonization.

TEP could be used as an important source of food by filter feeders such as protozoa and appendicularia and pico-euphasids allowing dissolved material to enter into the food web. Therefore, TEP can be used as food and, in the other hand; they can attract commensal bacteria and sequester micronutrients and toxins, which can be essential in the hygiene of hatcheries. In fact, Wotton (2005, 2011) observed that the reduction of TEP in the water column is mostly due to marine invertebrate.

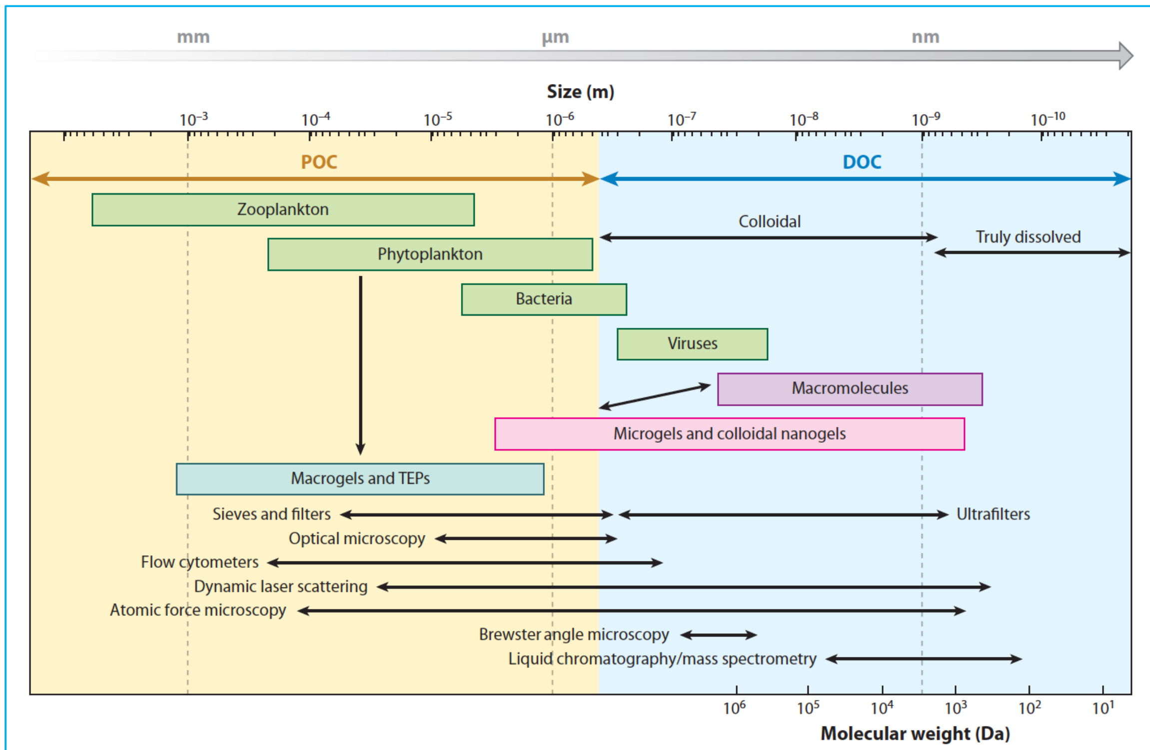


Fig. 1.2. Size scale of the different organic matter components found in seawater. Material retained by seawater filtration at a 0.2-µm cutoff pore is regarded as carbon-containing particulate stock or particulate organic carbon (POC). The fraction that passes through the filter is labeled as dissolved organic carbon (DOC)–containing, and includes colloidal and truly dissolved materials. POC comprises, besides marine microorganisms, transparent exopolymer particles (TEPs). This figure is taken from Verdugo (2012).

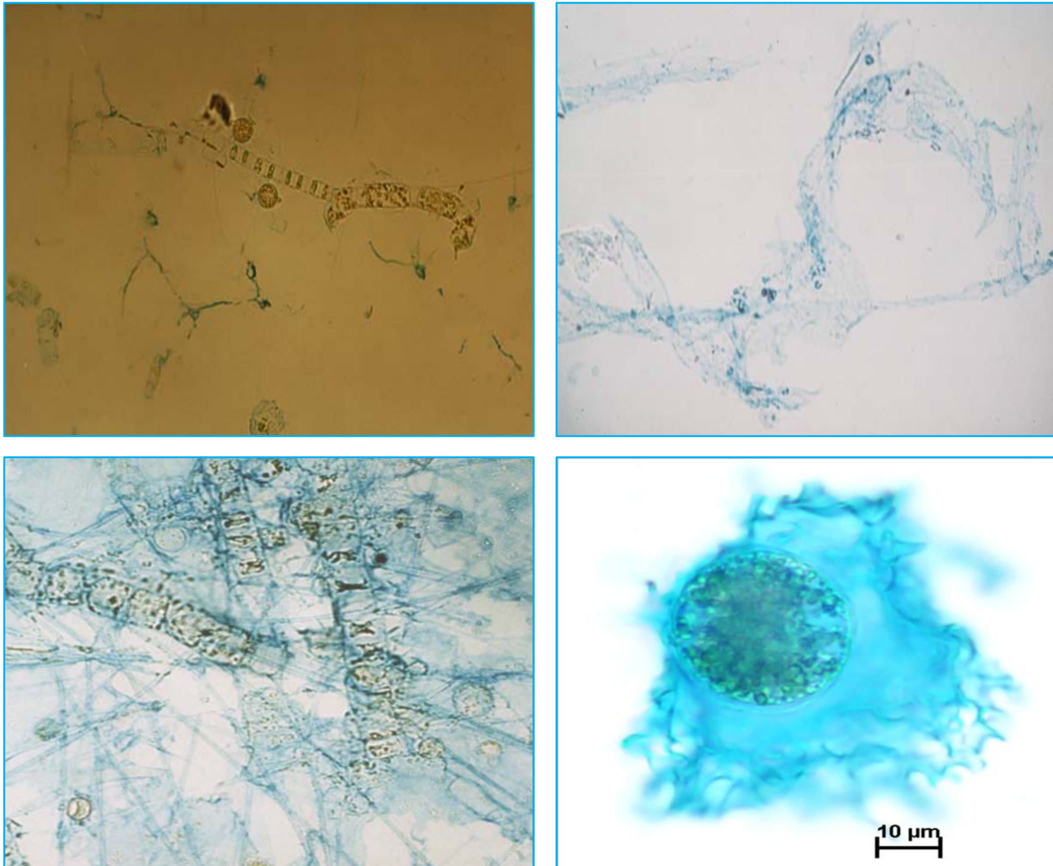


Fig. 1.3. Exopolymer particles stained with alcian blue. a) small particles, b) large fibrils, c) matrix of phytoplankton cells and aggregates (Passow and Berman, 2011) and d) TEP excreted by *Lepidodinium chlorophorum* (taken from Claquin *et al*., 2008).

Dissolved nutrients and organic matter

Operationally ''dissolved'' and ''particulate'' phases are defined by using 0.2-0.45 µm pore-size filters. The dissolved fraction passes this pore size, whereas the particulate fraction is retained on the filter. Chemical species in seawater are classified into four categories conservative elements, bioactive elements, adsorbed or scavenged elements and gases.

Mineral nutrients are bioactive elements, since they are affected by biota activity. These elements are nutrients required for phytoplankton growth such as PO_4^{3-}, NO_3^-, HCO_3^- and oxygen consumed during respiration. Some plankton species also need Si and Ca and trace metals (e.g., Fe) necessary for plankton growth or for shell or plaques formation. HCO_3^- is a major ion in seawater, while the micronutrients PO_4^{3-}, NO_3^- and SiO_3^{2-1} have concentrations that range from nano-to micromoles per kilogram of seawater. The bioactive metals are trace elements having concentrations in the range of nano- or picomoles per kilogram of seawater.

Dissolved organic matter (DOM) is a pool of diverse and complex molecules that are, especially in aquaculture, poorly characterized. The simplest of organic substances are hydrocarbons contain only carbon chains with hydrogen. In essence, hydrocarbons are classified based upon whether the carbon chain includes rings, double bonds, or branches. Straight-chain hydrocarbons are called aliphatic; rings are termed cyclic. Then, they are subdivided into aromatic and alicyclic, depending on whether or not they contain multiple carbon double bonds. Aliphatic compounds are further characterized as either depending on the presence or absence of double bonds. Saturated or unsaturated hydrocarbons have only CC and CH single bonds and number of hydrogen atoms per carbon (e.g., the maximum possible thus contain carbons are saturated with hydrogen). Unsaturated hydrocarbons contain fewer parts owing to the introduction of multiple bonds (e.g., in alkenes and alkynes) or the formation of ring structures. Many other classes of organic compounds are formed from hydrocarbon backbones by the addition of characteristic groups of atoms called functional groups (Emerson & Hedges, 2008). Rings and double bonds are chromophoric sites that are able to absorb photons from a wide range of wavelengths.

The ocean hold one of the largest reservoirs of organic carbon and a vast amount of the

carbon stored in ocean waters is in dissolved form (Hansell *et al*., 2009). With the exception of viruses and very small bacteria, the different components of the dissolved organic matter (DOM) pool are non-living and too small to sink in the water column. This distinction between particulate organic matter (POM) and DOM is defined based on filter pore size and the ability of particles to gravitationally settle. Thus, small particles and colloids are included in DOM pool (Fig 1.2). These particles are small enough and their density difference with water is low and they are transported largely by the water flow along with the dissolved organic matter (Emerson & Hedges, 2008). Seawater colloids also are sufficiently large to be effectively separated from other DOM components by ultrafilters with pore sizes as small as a nanometer(Fig.1.2).

Dissolved organic matter (DOM) is the main substrate of the microbial heterotrophs, which are consumed by heterotrophic protozoa and micro-zooplankton forming the named microbial loop (Pomeroy *et al.,* 2007). Seawater DOM also affects light transmission in the ocean, is involved in photochemical reactions, complexes metals, and carries biological and geochemical information (Hansell & Carlson, 2002). In particular, organic substances strongly complex iron, which is limiting for photosynthesis in large areas of the ocean.

DOM encompasses a large fraction of chomophoric compounds, which absorb light in the ultraviolet (UV) and, to a lesser extent, in the visible range of the spectrum. This fraction of DOM is termed **chromophoric dissolved organic matter (CDOM)**(Fig.1.4). Therefore, CDOM is largely responsible for UV and blue light attenuation in marine ecosystems (Bricaud *et al*., 1981; Nelson & Siegel, 2013). Since CDOM absorption overlaps one of the chlorophyll *a* absorption peaks, CDOM can diminish the potential for primary productivity. This fact affects the algorithms used in remote sensing to determine ocean color and infer primary productivity (Carder *et al*., 1989; Siegel *et al*., 2005; Ortega-Retuerta *et al*., 2010). Remote sensing has been suggested as an excellent tool to monitor the impact of offshore aquaculture (Populus *et al*., 1995; Rajitha *et al*., 2007; Saitoh *et al*., 2011). However, the relation between aquaculture waterwaste and CDOM has been scantily explored (Ibarra *et al*., 2012; Nimptsch *et al*., 2015; Del Bel Belluz *et al*., 2016).

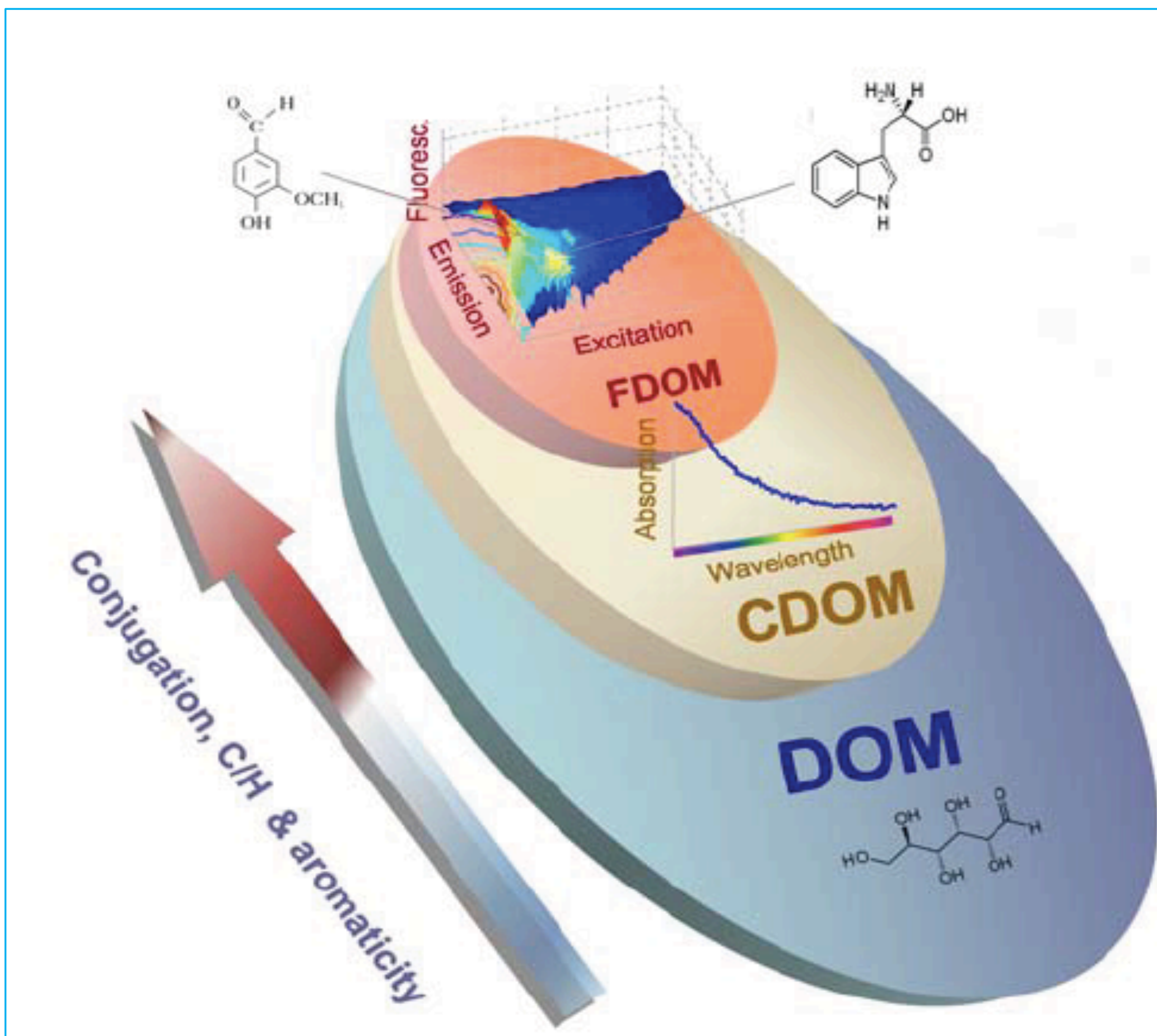


Fig. 1.4. Scheme of the chromophoric (CDOM) and fluorescent (FDOM) fraction of DOM in the total pool. The arrow indicates an increase in aromaticity, conjugation, and carbon to hydrogen (C/H) ratio. An example of CDOM absorption spectra and a FDOM excitation-emission matrix are shown. Structure of tryptophan, a natural amino acid, and vanillin, a constituent of lignin, are shown as examples of amino acid-like and humic-like fluorescent DOM, respectively. Taken from *Stedmon and Álvarez-Salgado*, 2011.

A fraction of CDOM compounds are also able to fluoresce. This last fraction is termed fluorescence dissolved organic matter (FDOM) (Fig.1.4). Absorption and fluorescence spectroscopy provides a comprehensive information on the nature of DOM pool in the ocean without an intrinsic chemical characterization. Chromophore and fluorophore compounds include aromatic amino acids (Yamashita & Tanoue, 2008), lignin phenols, and humic substances that are operationally characterized by their absorption and fluorescence properties (Coble, 1996).

Chromophoric Dissolved Organic Matter (CDOM) is also termed colored DOM. In seawaters, especially in places with high CDOM concentration (e.g., coastal water), CDOM seems brown color so it also called "Gelbstoff", "yellow substance" (Shifrin, 1988), or "gilvin" (Kirk, 1994). CDOM chromophores are specified by their optical properties including intensity (absorption coefficients or chromophore concentration) and shape (wavelengths across which it occurs). The chromophore concentration and the wavelength decay shape follow the Beer-Lambert law and electronic transition, respectively. Briefly, the aromatic chromophores with high conjugation and "-OR" group substitution are absorbing light at maximum wavelength (visible range) in comparison to less conjugated and simple chromophores.

CDOM in coastal waters is mainly originated from allochthonous sources such as terrestrial water or reverie inputs (Del Vecchio & Blough, 2002). Otherwise, it could be also produced by autochthonous sources such as phytoplankton extracellular release, excretion, sloppy feeding during grazing, viral cell lysis (Carlson *et al*., 2002). In surface oceanic water, CDOM normally is produced by primary producers (phytoplankton) (Romera-Castillo *et al.,* 2010) or associated bacteria (Steinberg *et al*., 2004; Ortega-Retuerta *et al.,* 2009). Interestingly, absorption properties of each source are different among them and, consequently, we can obtain information about DOM sources from its optical signatures. Depending on their molecular weight, DOM components can be consumed by heterotrophic bacteria (Raymond *et al*., 2000). Bacteria use extracellular enzyme to process DOM (Allison *et al*., 2012). It seems that bacteria preferentially consume fresh, with low chromophoric content DOM in surface waters. In the case of allochthonous DOM, bacteria need first to convert it into low molecular weight compounds, but a fraction of this DOC is exudated by bacteria with high

chromophoric content (Ortega-Retuerta *et al*., 2009; Yamashita & Tanoue, 2009; Catalá *et al*., 2015). CDOM can be also reduced by photobleaching (Micinski *et al*., 1993). Then, these photobleached compounds can enter into the microbial loop (Moran & Zepp, 1997) or be mediators for trace elements (Micinski *et al*., 1993).

In marine systems, CDOM is considered the dominant source of light absorption throughout the blue and especially in the UV spectral regions (Nelson & Siegel, 2013). This absorption of light contributes to its attenuation through the water column (Guéguen & Kowalczuk, 2013). In all natural waters the spectral absorption coefficient of water, a_t (λ), is defined as the sum of the absorption coefficient of pure water, a_w (λ), phytoplankton pigments contained in algae cells, a_{ph} (λ), non-algal particulate material, a_{nap} (λ), chromophoric dissolved organic matter (CDOM), a_g (λ), and inorganic salts dissolved in seawater, a_s (λ) (equation 1)

$$a_t(\lambda)=a_w(\lambda)+a_{ph}(\lambda)+a_{nap}(\lambda)+a_g(\lambda)+a_s(\lambda) \quad (1)$$

CDOM in sea and coastal waters is measured using spectrophotometers that contain one UV lamp and another visible lamp. CDOM absorption properties are mainly described by quantitative parameters such as the absorption coefficients a(λ) that are indicative of the quantity of chromophores (Nelson *et al*., 2010) and by qualitative parameters includes the spectral slopes (S) and the molar absorption coefficients as indicative of molecular structure. Spectral slopes describe the shape decay of absorption coefficient vs. wavelength (Twardowski *et al*., 2004). There are other CDOM parameters such as SUVA indexes (e.g., a_{254}/DOC or a_{325}/DOC) to account for the abundance of conjugated carbon double bonds (Weishaar *et al*., 2003). Each optical parameter provides information to describe CDOM. For example the spectral slope could be used to trace water origin (Vodacek *et al*., 1997), chemical composition and nature (Carder *et al*., 1989), degradation or photobleaching (Helms *et al*., 2008).

DOM has not a definite chemical composition and a detailed exploration of its composition is very difficult. Therefore, these optical parameters can offer an alternative approach to evaluate changes in the quantity and quality of DOM.

Fluorescence dissolved organic matter (FDOM) is the part of CDOM that emit fluorescence signal when it irradiated by UV light (Coble, 1996; 2007) (Fig.1.4). Organic matter fluorescence occurs when a loosely held electron is excited to a higher energy level by the absorption of energy, and some energy is lost from the excited electron by collision, non-radiative decay and other processes, prior to emission, where the electron returns to its original energy level (Hudson *et al*., 2007). Fluorescence properties of DOM can be used to obtain general information of the main fluorescent compounds that are present in the DOM pool. Amino acid- and humic-like compounds have very different fluorescence signatures and represent mostly bioavailable DOM and more biologically refractory DOM, respectively.

In aquatic environments, fluorescence concentration depends on various processes including allochthonous sources such as terrestrial water or riverine inputs (Del Vecchio & Blough, 2002), agriculture-intensive or industrial land use (Foden *et al*., 2008) that increases FDOM. On the other hand, bacterial consumption of amino acid-like compounds and photobleaching can reduce the concentration of specific fluorophores (Yamashita *et al.,* 2013).

Various techniques have been used to determine FDOM for instance Synchronous Fluorescence Spectroscopy and Excitation Emission matrix (EEM) spectroscopy. Coble (1996) popularized this technique due to its high sensitivity, precision and speed. Later, it has been applied in many studies worldwide both in fresh and marine ecosystem (Zhang *et al*., 2011; Chari *et al*., 2013; Milbrandt *et al*., 2010; Singh *et al*., 2010; Pitta *et al*., 2016; Fellman *et al*., 2010, wastewater (Cohen *et al*., 2014; Henderson *et al*., 2009) and more recently in aquaculture to trace the changes in DOM (Nimptsch *et al*., 2015; Hambly *et al*., 2015).

Excitation-Emission Matries (EEMs) spectroscopy involves the collection of multiple emission spectra at a range of excitation, which are concatenated into a matrix to form a three-dimensional matrix of fluorescence data. It was introduced at first by Weber (1961) and then applied by many researchers (Andersen & Bro, 2003; Ohno & Bro, 2006; Borisover *et al*., 2009; Kowalczuk *et al*., 2005; Coble *et al*., 1996, Zepp *et al*., 2004). The location of the excitation and emission peaks varies with the composition of DOM. Therefore, EEM spectra provide a comprehensive map (fingerprint) of fluorescence properties of DOM and it can be used to discriminate different fluorophores.

EEMs spectroscopy is more sensitive than absorption spectroscopy allowing to obtain more details of the DOM chemical and reactivity properties. Although, EEMs provide information about DOM, supplementary multivariate techniques are needed to obtain quantitative data. Parallel factor analysis (PARAFAC) is the multivariate data analysis more widely used by the DOM researchers (Stedmon & Bro, 2008). PARAFAC decompose EEMs output into individual fluorescent components (Stedmon *et al*., 2003). Then, PARAFAC provides comprehensive information related to relative quantity-quality of CDOM fluorescent components (Stedmon *et al*., 2003; Stedmon & Bro, 2008; Hall *et al*., 2005; Murphy *et al*., 2006).

Table 1.1. Spectral characteristics of the main FDOM peaks in marine waters.

Peak	Name	Ex wavelength (nm)	Em wavelength (nm)	Nature
1	T	270-280	340-360	Tryptophan-like, amino acid-like, autochthonous
2	B	270-280	300-310	Tyrosine-like, amino acid-like, autochthonous
3	-	260	282	Phenylalanine-like, amino acid-like, autochthonous
4	A	230-260	380-460	Terrestrial humic-like
5	C	320-360	420-480	Terrestrial humic-like
6	M	290-320	370-420	Marine humic-like

Several studies have used both EEMs and PARAFAC to identify CDOM fluorescent components in marine and freshwater ecosystems (Stedmon *et al*., 2000; Yamashita & Tanoue 2008; Zhang *et al*., 2011; Stubbins *et al*., 2014; Catalá *et al*., 2015), but only a few studies have been focused on wastewater monitoring (Li *et al*., 2014; Liu *et al*., 2014; Cohen *et al*., 2014). Among these last studies, just two studies were related to wastewater from aquaculture (Nimptsch *et al*., 2015; Hambly *et al*., 2015).

Generally, six types of fluorescence peaks have been identified in EEMs from natural and residual waters (Coble, 1996; Hudson *et al*., 2007; Guéguen & Kowalczuk, 2013) (Table 1.1.). These fluorescence peaks are: peak A (terrestrial humic), M (marine fulvic) and C (terrestrial fulvic) globally named as humic-like fluorescence group and peak B, T as amino acid-like fluorescence group.

The humic-like substances fluoresce at higher emission wavelengths (Ex/Em 250/435 nm for peak-A, Ex/Em 320/410 nm for peak-M and Ex/Em 340/440 nm for peak-C (Fig. 1.5). Peaks A and C were thought to represent terrestrial humics, and peak-M were thought to represent marine-derived humic substances.

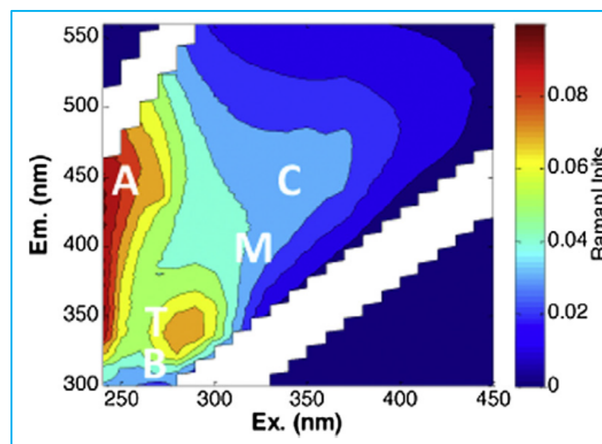


Figure 1.5. Example of a fluorescence excitation-emission matrix with the traditional peaks. Taken from Catalá *et al*. (2013). Peak details in Table 1.1.

Terrestrial humic-like materials display excitation and emission maxima at longer wavelengths than do marine humic-like materials, as would be predicted from their more aromatic chemical nature and presumed higher molecular weight (Coble, 2007). Tannins, lignin, polyphenols and melanins are some components responsible for the humic-like fluorescence (Coble, 2007). Humic substances can be sub-divided into three categories, chemically defined by solubility at different pH. Humic acids are insoluble in aqueous solution at pH lower than 2, but soluble at higher pH. Fulvic acids are soluble in water under all pH conditions. Humins are insoluble in water under any pH conditions (Aiken *et al*., 1985). Humic substances generated in aquatic systems have a fulvic acid nature, whereas allocthonous humic substances mostly generated in soils and

transported by fluvial and marine currents have a humic acid nature. The amino acid-like substances fluoresce at wavelengths characteristic of the aromatic amino acids tryptophan (Ex/Em 280 nm/350 nm, peak-T), tyrosine (Ex/Em 275 nm/305 nm, peak-B) (Coble, 1996) and phenylalanine (260/282) (Jørgensen *et al.*, 2011). The position of the amino acids in the proteins determines the intensity and Ex/Em wavelengths of the amino acid-like fluorescence peaks (Lakowicz, 2006). Tryptophan dominates the emission and is highly dependent upon polarity and/or local environment (Lakowicz, 2006). Tyrosine fluorescence is negligible when both tyrosine and tryptophan coexist in the same peptides because the emission energy of tyrosine is used as excitation energy for tryptophan (Creighton, 1993). Phenylalanine is generally present in higher concentration than the other two (Yamashita & Tanoue, 2009), although is not always detected due to its high bioavailability.

Sea cucumbers as extractive species in aquaculture tanks

Sea cucumbers are one of the most diverse groups of marine invertebrates inhabiting almost all marine environments. They can be found in shallow waters, in seagrass meadows or sandy bottoms as well as in deep benthic communities. There are almost more than one thousand species worldwide (Barnes, 1987). In the Mediterranean Sea, they have been widely studied in relation with *Posidonia oceanica* meadows as a key species recycling organic matter in sediments (Coulon & Jangoux, 1993; Costa *et al.*, 2014) and as potential in fisheries and aquaculture (González-Wangüemert & Borrero-Pérez, 2012; Ramón *et al.,* 2010; Domínguez-Godino *et al.,* 2015; Marquet *et al.*, 2017).

Sea cucumbers are highly demanded seafood for human consumption in some countries, particularly in Asia (Purcell *et al.*, 2013). In China, sea cucumbers are consumed as a delicatessen and luxury food (Fabinyi, 2012). These invertebrates have a high content in protein, important amino acids and bioactive components including antimicrobial and antitumor properties (Roggatz *et al.*, 2016; 2017; León-Palmero *et al.*, 2018; Tian *et al.*, 2005). Traditional Chinese medicine attributes healing properties to sea cucumbers (Purcell, 2014). Therefore, this high demand of sea cucumbers is leading to overfishing with the decline of sea cucumber populations not only in Pacific and Indian coasts (Purcell *et al.,* 2013), but also in the Mediterranean and Atlantic waters (González-Wangüemert *et al.,* 2018). Currently, more and more of this demand of sea cucumbers is supplied by

aquaculture (Purcell *et al.,* 2012; Eriksson *et al.,* 2012; Domínguez-Godino *et al.,* 2015). In addition to this commercial and economical value as seafood and biotechnological resources, sea cucumbers are excellent extractive species in mutitrophic aquaculture (Yokoyama, 2013; 2015; Zamora *et al.,* 2016).

Sea cucumbers are able to recycle large fractions of particulate waste through their feeding activities on detritus deposits when they are co-cultured with other species, playing a key role in bioremediation of wastewater (Paltzat *et al*., 2008; Slater & Carton, 2007; 2009; Slater *et al*. 2009; Yuan *et al*., 2013). Sea cucumbers are capable of consuming wastes from finfish cages (Hannah *et al*., 2013; Orr *et al*., 2014; Yokoyama, 2013; Yu *et al*., 2012), shrimp (Martínez-Porchas *et al*., 2010; Pitt *et al*., 2004), and mussel (Ren *et al*., 2012; Slater & Carton, 2007). Therefore, they are also important extractive species with a high capacity to consume waste particulate organic matter in sediment deposits (Nelson *et al*., 2012a, b; Yokoyama, 2013; 2015). Despite the effects of sea cucumbers on different components of the particulate organic matter has been studied, particularly in open waters under fish cages or mollusk rafts (Slater & Carton, 2009; Slater *et al*., 2009; Zamora & Jeff, 2011; Yokoyama, 2013;2015; Zhang *et al*., 2014a), their influence on the optical properties of the dissolved organic matter still remains unexplored (Zamora *et al*., 2016). In addition, recently Brothers *et al*. (2015) have demonstrated a direct uptake of free amino acids marked with stable isotopes (^{15}N and ^{13}C) in several tissues such as the respiratory trees, epidermis, and oral tentacles of a sea cucumber species (*Parastichopus californicus*) during the visceral regeneration. This alternative form of dietary supplementation has been related to the presence of a layer of bacteria in the tentacle epidermis and below the cuticle in deep-sea holothurians where the food is limiting (Roberts *et al.,* 1991). More recently, Lawrence *et al*. (2010) were able to detect and characterize these subcuticular bacteria using fluorescence in situ hybridation (FISH) (Figure 1.6.). The potential contribution of these symbiotic bacteria to DOM recycling is, to our knowledge, unknown.

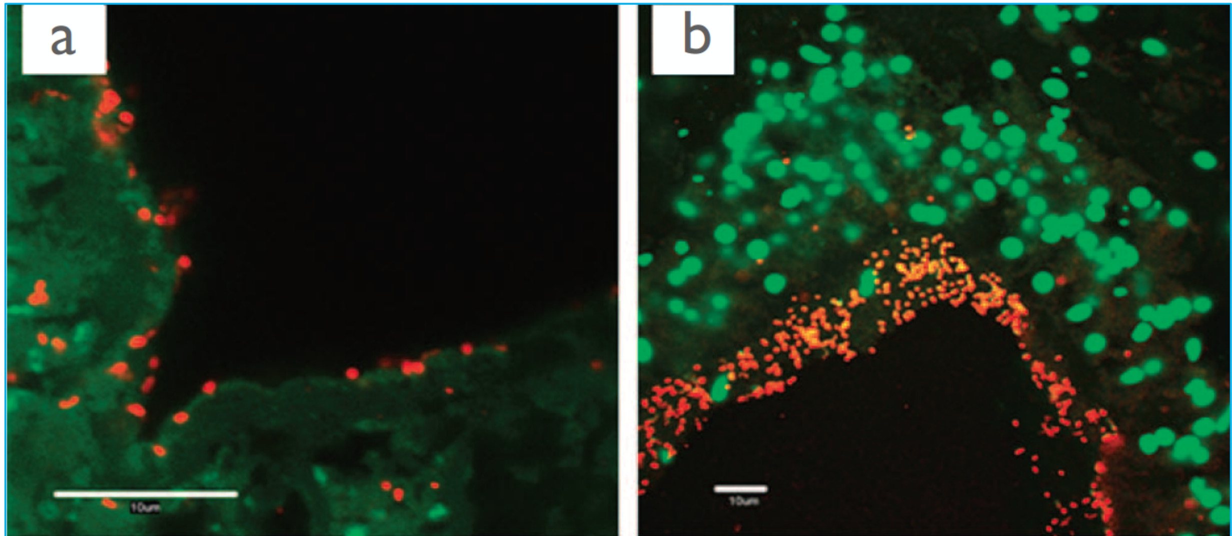


Figure 1.6. Confocal laser scanning microscope images showing subcuticular bacteria with fluorescence in situ hybridation (FISH) in *Stichopus mollis* (class Holothuroidea) with the probes EUB 338 (most bacteria) and ALF968 (Alphaproteobacteria). Fluorescently labeled bacteria are shown in red, and the autofluorescence of the sea cucumber tissue in green. Scale bar 10 µm. Images taken from Lawrence *et al*. (2010).

Objectives

The general objective of this PhD dissertation is to determine the role of sea cucumbers (*Holothuria tubulosa* and *H. forskali*) as extractive species in multitrophic aquaculture tanks with *Anemonia sulcata* as the primary species cultured. We explored the direct effects on particulate matter components as well as indirect effects on dissolved nutrients and organic matter components that affect water quality. To reach this objective we used two approaches: time-series monitoring in two big tanks with and without holothurians and short-term experiments in smaller tanks with variability sources under control to corroborate the observations obtained in the time series.

The specific objectives of this thesis were:

1) To determine the effects of holothurians on major nutrients specifically ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and total phosphorous (TP).

2) To determine the effects of holothurians on particulate, detrital components such as total organic carbon (TOC), particulate organic matter (POM), bacteria, phytoplankton cells (chlorophyll *a* as surrogate), and transparent exopolymer particles (TEP).

3.To determine the effects of holothurians on dissolved organic matter optical properties; specifically on the absorption coefficients and the spectral slopes of chromophoric dissolved organic matter (CDOM) and assess its importance for water transparency in the aquaculture effluents.

4. To determine the effects of holothurians on fluorescent dissolved organic matter (FDOM); specifically on the different humic- and amino acid- like fluorophores and assess its importance to trace organic matter recycling and alternative nutritional supplementation of amino acids.

References

Aiken GR, McKnight DM, Wershaw RL, MacCarthy P. 1985. An introduction to humic substances in soil, sediment, and water. In: Aiken GR, McKnight DM, Wershaw RL, MacCarthy P, Wiley, ed. *Humic Substances in Soil, Sediment, and Water: Geochemistry, Isolation and Characterization*. New York: Wiley-lnterscience, 1–9.

Ajin AM, Silvester R, Alexander D, Nashad M, Abdulla MH. 2016. Characterization of blooming algae and bloom-associated changes in the water quality parameters of traditional *pokkali cum prawn* fields along the South West coast of India. *Environmental Monitoring and Assessment* **188**:145 DOI 10.1007/s10661-016-5133-6.

Allison SD, Chao Y, Farrara JD, Hatosy S, Martiny AC. 2012. Fine scale temporal variation in marine extracellular enzymes of coastal southern California. *Frontiers in Microbiology* 3: 301 DOI 10.3389/fmicb.2012.00301.

Andersen CM, Bro R. 2003. Practical aspects of PARAFAC modeling of fluorescence excitation-emission data. *Journal of Chemometrics* **17**:200-215 DOI 10.1002/cem.790.

Apostolaki ET, Tsagaraki T, Tsapakis M, Karakassis I. 2007. Fish farming impact on sediments and macrofauna associated with sea grass meadows in the Mediterranean. *Estuarine, Coastal and Shelf Science* **75**:408-416 DOI 10.1016/j.ecss.2007.05.024.

Bar-Zeev, E., Berman-Frank, I., Girshevitz, O., & Berman, T. 2012. Revised paradigm of aquatic biofilm formation facilitated by microgel transparent exopolymer particles. Proceedings of the National Academy of Sciences, 109(23), 9119-9124.

Barnes RD. 1987. Invertebrate zoology. In: Bhatnagar I, Kim SK, ed. Immense essence of excellence: marine microbial bioactive. Philadelphia: Saunders College Publishing, 100– 5424–5430.

Bhatnagar I, Kim SK. 2010. Immense essence of excellence: Marine Microbial Bioactive Compounds. *Marine Drugs* **8**: 2673-2701 DOI 10.3390/md8102673.

Black KD. 2001. Environmental Impacts of Aquaculture. *Aquaculture Research* **28**: 829- 383 839.

Borisover M, Laor Y, Parparov A, Bukhanovsky N, Lado M. 2009. Spatial and seasonal patterns of fluorescent organic matter in Lake Kinneret (Sea of Galilee) and its catchment basin. *Water Research* **43**: 3104-3116 DOI 10.1016/j.watres.2009.04.039.

Bortone G. 2009. Integrated anaerobic/aerobic biological treatment for intensives swine production. *Bioresource Technology* **100**: 5424–5430 DOI 10.1016/j.biortech.2008.12.005.

Bostock J, McAndrew B, Richards R, Jauncey K, Telfer T, Lorenzen K, Little D, Ross L, Handisyde N, Gatward I, Corner R. 2010. Aquaculture: global status and trends. *Philosophical Transaction of the Royal Society* **365**: 2897–2912 DOI 10.1098/rstb.2010.0170.

Boyd CE, Massaut L. 1999. Risks associated with the use of chemicals in pond aquaculture. *Aquacultural Engineering* **20**:113–132 DOI 10.1016/S0144-8609(99)00010-2.

Bricaud A, Morel A, Prieur L. 1981. Absorption by dissolved organic matter of the sea (yellow substance) in the UV and visible domains. *Limnology and Oceanography* **26:** 43-53 DOI 10.4319/lo.1981.26.1.0043.

Brothers CJ, Lee RW, Nestler JR. 2015. The uptake of dissolved organic material by the sea cucumber *Parastichopus californicus* (Stimpson) and its potential role in visceral regeneration. Journal of Experimental Marine Biology and Ecology **469**:69–75 DOI 10.1016/j.jembe.2015.04.016.

Buhmann AH, Papenbrock J. 2013. Biofiltering of aquaculture effluents by halophytic plants: Basic principles, current uses and future perspectives. *Environmental and Experimental Botany* **92**:122-133 DOI 10.1016/j.envexpbot.2012.07.005.

Camargo JA, Alonso A, Salamanca A. 2005. Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. *Chemosphere* **58**:1255–1267 DOI 10.1016/j.chemosphere.2004.10.044.

Carder KL, Steward RG, Harvey GR, Ortner PB. 1989. Marine humic and fulvic acids: their effects on remote sensing of ocean chlorophyll. *Limnology and Oceanography* **34**: 68–81 DOI 10.4319/lo.1989.34.1.0068.

Carlson AC. 2002. Production and removal processes. In: Hansell DA, Carlson CA, ed. *Biogeochemistry of Marine Dissolved Organic Matter*. San Diego: Academic Press, 91–151.

Catalá TS, Mladenov N, Echevarría F, Reche I. 2013. Positive trends between salinity and chromophoric and fluorescent dissolved organic matter in a seasonally inverse estuary. *Estuarine, Coastal and Shelf Science* **133**: 206–216 DOI 10.1016/j.ecss.2013.08.030.

Catala ´TS, Reche I, Fuentes-Lema A, Romera-Castillo C, Nieto-Cid M, Ortega-Retuerta E, CalvoE, A ´lvarez M, Marrase ´C, Stedmon CA, Anto ´n A ´lvarez-Salgado X. 2015. Turnover time of fluorescent dissolved organic matter in the dark global ocean. *Nature Communication* **6**:5986 DOI 10.1038/ncomms6986.

Chari NVHK, Keerthi S, Sarma NS, Pandi SR, Chiranjeevulu G, Kiran R, Koduru, U. 2013. Fluorescence and absorption characteristics of dissolved organic matter excreted by phytoplankton species of western Bay of Bengal under axenic laboratory condition. *Journal of Experimental Marine Biology and Ecology* **445**:148–155 DOI 10.1016/j.jembe.2013.03.015.

Chen S, Timmons MB, Aneshansley DJ, Bisogni JJ. 1993. Suspended solids characteristics from recirculating aquaculture systems and design implications. *Aquaculture* **112**: 143–155 DOI 10.1016/0044-8486(93)90440-A.

Chopin T, Cooper JA, Reid G, Cross S, Moore C. 2012. Open-water integrated multi-trophic aquaculture: environmental biomitigation and economic diversification of fed aquaculture by extractive aquaculture. *Reviews in Aquaculture* **4**: 209-220 DOI 10.1111/j.1753-5131.2012.01074. x.

Claquin P, Probert I, Lefebvre S, Veron B. 2008. Effects of temperature on photosynthetic parameters and TEP production in eight species of marine microalgae. *Aquatic Microbial Ecology* **51**:1–11.

Coble PG. 1996. Characterization of marine and terrestrial DOM in seawater using excitation– emission matrix spectroscopy. *Marine Chemistry* **51**:325–346 DOI 10.1016/0304-4203(95)00062- 3.

Coble PG. 2007. Marine optical biogeochemistry: the chemistry of ocean color. *Chemical Reviews* **107**:402–418 DOI 10.1021/cr050350+.

Codling ID, Doughty R, Henderson A, Naismith I. 1995. Strategies for Monitoring Sediments and Fauna Around Cage Fish Farms. UK: Scotland and Northern Ireland Forum for Environmental Research (SNIFFER).

Cohen E, Levy GJ, Borisover M. 2014. Fluorescent components of organic matter in wastewater: Efficacy and selectivity of the water treatment. *water research* **55**:323-334 DOI 10.1016/j.watres.2014.02.040.

COM. 2013. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions. Blue Growth: opportunities for marine and maritime sustainable growth. Brussels, 10.9.2013.

CostaV, Mazzola A, Vizzini S. 2014. *Holothuria tubulosa* Gmelin 1791 (Holothuroidea, Echinodermata) enhances organic matter recycling in *Posidonia oceanica* meadows. *Journal of Experimental Marine Biology and Ecology* **461**: 226–232 DOI 10.1016/j.jembe.2014.08.008.

Coulon P, Jangoux M. 1993. Feeding rate and sediment reworking by the holothuroid *Holothuria tubulosa* (Echinodermata) in a Mediterranean seagrass bed off Ischia Island, Italy. *Marine Ecology Progress Series* **92**: 201- 204 Available at: http://www.jstor.org/stable/24832630.

Crab R, Avnimelech Y, Defoirdt T, Bossier P, Verstraete W. 2007. Nitrogen removal techniques in aquaculture for a sustainable production. *Aquaculture* **270**:1-14 DOI 10.1016/j.aquaculture.2007.05.006.

Creighton TE. 1993. Proteins, Structure and Molecular Properties. New York: Freeman.

Da CT, Phuoc LH, Duc HN, Troell M, Berg H. 2015. Use of Wastewater from Striped Catfish (*Pangasianodon hypophthalmus*) Pond Culture for Integrated Rice-Fish Vegetable Farming systems in the Mekong Delta, Vietnam. *Agroecology and sustainable food system* **39**:580-597 DOI 10.1080/21683565.2015.1013241.

Del Bel Belluz J, Costa M, Reid G, Cross S. 2016. Bio-optical variability at a Vancouver Island aquaculture site. *Limnology and Oceanography* **61**:1686–1704 DOI 10.1002/lno.10332.

Del Vecchio R, Blough NV. 2002. Photobleaching of chromophoric dissolved organic matter in natural waters: Kinetics and modeling. *Marine Chemistry* **78**:231–253 DOI 10.1016/S0304- 4203(02)00036-1.

Diana JS, Egna HS, Chopin T, Peterson MS, Cao L, Pomeroy R, Verdegem M, Slack WT, Bondad-Reantaso MG, Cabello F. 2013. Responsible Aquaculture in 2050: Valuing Local Condition and Human Innovation will be Key to Success. *Bioscience* **4**:255-262 DOI 10.1525/bio.2013.63.4.5.

Dodds WK. 2006. Nutrients and the 'dead zone': the link between nutrient ratios and dissolved oxygen in the northern Gulf of Mexico. *Frontiers in Ecology and the Environment* **4**: 211–217 DOI 10.1890/1540-9295(2006)004[0211: NATDZT]2.0.CO;2.

Dominguz-Godino JA, Slater MJ, Hannon C, González-Wangüemert M. 2015. A new especies of sea cucumber ranching and aquaculture : Breeding and rearing of *Holothuria arguinensis*. Aquaculture **438:** 122-128 DOI 10.1016/j.aquaculture.2015.01.004.

Emerson, S., & Hedges, J. 2008. Chemical oceanography and the marine carbon cycle. Cambridge University Press.

Eriksson, H., Torre-Castro, M., Olsson, P., 2012. Mobility, expansion and manage- ment of a multi-species scuba diving fishery in East Africa. *PloS One* **7:** e35504 DOI 10.1371/journal.pone.0035504.

Fabinyi, M., 2012. Historical cultural and social perspectives on luxury seafood consumption in China. *Environmental Conservation* **39:** 83–92 DOI 10.1017/S0376892911000609.

Fellman JB, Hood E, Spencer RGM. 2010. Fluorescence spectroscopy opens new windows into dissolved organic matter dynamics in freshwater ecosystems: a review. *Limnology and Oceanography* **55**:2452–2462 DOI 10.4319/lo.2010.55.6.2452.

Foden J, Sivyer DB, Mills DK, Devlin MJ. 2008. Spatial and temporal distribution of chromophoric dissolved organic matter (CDOM) fluorescence and its contribution to light attenuation in UK waterbodies. *Estuarine, Coastal and Shelf Science* **79**:707–717 DOI 10.1016/j.ecss.2008.06.015.

Fontenot Q, Bonvillain C, Kilgen M, Boopathy R. 2007. Effects of temperature, salinity, and carbon: nitrogen ratio on sequencing batch reactor treating shrimp aquaculture wastewater. Bioresource Technology **98**:1700–1703 DOI 10.1016/j.biortech.2006.07.031.

Food and Agriculture Organization of the United Nations (FAO). 2016. The State of World Fisheries and Aquaculture 2016. www.fao.org/documents/card/es/c/2c8bcf47-2214-4aeb-95b0-62ddef8a982a.

Froehlich, H. E., Gentry, R. R., & Halpern, B. S. 2017. Conservation aquaculture: Shifting the narrative and paradigm of aquaculture's role in resource management. Biological Conservation **215:** 162-168 DOI 10.1016/j.biocon.2017.09.012.

Frouin P. 2000. Effects of anthropogenic disturbances of tropical soft-bottom benthic communities. *Marine Ecology Progress Series* **194**:39–53 Available at: http://www.jstor.org/stable/24855650.

González-Wangüemert, M., Borrero-Pérez, G., 2012. A new record of Holothuria arguinensis colonizing the Mediterranean Sea. Marine Biodiversity Records **5:** e105 DOI 10.1017/S1755267212000887.

González-Wangüemert, M., Domínguez-Godino, J. A., & Cánovas, F. 2018. The fast development of sea cucumber fisheries in the Mediterranean and NE Atlantic waters: From a new marine resource to its over-exploitation. *Ocean & Coastal Managemen*t **151**: 165-177 DOI 10.1016/j.ocecoaman.2017.10.002.

Guéguen C, Kowalczuk P. 2013. Colored dissolved organic matter in frontal zones. In Chemical Oceanography of Frontal Zones. *The handbook of Environmental Chemistry* DOI 10.1007/698_2013_244.

Hall GJ, Clow KE, Kenny JE. 2005. Estuarial fingerprinting through multidimensional fluorescence and multivariate analysis. *Environmental Science & Technology* **39**:7560-7567 DOI 10.1021/es0503074.

Halpern BS, Walbridge S, Selkoe KA, Kappel CV, Micheli F, D'Agrosa C, Bruno JF, Casey KS, Ebert C, Fox HE, Fujita R, Heinemann D, Lenihan HS, Madin EMP, Perry MT, Selig ER, Spalding M, Steneck R, Watson R. 2008. A global map of human impact on marine ecosystems. *Science Careers* **319**: 948–952 DOI 10.1126/science.1149345.

Hambly AC, Arvin E, Pedersen L-F, Pedersen PB, Seredynska-Sobecka B, Stedmon CA. 2015. Characterizing organic matter in recirculating aquaculture systems with fluorescence EEM spectroscopy. *Water Research* **83**:112-120 DOI 10.1016/j.watres.2015.06.037.

Hannah L, Pearce CM, Cross SF. 2013. Growth and survival of California sea cucumbers (*Parastichopus californicus*) cultivated with sablefish at an integrated Multitrophic aquaculture site. *Aquaculture* **406–407**: 34–42 DOI 10.1016/j.aquaculture.2013.04.022.

Hansell DA, Carlson CA, 2002. ed. Biogeochemistry of Marine Dissolved Organic Matter. San Diego: Academic Press

Hansell, D. A., Carlson, C. A., Repeta, D. J. & Schlitzer, R. 2009. Dissolved organic matter in the ocean. A controversy stimulates new insights. Oceanography 22, 202–211.

Helms JR, Stubbins A, Ritchie JD, Minor EC, Kieber DJ, Mopper K. 2008. Absorption spectral slopes and slope ratios as indicators of molecular weight, source, and photobleaching of chromophoric dissolved organic matter. *Limnology and Oceanography* **53**:955–969 DOI 10.4319/lo.2008.53.3.0955.

Henderson RK, Baker A, Murphy K, Hambly A, Stuetz RM, Khan SJ. 2009. Fluorescence as a potential monitoring tool for recycled water systems: a review. *Water Research* **43**:863-881 DOI 10.1016/j.watres.2008.11.027.

Hickey CM. 2009. A review of nitrate toxicity to freshwater aquatic species. Environment Canterbury. Report No. R09/57 ISBN 978-1-86937-997-1. *Available at: www.ecan.govt.nz.*

Hudson N, Baker A, Reynolds D. 2007. Fluorescence analysis of dissolved organic matter in natural, waste and polluted waters—A review. *River Research and Application* **23:**631–649 DOI 10.1002/rra.1005.

Ibarra DA, Cembella A, Grant, J. 2012. Attenuation of sunlight measured from moored radiometers to assess depletion of suspended particles caused by bivalve aquaculture. *Limnologyand Oceanography Methods* **10**:1051–1069 DOI 10.4319/lom.2012.10.1051.

Jørgensen L, Stedmon CA, Kragh T, Markager S, Middelboe M, Søndergaard M. 2011. Global trends in the fluorescence characteristics and distribution of marine dissolved organic matter. *Marine Chemistry* **126**:139–48 DOI 10.1016/j.marchem.2011.05.002.

Kalantzi I, Karakassis I. 2006. Benthic impacts of fish farming: meta-analysis of community and geochemical data. *Marine Pollution Bulletin* **52**: 484-493 DOI 10.1016/j.marpolbul.2005.09.034.

Kirk JTO. 1994. Light and Photosynthesis in Aquatic Ecosystems. Cambridge: University Press.

Klinger D, Naylor R. 2012. Searching for solutions in aquaculture: charting a sustainable course. *The Annual Reviews of Environment and Resources* **37**:247-276 DOI 10.1146/annurev-environ- 021111-161531.

Kowalczuk P, Ston-Egiert J, Cooper WJ, Whitehead RF, Durako MJ. 2005. Characterization of chromophoric dissolved organic matter (CDOM) in the Baltic Sea by excitation emission matrix fluorescence spectroscopy. *Marine Chemistry* **96**:273–292 DOI 10.1016/j.marchem.2005.03.002.

Kutti T, Hansen PK, Ervik A, Hqsater T, Johannessen P. 2007. Effects of organic effluents from a salmon farm on a fjord system. II. Temporal and spatial patterns in infauna community composition. *Aquaculture* **262**: 355–366 DOI 10.1016/j.aquaculture.2006.10.008.

Lakowicz JR. 2006. Principles of Fluorescence Spectroscopy. *Journal of biomedical optics* **13**:029901 DOI 10.1117/1.2904580.

Lawrence, S. A., O'Toole, R., Taylor, M. W., & Davy, S. K. 2010. Subcuticular bacteria associated with two common New Zealand Echinoderms: characterization using 16S rRNA sequence analysis and fluorescence in situ hybridization. *The Biological Bulletin* **218**: 95-104.

Lee H, Shoda M. 2008. Removal of COD and color from livestock wastewater by the Fenton method. *Journal of Hazardous Materials* **153**:1314-1319 DOI 10.1016/j.jhazmat.2007.09.097.

León-Palmero, E., Joglar, V., Álvarez, P. A., Martín-Platero, A., Llamas, I., & Reche, I. (2018). Diversity and antimicrobial potential in sea anemone and holothurian microbiomes. *PloS one* **13:** 0196178 DOI 10.1371/journal.pone.0196178.

Lewis JR WM, Morris DP. 1986. Toxicity of nitrite to fish: a review. *Transactions of the American Fisheries Society* **115**: 183–195 DOI 10.1577/1548-8659.

Li WT, Chen SY, Xu ZX, LiY, Shuang CD, Li AM. 2014. Characterization of Dissolved Organic Matter in Municipal Wastewater Using Fluorescence PARAFAC Analysis and Chromatography Multi-Excitation/Emission Scan: A Comparative Study. *Environmental Science & Technology* **48:**2603–2609 DOI 10.1021/es404624q.

Liu X, Zhang Y, Shi K, Zhu G, Xu H, Zhu M. 2014. Absorption and fluorescence properties of chromophoric dissolved organic matter: implications for the monitoring of water quality in a large subtropical reservoir. *Environmental Science and Pollution Research* **21**:14078–14090. *Available at:* https://link.springer.com.

Marquet, N., Conand, C., Power, D. M., Canário, A. V., & González-Wangüemert, M. 2017. Sea cucumbers, Holothuria arguinensis and H. mammata, from the southern Iberian Peninsula: variation in reproductive activity between populations from different habitats. Fisheries Research, 191, 120-130.

Martínez-Porchas M, Martínez-Córdova LR, Porchas-Cornejo MA, López-Elías JA. 2010. Shrimp polyculture: a potentially profitable, sustainable, but uncommon aqua cultural practice. *Reviews in Aquaculture* **2**:73–85 DOI 10.1111/j.1753-5131.2010.01023. x.

Micinski E, Ball L, Zafiriou O. 1993. Photochemical oxygen activation: superoxide radical detection and production rates in the Eastern Caribbean. *Journal of Geophysical Research* **98**:2299-2306 DOI 10.1029/92JC02765.

Milbrandt EC, Coble PG, Conmy RN, Martignette AJ, Siwicke JJ. 2010. Evidence for the production of marine fluorescence dissolved organic matter in coastal environments and a possible mechanism for formation and dispersion. *Limnology and Oceanography* **55**:2037–2051 DOI 10.4319/lo.2010.55.5.2037.

Moran MA, Zepp RG. 1997. Role of photoreactions in the formation of biologically labile compounds from dissolved organic matter. *Limnology and Oceanography* **42**:1307–1316 DOI 10.4319/lo.1997.42.6.1307.

Murphy, KR, Ruiz GM, Dunsmuir WTM, Waite TD. 2006. Optimized parameters for rapid fluorescence-based verification of ballast water exchange by ships. *Environmental Science & Technology* **40**:2357-2362 DOI 10.1021/es0519381.

Nelson EJ, MacDonald BA, Robinson SMC. 2012a. The absorption efficiency of the suspension-feeding sea cucumber, *Cucumaria frondosa*, and its potential as an extractive integrated multi-trophic aquaculture (IMTA) species. *Aquaculture* **370–371**:19–25 DOI 10.1016/j.aquaculture.2012.09.029.

Nelson EJ, MacDonald BA, Robinson SMC. 2012b. A Review of the Northern Sea Cucumber *Cucumaria frondosa* (Gunnerus,1767) as a Potential Aquaculture Species. *Reviews in Fisheries Science* **20**:212–219 DOI 10.1080/10641262.2012.719043.

Nelson NB, Siegel DA, Carlson CA, Swan CM, 2010. Tracing global biogeochemical cycles and meridional over tuning circulation using chromophoric dissolved organic matter. *Geophysical Research Letter* **37**: L03610 DOI 10.1029/2009GL042325.

Nelson NB, Siegel DA. 2013. The global distribution and dynamics of chromophoric dissolved organic matter. *Annual Review of Marine Science* **5**:447-476 DOI 10.1146/annurev-marine- 120710-100751.

Nickell LA, Black KD, Hughes DJ, Overnell J, Brand T, Nickell TD, Breyer E, Harvey MS. 2003. Bioturbation, sediment fluxes and benthic community structure around a salmon cage farm in Loch Creran, Scotland. *Journal of Experimental Marine Biology and Ecology* **285 -286**: 221 – 233 DOI 10.1016/S0022-0981(02)00529-4.

Nimptsch J, Woelfl S, Osorio S, Valenzuela J, Ebersbach P, Tuempling WV, Palma R, Encina F, Figueroa D, Kamjunke N, Graeber D. 2015. Tracing dissolved organic matter (DOM) from land-based aquaculture systems in North Patagonian streams. *Science of the Total Environment* **537**:129–138 DOI 10.1016/j.scitotenv.2015.07.160.

Ohno T, Bro R. 2006. Dissolved organic matter characterization using multiway spectral decomposition of fluorescence landscapes. *Soil Science Society of America Journal Abstract - Soil Chemistry* 70:2028-2037 DOI 10.2136/sssaj2006.0005.

Oladoja NA, Adelagun ROA, Ahmad AL, Ololade IA. 2015. Phosphorus recovery from aquaculture wastewater using thermally treated gastropod shell. *Process Safety and Environmental Protection* **98**:296-308 DOI 10.1016/J.PSEP.2015.09.006.

Orr LC, Curtis DL, Cross SF, Gurney-Smith H, Shanks A, Pearce CM. 2014. Ingestion rate, absorption efficiency, oxygen consumption, and fecal production in green sea urchins (*Strongylocentrotus droebachiensis*) fed waste from sablefish (*Anoplopoma fimbria*) culture. *Aquaculture* **422–423**:184–192 DOI 10.1016/j.aquaculture.2013.11.030.

Ortega-Retuerta E, Duarte CM, Reche I. 2010. Significance of Bacterial Activity for the distribution and dynamics transparent exopolymer particles in the Mediterranean Sea. *Microbial Ecology* **59**:808–818 DOI 10.1007/s00248-010-9640-7.

Ortega-Retuerta E, Frazer TK, Duarte CM, Ruiz-Halpern S, Tovar-Sánchez A, Arrieta JM, Reche, I. 2009. Biogeneration of chromophoric dissolved organic matter by bacteria and krill in the Southern Ocean. *Limnology and Oceanography* **54**:1941–1950 DOI 10.4319/lo.2009.54.6.1941.

Paltzat DL, Pearce CM, Barnes PA, McKinley RS. 2008. Growth and production of California sea cucumbers co-cultured with suspended Pacific oysters. *Aquaculture* **275**:124–137 DOI 10.1016/j.aquaculture.2007.12.014.

Partensky, F., Hess, W. R., and Vaulot, D. 1999. Prochlorococcus, a marine photosynthetic prokaryote of global significance. Microbiol. Mol. Biol. Rev. 63, 106–127.

Passow U, Berman U. 2011. Transparent Exopolymer Particles (TEP): A frequently ignored, but extremely active component of NOM. Marine Science Institute, University of California Santa Barbara, NOM- conference.

Passow U. 2002. Transparent exopolymer particles (TEP) in aquatic environments. Progress in Oceanography, 55: 287 – 333.

Pitt R, Duy NDQ, Duy TV, Long HTC. 2004. Sandfish (*Holothuria scabra*) with shrimp (*Penaeus monodon*) co-culture tank trials. *SPC Beche-de-mer Information Bulletin* **20**:12-22. *Available at: https://www.researchgate.net*.

Pitta E, ZeriC, Tzortziou M, Mousdis G, Scoullos M. 2016. Seasonal variations in dissolved organic matter composition using absorbance and fluorescence spectroscopy in the Dardanelles Straits North Aegean Sea mixing zone. *Continental Shelf Research* **149**:82-95 DOI 10.1016/j.csr.2016.07.013.

Pomeroy, L. R., leB. Williams, P. J., Azam, F., & Hobbie, J. E. 2007. The microbial loop. Oceanography, 20(2), 28-33.

Populus J, Hastuti W, Martin J-L M, Guelorget O, Sumartono B Wibowo A. 1995. Remote sensing as a tool for diagnosis of water quality in Indonesian seas. *Ocean & Coastal Management* **27**:197-215 DOI 10.1016/0964-5691(95)00033-X.

Purcell SW, Mercier A, Conand C, Hamel JF, Toral-Granda MV, Lovatelli A, Uthicke S. 2013. Sea cucumber fisheries: global analysis of stocks, management measures and drivers of overfishing. *Fish and Fisheries* **14**:34–59 DOI 10.1111/j.1467-2979.2011.00443. x.

Purcell, S.W., 2014. Value, market preferences and trade of beche-de-mer from Pacific Island sea cucumbers. Plos One 9 (4), e95075. https://doi.org/10.1371/journal.pone.0095075.

Purcell, S.W., Hair, C.A., Mills, D.J., 2012. Sea cucumber culture farming and sea ranching in the tropics: progress problems and opportunities. Aquaculture 368, 68–81.

Rajitha K, Mukherjee CK, Vinu Chandran R. 2007. Applications of remote sensing and GIS for sustainable management of shrimp culture in India. *Aquacultural Engineering* **36**: 1–17 DOI 10.1016/j.aquaeng.2006.05.003.

Ramón, M., Lleonart, J., Massutí, E., 2010. Royal cucumber (*Stichopus regalis*) in the northwestern Mediterranean: distribution pattern and fishery. Fisheries Research **105**: 21–27 DOI 10.1016/j.fishres.2010.02.006.

Raymond PA, Bauer JE. 2000. Bacterial consumption of DOC during transport through a temperate estuary. *Aquatic Microbial Ecology* **22**:1–12 DOI 10.3354/ame022001.

Read P, Fernandes T. 2003. Management of environmental impacts of marine aquaculture in Europe. *Aquaculture* **226**: 139–163 DOI 10.1016/S0044-8486(03)00474-5.

Ren JS, Stenton-Dozey J, Plew DR, Fang J, Gall M. 2012. An ecosystem model for optimizing production in integrated multitrophic aquaculture systems. *Ecological Modeling* **246**:34-46 DOI 10.1016/j.ecolmodel.2012.07.020.

Roberts, D., D. S. M. Billett, G. McCartney, and G. E. Hayes. 1991. Procaryotes on the tentacles of deep-sea holothurians: a novel form of dietary supplementation. *Limnology and Oceanography*. **36**: 1447–1452 DOI 10.4319/lo.1991.36.7.1447.

Roggatz, C., González-Wangüemert, M., Pereira, H., Rodrigues, M., da Silva, M., Barreira, L.M., Varela, J., Custodio, L., 2016. First report of the nutritional profile and antioxidant potential of Holothuria arguinensis, a new resource for aquaculture in Europe. *Natural Product Research* **30:** 2034–2040 DOI 10.1080/14786419.2015.1107555.

Roggatz, C., González-Wangüemert, M., Pereira, H., Vizzeto-Duarte, C., Rodrigues, M., Barreira, L., da Silva, M., Varela, J., Custodio, L., 2017. A first glance nto the nutritional properties of the sea cucumber Parastichopus regalis from the Mediterranean Sea (SE Spain). *Natural Product Research* DOI 10.1080/ 14786419.2017.1331224.

Romera-Castillo C, Sarmento H, Alvarez-Salgado XA, Gasol JM, Marrasé C. 2010. Production of chromophoric dissolved organic matter by marine phytoplankton. *Limnology and Oceanography* **55**: 446-454 DOI 10.4319/lo.2010.55.1.0446.

Ruiz-Zarzuela I, Halaihel N, Balacázar J, Ortega C, Vendrell D, Pérez T, Alonso J, de Blas I. 2009. Effect of fish farming on the water quality of rivers in northeast Spain. *Water Science and Technology* **60**: 663–671 DOI 10.2166/wst.2009.435.

Saitoh S-I, Mugo R, Radiarta IN, Asaga S, Takahashi F, Hirawake T, Ishikawa Y, Awaji T, In T, Shima S. 2011. Some operational uses of satellite remote sensing and marine GIS for sustainable fisheries and aquaculture. *ICES Journal of Marine Science* **68**:687–695 DOI 10.1093/icesjms/fsq190.

Shifrin KS. 1988. Physical Optics of Ocean Water. New York: American Institute of Physics.

Siegel, DA, Maritorena S, Nelson NB, Behrenfeld MJ, McClain CR. 2005. Colored dissolved organic matter and its influence on the satellite-based characterization of the ocean biosphere. *Geophysics Research Letter* **32**: L20605 DOI 10.1029/2005GL024310.

Singh S, D'Sa E, Swenson E. 2010. Seasonal variability in CDOM absorption and fluorescence properties in the Barataria Basin, Louisiana, USA. *Journal of Environmental Sciences* **22**: 1481–1490 DOI 10.1016/S1001-0742(09)60279-5.

Slater MJ, Carton AG. 2007. Survivorship and growth of the sea cucumber *Australostichopus mollis* (Hutton 1872) in polyculture trials with green- lipped mussel farms. *Aquaculture* **272**: 389–398 DOI 10.1016/j.aquaculture.2007.07.230.

Slater MJ, Carton, AG. 2009. Effect of sea cucumber (*Australostichopus mollis*) grazing on coastal sediments impacted by mussel farm deposition. *Marine Pollution Bulletin* **58**:1123-1129 DOI 10.1016/j.marpolbul.2009.04.008.

Slater MJ, Jeffs AG, Carton AG. 2009. The use of the waste from green-lipped mussels as a food source for juvenile sea cucumber, *Australostichopus mollis*. *Aquaculture* **292**:219–224 DOI 10.1016/j.aquaculture.2009.04.027.

Stedmon CA, Álvarez-Salgado XA. 2011. Shedding Light on a Black Box: UV-Visible Spectroscopic Characterization of Marine Dissolved Organic Matter. In: Jiao N, Azam F, Sean S, ed. *Microbial Carbon Pump in the Ocean*. Science/AAAS, 62–63 DOI 10.1126/science.opms.sb0001.

Stedmon CA, Bro R. 2008. Characterizing dissolved organic matter fluorescence with parallel factor analysis: a tutorial. *Limnology and Oceanography Methods* **6**:572–579 DOI 10.4319/lom.2008.6.572.

Stedmon CA, Markager S, Bro R. 2003. Tracing dissolved organic matter in aquatic environments using a new approach to fluorescence spectroscopy. *Marine Chemistry* **82**:239-254 DOI 10.1016/S0304-4203(03)00072-0.

Stedmon CA, Markager S, Kaas H. 2000. Optical properties and signatures of chromophoric dissolved organic matter (CDOM) in Danish coastal waters. *Estuarine Coastal and Shelf Science* **51**:267–278 DOI 10.1006/ecss.2000.0645.

Steinberg DK, Nelson NB, Carlson CA, Prusak AC. 2004. Production of chromophoric dissolved organic matter (CDOM) in the open ocean by zooplankton and the colonial cyanobacterium *Trichodesmium spp*. *Marine Ecology Progress Series* **267**:45–56. *Available at:* http://www.int-res.com.

Stubbins A, Lapierre J-F, Berggren M, Prairie YT, Dittmar T, del Giorgio PA. 2014. What's in an EEM? Molecular Signatures Associated with Dissolved Organic Fluorescence in Boreal Canada. *Environment Science Technology* **48**:10598–10606 DOI 10.1021/es502086e.

Tian, F., Zhang, X., Tong, Y., Yi, Y., Zhang, S., 2005. A newsulfated saponin fromsea cucumber exhibits anti-angiogenic and antitumor activities in vivo and in vitro. Cancer Biol. Ther. 4, 874–882.

Tilley DR, Badrinarayanan H, Rosati R, Son J. 2002. Constructed wetlands as recirculation filters in large-scale shrimp aquaculture. *Aquacultural Engineering* **26**:81-109 DOI 10.1016/S0144-8609(02)00010-9.

Twardowski MS, Boss E, Sullivan JM, Donaghay PL. 2004. Modeling the spectral shape of absorption by chromophoric dissolved organic matter. *Marine Chemistry* **89**:69-88 DOI 10.1016/j.marchem.2004.02.008.

Valliappan K, Sun W, Li Z. 2014. Marine actinobacteria associated with marine organisms and their potentials in producing pharmaceutical natural products. *Applied Microbiology and Biotechnology* **98**:7365–7377 DOI 10.1007/s00253-014-5954-6.

Vardi A., Haramaty L., Van Mooy B. A. S., Fredricks H. F., Kimmance S. A., Larsen A., Bidle K. D. 2012. Host-virus dynamics and subcellular controls of cell fate in a natural coccolithophore population. PNAS, 109 (47): 19327 - 19332.

Verdugo P. 2012. Marine Microgels. *Annual Reviews of Marine Science* 4:375–400 DOI 10.1146/annurev-marine-120709-142759.

Vodacek A, Blough NV, DeGrandpre MD, Peltzer ET, Nelson RK. 1997. Seasonal variation of CDOM and DOC in the Middle Atlantic Bight: terrestrial inputs and photooxidation. *Limnology and Oceanography* **42**:674–686 DOI 10.4319/lo.1997.42.4.0674.

Weber G. 1961. Enumeration of components in complex systems by fluorescence spectrophotometry. *Nature* **90**: 27–29 DOI 10.1038/190027a0.

Weishaar JL, Aiken GR, Bergamaschi BA, Fram MS, Fujii R, Mopper K. 2003. Evaluation of specific ultraviolet absorbance as an indicator of the chemical composition and reactivity of dissolved organic carbon. *Environmental Science and Technology* **37**:4702–4708 DOI 10.1021/es030360x.

Weston DP. 1990. Quantitative examination of macrobenthic community changes along an organic enrichment gradient. *Marine Ecology Progress Series* **61**:233-244. *Available at:* http://www.int-res.com.

Wotton RS. 2005. The essential role of exopolymers (EPS) in aquatic systems. In: Barnes H, Gibson RN, Atkinson RJA, Gordon JDM, ed. Oceanography and Marine Biology. New York (NY): Taylor and Francis Group (CRC), 57–9.

Wotton RS. 2011. EPS (Extracellular Polymeric Substances), silk, and chitin: vitally important exudates in aquatic ecosystems. *Journal of the North American Benthological Society* **30**:762- 769 DOI 10.1899/10-120.1.

Yamashita Y, Nosaka Y, Suzuki K, Ogawa H, Takahashi K, Saito H. 2013. Photobleaching as a factor controlling spectral characteristics of chromophoric dissolved organic matter in open ocean. *Biogeosciences* **10**:7207–7217 DOI 10.5194/bg-10-7207-2013.

Yamashita Y, Tanoue E. 2008. Production of bio-refractory fluorescent dissolved organic matter in the ocean interior. *Nature* **1**:579–582 DOI 10.1038/ngeo279.

Yamashita Y, Tanoue E. 2009. Basin scale distribution of chromophoric dissolved organic matter in the Pacific Ocean. *Limnology and Oceanography* **54**:598–609 DOI 10.4319/lo.2009.54.2.0598.

Yokoyama H. 2002. Impact of fish and pearl farming on the benthic environment in Gokash bay; evaluation from seasonal fluctuation of the macrobenthos. *Fisheries Science* **68**:258-268 DOI 10.1046/j.1444-2906.2002.00420.x.

Yokoyama H. 2013. Growth and food source of the sea cucumber *Apostichopus japonicus* cultured below fish cages. Potential for integrated Multitrophic aquaculture. *Aquaculture* **372–375**: 28–38 DOI 10.1016/j.aquaculture.2012.10.022.

Yokoyama H. 2015. Suspended culture of the sea cucumber Apostichopus japonicus below a Pacific oyster raft potential for integrated multi-trophic aquaculture. *Aquaculture Research* **46**: 825–832 DOI 10.1111/are.12234.

Yu Z, Zhou Y, Yang H, Hu C. 2012. Bottom culture of the sea cucumber *Apostichopus japonicus* in a fish farm, southern China. *Aquaculture Research* **45**:1434–1441 DOI 10.1111/are.12089.

Yuan X, Yang H, Meng L, Wang L, LiY. 2013. Impacts of temperature on the scavenging efficiency by the deposit feeding holothurian *Apostichopus japonicus* on a simulated organic pollutant in the bivalve macroalage polyculture from the perspective of nutrient budgets. *Aquaculture* **406–407**: 97–104 DOI 10.1016/j.aquaculture.2013.05.009.

Zamora LN, Jeffs AG. 2011. Feeding, selection, digestion and absorption of the organic matter from mussel waste by juveniles of the deposit-feeding sea cucumber, *Australostichopus mollis*. *Aquaculture* **317**:223–228 DOI 10.1016/j.aquaculture.2011.04.011.

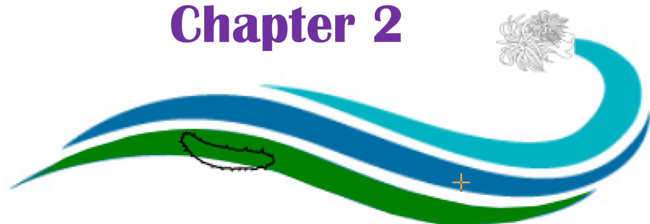
Zamora LN, Yuan X, Carton AG, Slater MJ. 2016. Role of deposit-feeding sea cucumbers in integrated multitrophic aquaculture: progress, problems, potential and future challenges. *Reviews in Aquaculture* **0**:1-18 DOI 10.1111/raq.12147.

Zepp RG, Sheldon WM, Moran MA. 2004. Dissolved organic fluorophores in southeastern US coastal waters: correction method for eliminating Rayleigh and Raman scattering peaks in excitation-emission matrices. *Marine Chemistry* **89**:15–36 DOI 10.1016/j.marchem.2004.02.006.

Zhang L, Gao Y, Zhang T,Yang H, Xu Q, Sun L, Yu Z. 2014a. A new system for bottom co- culture of the scallop, *Patinopecten yessoensis*, with the sea cucumber, *Apostichopus japonicus*, and the sea urchin, *Anthocidaris crassispina*, in shallow water in China. *Aquaculture International* **22**:1403–1415 DOI 10.1007/s10499-014-9755-9.

Zhang Q, Achal V, Xu Y, Xiang WN. 2014b. Aquaculture Wastewater quality improvement by water Spinach (*Ipomoea aquatica Forskall*) floating bed and ecological benefit assessment in ecological agriculture district. *Aquacultural Engineering* **60**:48-55 DOI 10.1016/j.aquaeng.2014.04.002.

Zhang Y, Yin Y, Feng L, Zhu G, Shi Z, Liu X. 2011. Characterizing chromophoric dissolved organic matter in Lake Tianmuhu and its catchment basin using excitation-emission matrix fluorescence and parallel factor analysis. *water research* **45**:5110-5122 DOI 10.1016/j.watres.2011.07.014.



Material & Methods

Material and Methods:

Study tanks and sampling

Time-series in the big tanks

We monitored for one year two aquaculture tanks at iMareNatural S.L. facilities (http://www.imarenatural.com) in Southern Spain (36°44′38″ N, 3°35′59″ W). Water samples from each tank were collected biweekly from July 2013 to August 2014. Each tank (50,000 liters of capacity) was connected directly with the coastal water by one inlet pipe (inlet waters) and the water from each tank was released by one outlet pipe located in the bottom of the tank (effluent). The seawater was pumped into the tanks at a continuous flow of 1,200 l h^{-1}. Therefore, water residence time in the tanks was ca. 42 hours. In one of the tanks, 811 ± 125 individuals of the primary species, the sea anemone *Anemonia sulcata*, and 93 ± 3 adults of sea cucumbers *Holothuria tubulosa* (≈ 80 %) and *H. forskali* (≈ 20 %) were included (hereafter designated as + *holothurian* tank). In the other tank only 690 ± 87 individuals of the primary specie were included (hereafter designated as – *holothurian* tank). Sea anemones were placed on floating plastic boxes in the surface of the tanks and holothurians were free in the bottom and walls of the tanks. Sea anemones were fed with about 900-1800 g of fresh chopped fish, mainly *Scomber scombrus* (Van-Praët, 1985; Chintiroglou & Koukouras, 1992) twice per week. *Anemonia sulcata* was selected as the primary species because is a very palatable species, highly demanded for catering in Southern Spain. In addition, the company iMare Natural S.L. is also involved in the study of this species due to its pharmacological potential (http://www.tascmar.eu).

The sea cucumbers were caught from offshore seabed of the aquaculture facilities in Granada coastal water (Salobreña zone). Sea anemones were collected from floating cages of mussel in the same place. Then, they transported by using small plastic containers to iMare Natural S.L. center (previous authorization from the Ministry of Agriculture and Fisheries of the Junta de Andalucía, Spain).

Anemonia sulcata individuals were kept in aquariums of 90 liters to control their sexual reproduction. Once, the sea anemones spawn, the fertilized eggs were removed and introduce them into 30 liters aquariums to promote the larval development. Larval individuals were fed

with zooplankton. When sea anemones reached the juvenile size (about 4 cm), they were transferred to the big-size culture tanks. Sea anemones were placed in floating structures (plastic boxes) in the surface of tanks (+ holothurian tank) and fattened with fresh chopped fish (mainly *Scomber scombrus*) (Fig. 2.1).

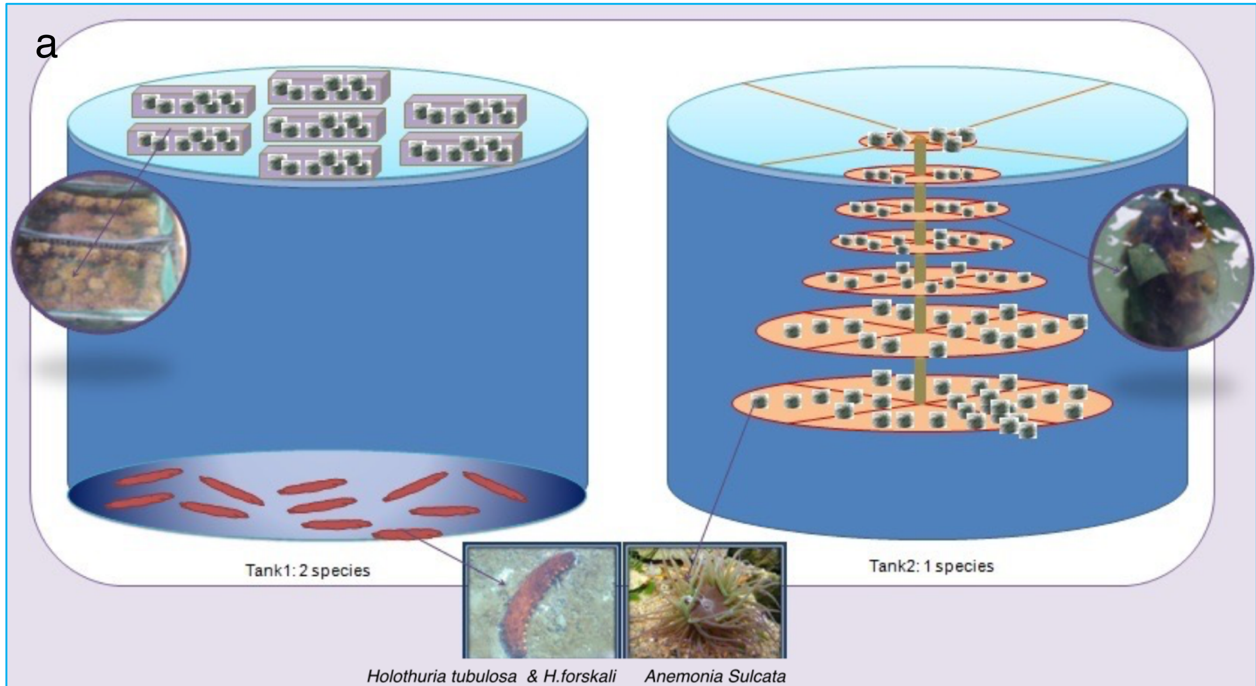


Fig. 2.1. a) Schematic representation of + *Holothurian* tank and – *Holothurian* tank during time-series in the big tanks, b) *H. tubulosa*

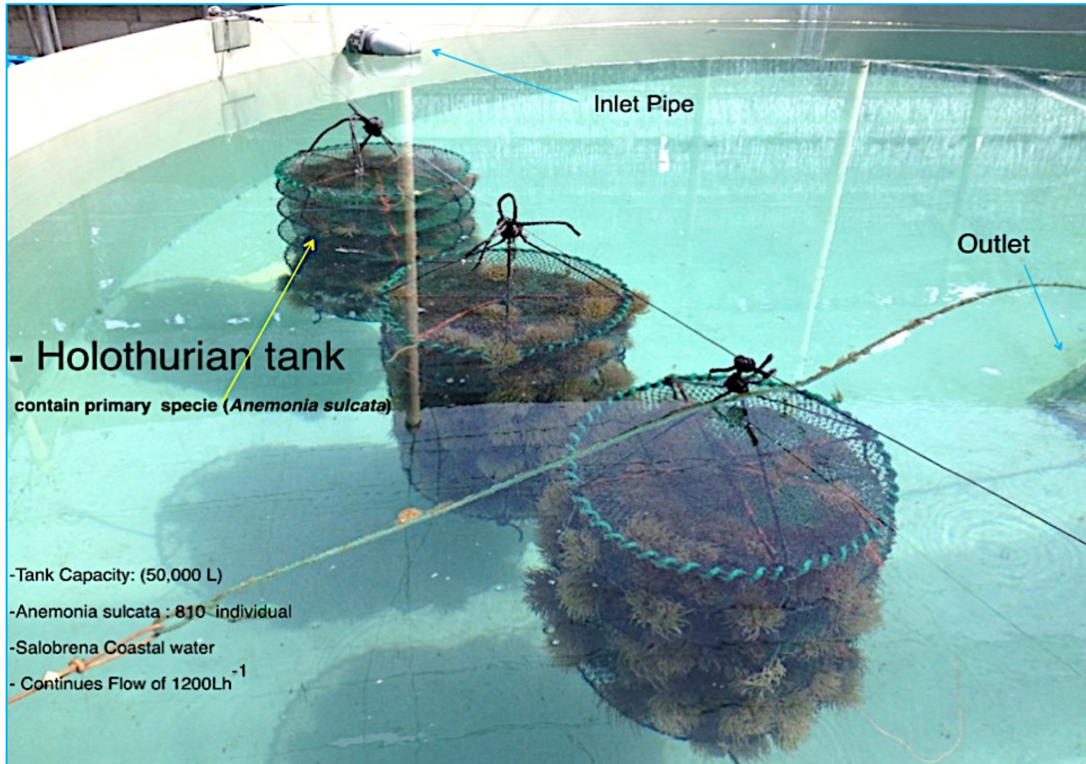


Fig. 2.2. – *Holothurian* tank during time-series in the big tanks

Short-term experiments

To corroborate the statistical significance of the results obtained in the time-series, we performed three short-term experiments (Fig.2.3a). Each experiment consisted of seven tanks of 300 liters that contained 80 individuals of *A. sulcata* per tank and included two treatments: +holothurians (+H) and –Holothurian (-H). At the initial time, in three of tanks (+H tanks) we transplanted 10 individuals of H. *tubulosa* in each tank (Fig.2.3b). The other four tanks (-H tanks) contained only 80 individuals of *A. sulcata* . Each experiment lasted 3 days (ca. 72 h).The experiment 1 was carried out from 6th to 9th October 2017, the experiment 2 from 27th to 30th October 2017, and the experiment 3 from 3th to 6th November 2017.To analyze the samples we followed the same procedures used in the time- series and they are describe in the next sections.

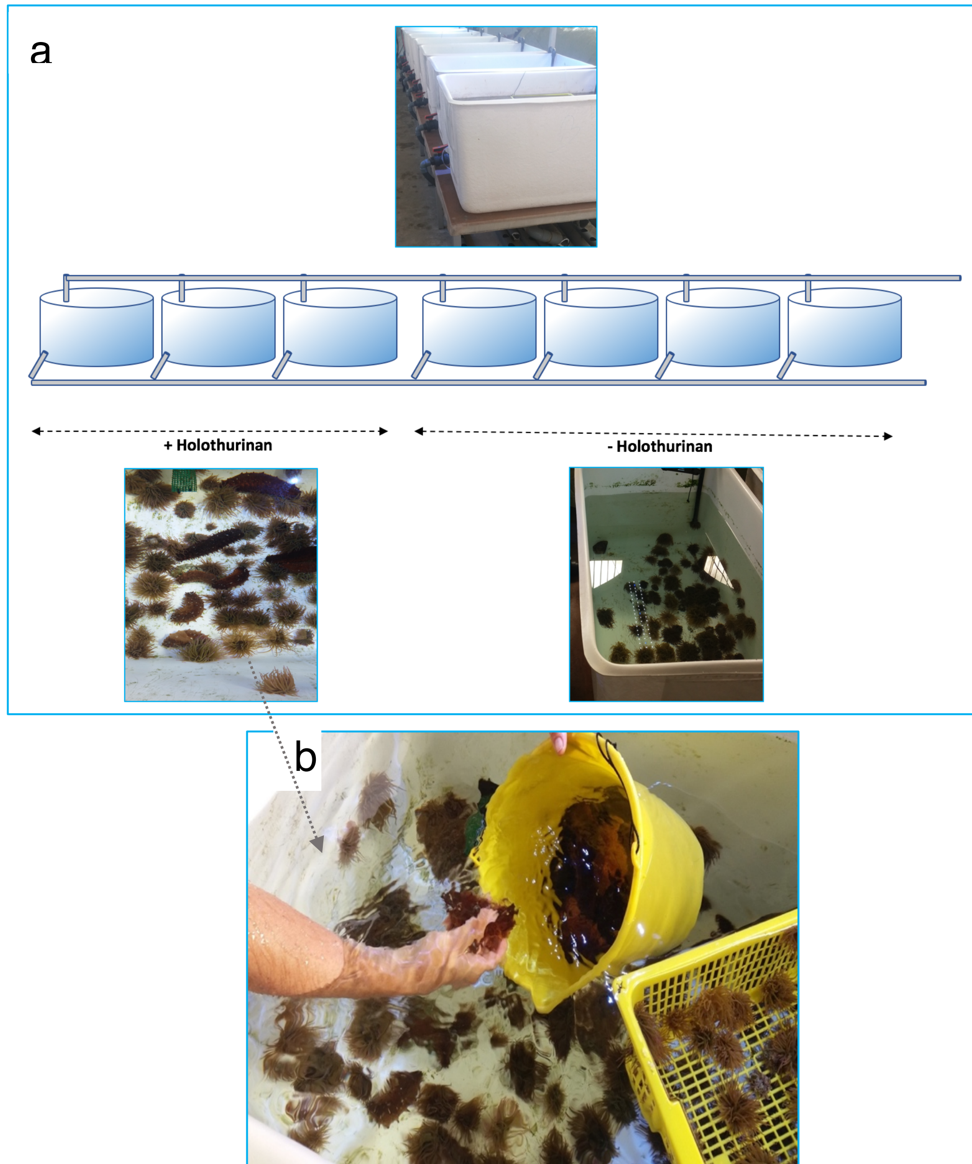


Fig. 2.3. Scheme of + Holothurian tank and – Holothurian tank (a) and transplanting *H. tubulosa* to + Holothurian tanks during the short-term experiments (b)

Water sample analysis

For the time-series and and the short-term experiments, water samples were taken from the tanks center, transferred to sampling bottles and immediately placed in an icebox for their transport to the laboratory. Sampling bottles were acid-clean and rinsed several times with seawater. We used small amber bottles previously combusted at 500ºC for the total organic carbon (TOC) samples. Once in the laboratory (about one hour from the tanks), samples for dissolved nutrients were filtered through Whatman GF/F filters and the filtrates stored at -20 ºC until analysis. The samples for prokaryote abundance were fixed with paraformaldehyde 1% and glutaraldehyde 0.05% and then immediately stored at -80 °C.

Environmental parameters as temperature (°C), pH, salinity (psu), total dissolved solid (TDS), and conductivity (mS cm^{-1}) were measured *in situ* using a Multi-parameter HANNA probe (HI9828 model).

Major nutrients analysis

Analyses of mineral nutrients were performed by triplicate following the standard methods (APHA, 1992). We measured ammonium, nitrate, nitrite, total nitrogen (TN), soluble reactive phosphorous (SRP) and total phosphorous (TP). The dissolved nutrients were previously filtered using Whatman GF/F filters; whereas the samples for total nutrients were not filtered.

Ammonium

To determine the concentration of ammonium we used a commercial kit model HC243987. We prepared the calibration curve from stock solutions of 1000 mg/l of $SO_4(NH_4)_2$ and 50 mg/l) and then we made serial dilutions to 0.01, 0.1, 0.4, 0.8, 2, 4, 8, and 10 μg/l (Fig.2.4a). The calibration curve allows calculating the sample concentration from absorbance measurements. To convert μg/l into μmol-N/l we divided by 14 unit. This method is analogous to EPA 350.1, APHA 4500-NH3 D, ISO 7150/1, and DIN 38406 E5.

The procedure with the samples was to take 5 ml-aliquots of the samples in test tubes and, then, we added 600-μl of reagent 1 (NH_4^-1) and we mixed well. In next step, we added 0.1 ml of solution sodium hydroxides 5M to increase pH around 4-13. Then, we added 1 cap of reagent 2 (NH_4^-2) and we mixed well. After 5-minute we added 3 drops of the reagent 3 ($NH_4$3). Then, the samples were mixed well with a

vortex and transfered to a 1cm-cuvette and we measured the absorbance at 690 nm with a UV/VIS Perkin-Elmer spectrophotometer at room temperature (20-30 ºC).

Nitrate

To determine the concentration of nitrate we used ultraviolet spectrophotometric method (APHA 1992). Samples were filtered by Whatman GF/F glass fiber filter with 0. 7 µm of pore size. This method is reliable only for waters with low content in dissolved organic carbon (DOC) since both compounds can overlap their absorbances in the UV band. Therefore, an absorbance correction at wavelength 275 nm is needed to determine accurately nitrate concentration.

We made the calibration curve using by standard solutions of 1000 μmol/l and 100 μmol/l of KNO_3 and, then, we prepared serial dilutions of 0.05, 0.1, 0.5, 0.8, 1, 2, 3 μmol/l (Fig.2.4b).

To determine the nitrate concentration in the,samples we added 0.5 ml of HCL 1M in 25 ml of samples and mixed well and we measured the absorbance at 220 nm and 275nm in a UV/Vis Perkin Elmer spectrophotometer with a 10 cm cuvette applied during working with equipment.

Nitrite

We determined the concentration of Nitrite (NO_2^-) spectrophotometrically through the formation of a reddish purple azodye produced at pH 2.0 to 2.5 by coupling diazotized sulfanilamide with N-(1-naftile)-ethylendiamine dihydrochloride.

We prepared the calibration curve from stock solutions of 100 mg/l and 100 μg/l of $NaNO_2$ and, then, we performed serial dilutions of 0.005, 0.05, 0.1, 0.5, 0.8, 1, 2, 3 μg/l (Fig.2.4c). To convert µg/l into μmol/l we divided by 14. The $NaNO_2$ was dehydrated for 1 hour in the oven at 105 ºC before any calibration curve measurements.

We determined the nitrite concentration using the solution A (1gr of Sulfanil amid + 100 ml of HCL 10%) and the solution B (0.1 gr of Dicloridrate N-(1-naftile)-ethylendiamine 0.1 % + 100 ml of distillated water). Diazotization of sulfanilamide in acid medium and coupling with N-(1-Naphthyl)-ethylene diamine allow to determine nitrite from reddish purple color via colorimetric methods. In the laboratory, 0.5 ml of solution A was added to 25 ml of each samples and were mixed well. Then, we added 0.5 ml of the solution B, mixed well and waited for 10 minutes. Finally, we measured the absorbance at 543 nm using an

UV/VIS Perkin-Elmer spectrometer with a 10 cm-cuvette.

Total Nitrogen

Total Nitrogen (TN) was measured as nitrate after oxidation to nitrate under alkaline conditions at 100-110 °C with sodium persulfate. We prepared the calibration curve from standard solutions of 1mmol/l and 100 μm/l and, then, we performed serial dilution of 1, 5, 10, 20, 40, 80 μmol/l of KNO_3 (Fig.2.5a).

The oxidative mix solution (OS) was prepared with 50 gr/l of potassium persulfate, 30 gr/l of boric acid, and 14 gr/l of sodium hydroxide in distillated water. This solution was kept at room temperature during all measurements. Later, we added 3.5 ml of OS in 25 ml of sample and autoclaved for 30 min at 120 ºC. Then, the samples were treated with 0.5 ml of HCL 1M and, like for nitrate measurements, the absorption at 220 nm and 275 nm were measured.

Total Phosphorous (TP) and Soluble Reactive Phosphorous (SRP)

SRP and TP were spectrophotometrically determined using the procedure of the ascorbic acid (Murphy & Riley, 1962). We used several reagents: ammonium molybdate and antimony potassium tartrate react in acid medium with orthophosphate to form a heteropoly acid-phosphomolybdic acid, that is reduced to intensely colored molybdenum blue by ascorbic acid. All bottles were washed with sulfuric acid (5-10%) and rinsed several times with distillated water to remove any potential contamination.

We prepared the calibration curve from stock solutions of 1mmol/l and 100 μmol/l and then we performed serial dilutions of 0.05, 0.01, 0.1, 0.4, 0.8, 1, 2, μmol of KH_2PO_4 for TP (Fig.2.5b) and 25 μmol/ for SRP with serial dilutions of 0,009, 0.05, 0.1, 0.8, 1 μmol of KH_2PO_4 (Fig.2.5c).

For TP analysis, the mixed solution contained solution A (SA= 27gr ascorbic acid + 700 ml distilled water), solution B (SB= 15 gr ammonium molybdate + 500 ml distillated water), solution C (SC= 140 ml sulfuric acid + 900 ml distilled water) and solution D (SD= 0.34 gr of antimony potassium tartrate + 250 ml distilled water). This mixed solution has a yellow color. Normally, this solution is stable for 4 hours. For this reason, we used it immediately after preparation. In next step, we added 0.1 g potassium persulfate for each 10 ml samples. Later, sample bottles were

autoclaved for 30 min at 120 ^{0}C (Fig.2.6f). When sample bottles, were cooled at room temperature, we added 1 drop of phenolphthalein, 3-4 drop sodium hydroxide 6 N (during this stage solution color changed to pink) (Fig.2.6g, h). Then, we added 2 ml of the mixed solution (as explained before). After 5-10 minutes, we measured at 885 nm by using cuvette 1 cm for TP and 10 cm for SRP analysis,

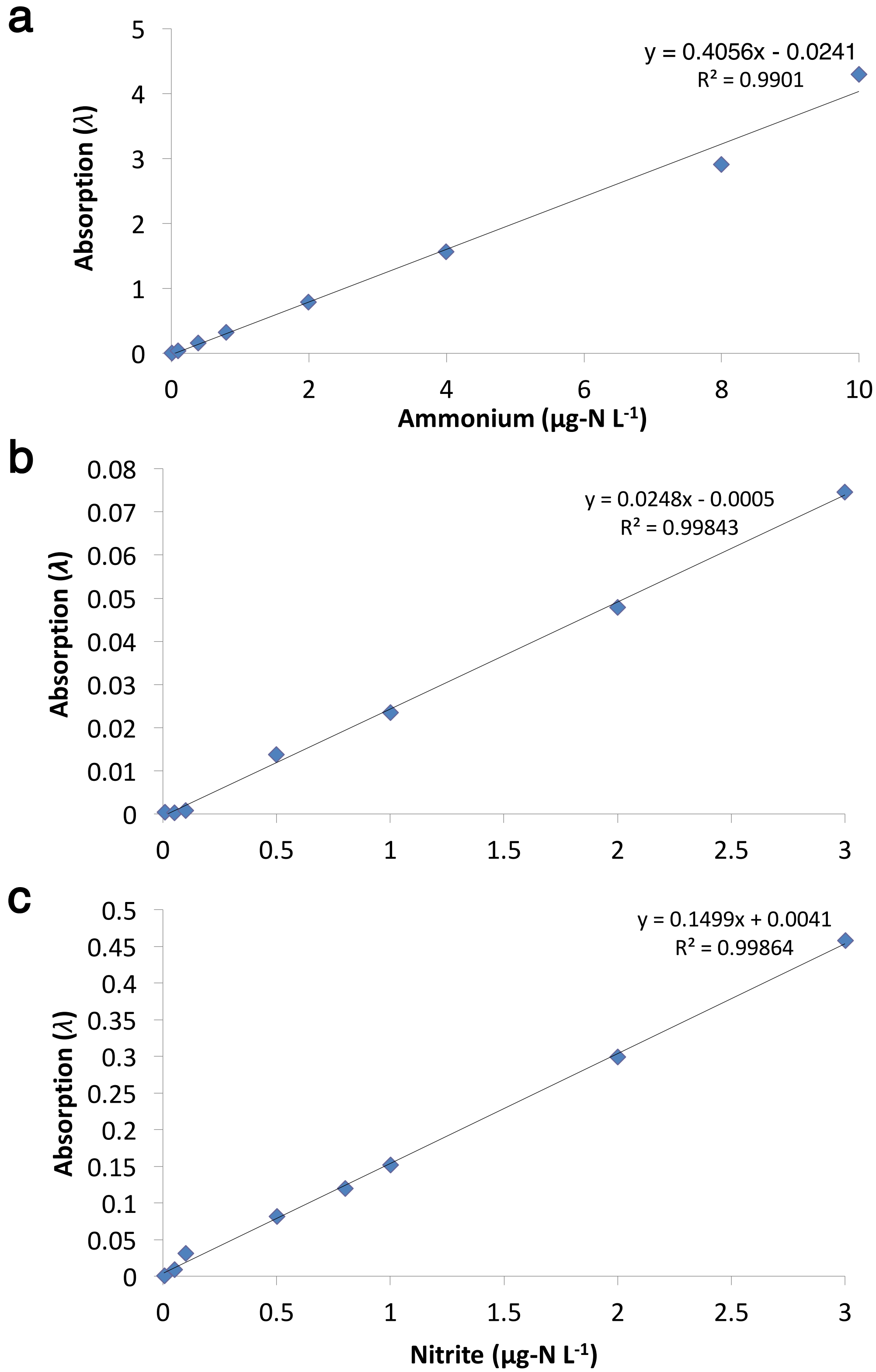


Fig.2.4. Calibration curves of (a) ammonium, (b) nitrate, and (c) nitrite.

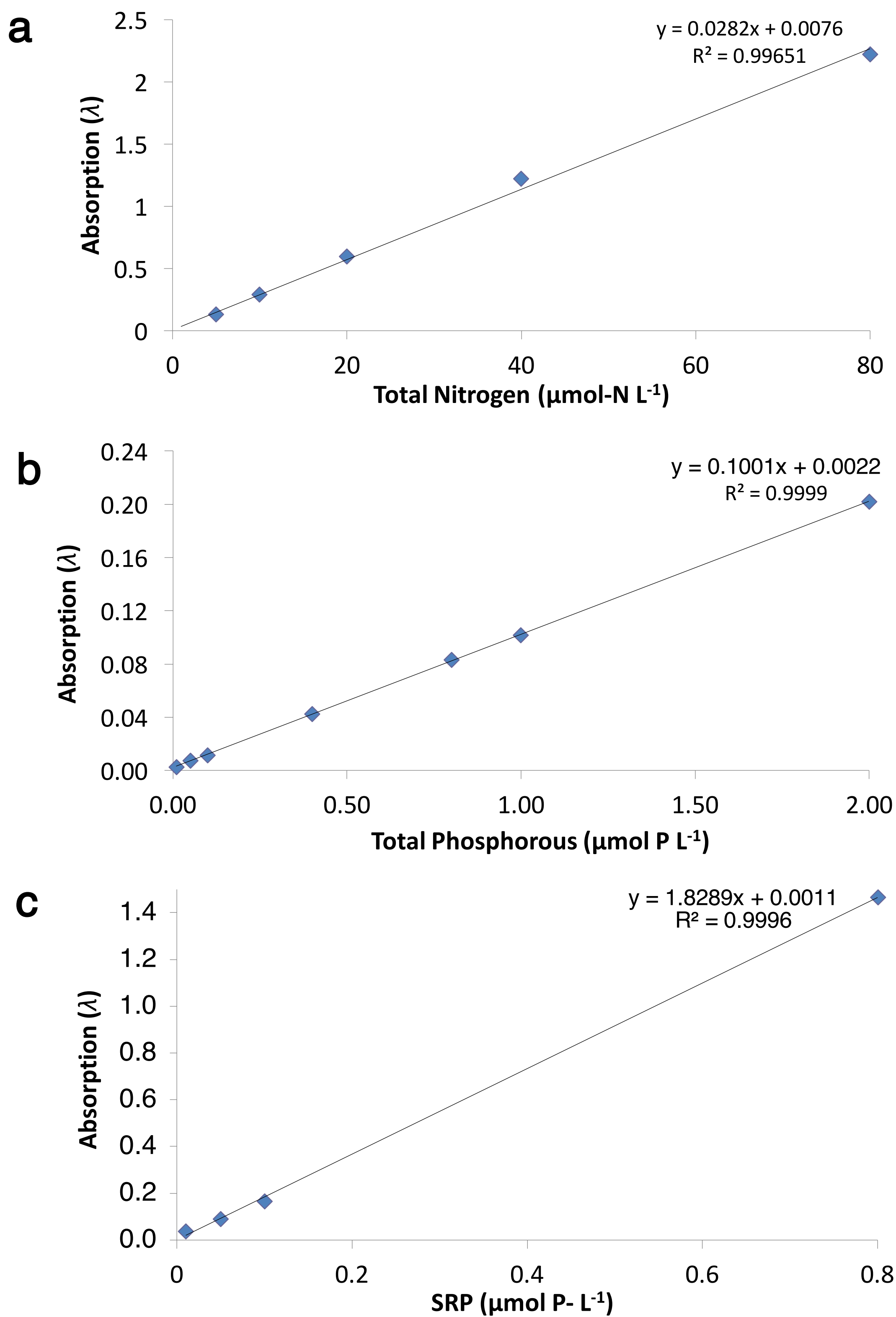


Fig.2.5. Calibration curves of (a) Total Nitrogen (TN), (b) total phosphorous (TP) and (c) soluble reactive phosphorus (SRP).

Fig.2.6. Ammonium calibration curve using kit model HC243987(a), ammonium measurements(b), nitrite, total Phosphorous and Soluble Reactive Phosphorous calibration curve (c, d and e, respectively). After adding Potassium Persulfate, bottle autoclaved for 120 ºC for 30 for TP nor for SRP measurements(f) using the ascorbic acid (Murphy and Riley, 1962). The color of TP samples bottle, when added 1 drop of Phenolphthalein and then 3-4 drop Sodium hydroxide (6 N), changed to pink color(g-h).

Particulate organic components

Total Organic Carbon (TOC)

Total organic carbon (TOC) concentration was measured by a high-temperature catalytic oxidation as non-purgable organic carbon using a Shimadzu TOC-V CSN (Fig.2.7a).

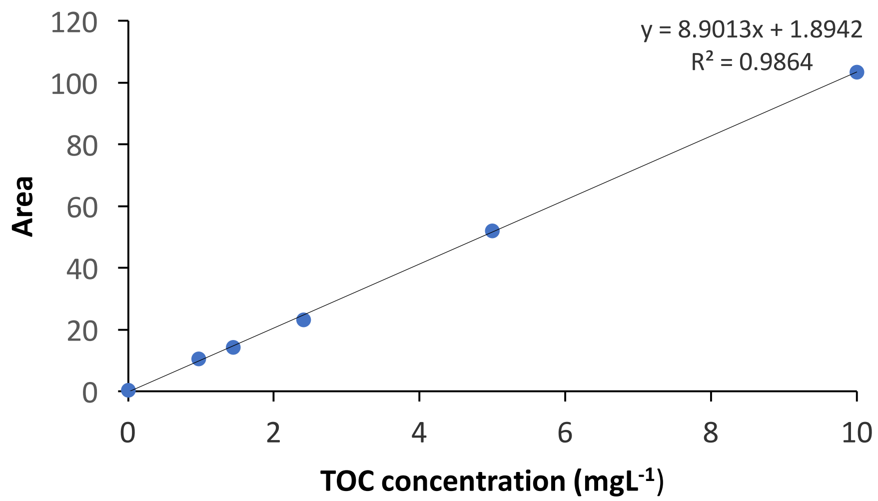
The principle of TOC analysis is a complete combustion catalytic oxidation of organic compounds to carbon dioxide followed by quantitative measurement of the CO_2 by Non-Dispersive Infra-Red (NDIR) detectors. Previously, all the inorganic carbon content in the water was removed by sparkling with CO_2^- free gas after acidification of the sample with phosphoric acid (Sharp & Peltzer, 1993).

Samples were taken in dark glass vials previously cleaned with (HCL), rinsed several times with Mili-Q water and precombusted at 450-500 °C for at least 2 hr. To reduce sample pH to ca. 2 and purge dissolved CO_2, we added 100μL of H_3PO_4 85% in each 100 ml of sample. This pH reduction of the samples also preserved the samples. Samples were stored at dark at low temperature (i.e., 2-4°C) in refrigerator until analysis.

We prepared the calibration curve from a stock solution of potassium hydrogen phthalate. We weighted 0.021 gr of C8H5KO4 and dissolved into 100 ml of Milli-Q water. The TOC analyzer has an internal calibration curve using serial dilutions from the stock solution. In addition, we also run the next dilutions to check routinely its performance 0, 1, 1.4, 2.4, 5 and 10 ppm prepared (Fig.2.7b, c). Three injections were analyzed for each sample and the blanks (Milli-Q water). Equipment was set up to inject 100 μl three times to automatically wash the syringe.

a

b



c

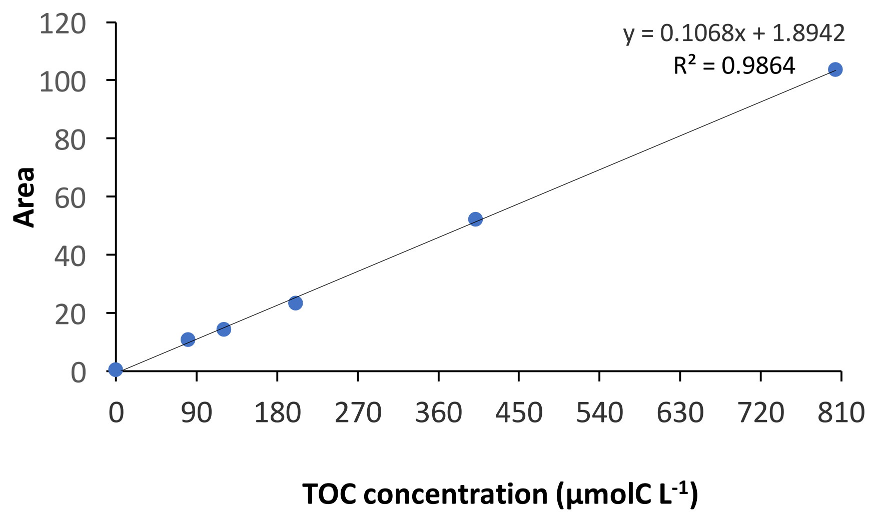


Fig.2.7. Shimadzu TOC-V CSN used for TOC measurements(a), TOC calibration curve (b and c)

Particle Organic Matter and Particle Matter:

Particle Organic Matter (POM) and Particle Matter (PM) measurements were determined by using Byers method (1978). To determine the concentration of PM and POM between 1500 and 2000 ml were filtered through pre-combusted (500°C for 4 h) Whatman GF/F glass fiber filter with 0.7 μm nominal pore size (Fig.2.8). The filters containing PM were dried at 60°C for >24 h and reweighed to determine the PM mass. Then, the filters were combusted at 500°C for 6 h and reweighed again to determine the mineral residue (equation1). POM was obtained after the subtraction of the mineral residue (equation 2) to the PM.

Equation1:

$$\mathrm{PM\ (mg/L)} = \frac{\mathrm{A\ (mg) - F\ (mg)}}{\mathrm{VF\ (L)}}$$

Equation 2:

$$\mathrm{POM\ (mg/L)} = \frac{\mathrm{A\ (mg) - B\ (mg)}}{\mathrm{VF\ (L)}}$$

Where:

A: Filter weight before combustion

F: Empty filter weight

B: Filter weight after combustion

VF: Volume of filtered water

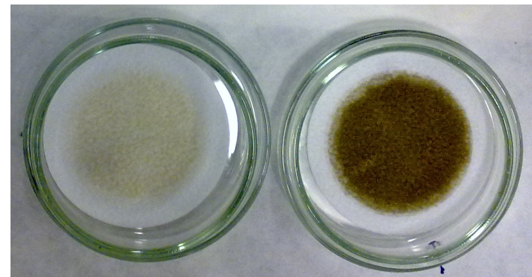


Fig.2.8. POM obtained by filtering 2-liter sample waters (- *Holothurian* tank, right)

Chlorophyll *a*:

The concentration of chlorophyll-*a* was determined spectrophotometrically after pigment extraction with methanol (APHA, 1992). In the laboratory, usually a volume of two liters of water from the tanks was filtered through Whatman GF/F glass fiber filter with 0.7 µm nominal pore size covered with aluminum foil and frozen at -20 ºC until analysis. Pigments were extracted with methanol during 24h at 2-4ºC. To avoid potential scattering in the absorption measurements due to small filter pieces during the extraction procedure a second filtration was performed. For each sample an absorbance scan from 400 nm to 750 nm was made with 1cm-cuvette using a spectrophotometer UV/VIS Perkin Elmer (Fig.2.9). To obtain the concentration of chlorophyll-*a* we used the absorbance at 665 nm that was corrected for turbidity using the absorption at 750 nm when it was needed. Chlorophyll-a concentration was calculated as described by Talling (1984) (equation 3).

$$\text{Chlorophyll } a\ (\mu g/L) = f\, A_{665} * \left(\frac{V}{B}\right)$$

Where:

f: Specific absorbance coefficient =13.9

A: Corrected absorbance at wavelength 665 nm (A at 665 nm - A at 750 nm).

V: Methanol Volume (ml)

B: Filtered water samples (L)

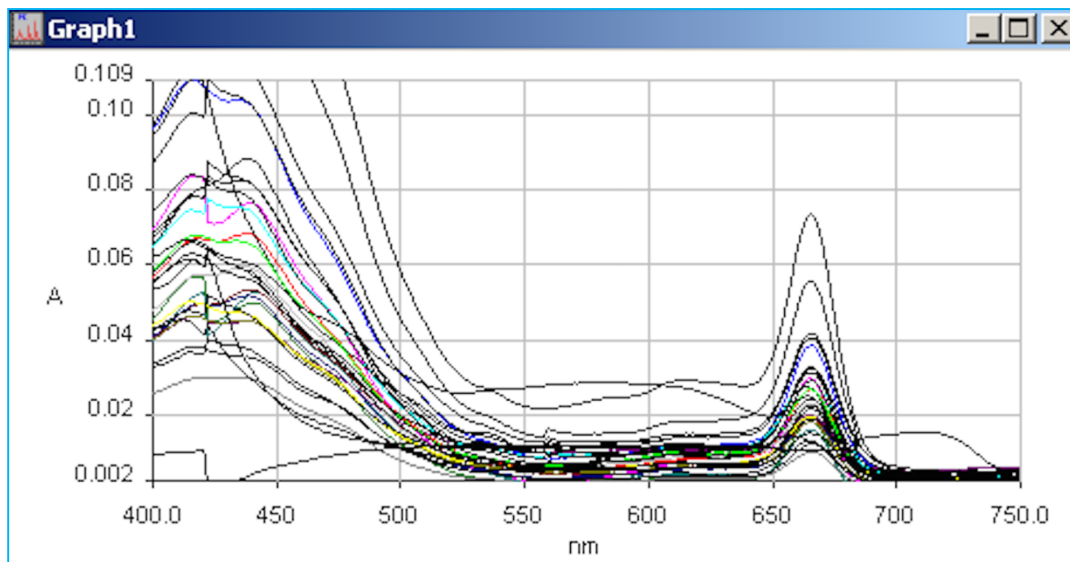


Fig.2.9. Chlorophyll *a* obtained by spectrophotometer

Prokaryotes (bacteria and cyanobacteria)

The prokaryotic abundance was determined using flow cytometry (Gasol & del Giorgio, 2000). To count bacteria and cyanobacteria we used a flow cytometer FACScalibur Becton Dickinson (Fig.2.10). FACScalibur was equipped with four detectors (Photomultipliers) with band pass filters include 530 nm (green fluorescence e.g., SyberGreen), 585 nm (e.g., Phycoerythrin), 661 nm (e.g., Allophycocyanin excited by 635 laser), >670 nm (e.g., chlorophyll *a*) and also equipped with a laser emitting at 488 nm. Briefly, samples go through a constant point in front of a laser beam then, due to the particles the light is dispersed and fluorescence is emitted. After that laser excitation by the particles the emissions are gathering in photomultipliers and finally, sent them to the computer. Data were processed using Cell quest software. Total prokaryotic abundance was detected by their signature in bivariate plots of Side Scatter (SSC) vs. FL1 (green fluorescence). Orange fluorescence was detected in the FL2 channel, and red fluorescence in the FL3 channel. In addition, for Cyanobacteria abundance (mostly *Prochlorococcus* and *Synechococcus* cells) another bivariate plot contains FL1 vs. FL3 (or/and FL3 vs. SSC) was set. *Synechococcus* individuals are detected by their signature in a plot of orange fluorescence (FL2) vs. red fluorescence (FL3). *Prochlorococcus* have a lower FL3 signal and no FL2 signal (Fig.2.11). Heterotrophic bacteria abundance was obtained by the subtraction of the autotrophic bacteria abundances from the total abundance of prokaryotes. Standard settings for pico-phytoplankton and bacteria in cell quest software programmed for FSC: E01, SSC: 367-400, FL1: 400, FL2: 505-580, FL3: 590-623 (Threshold at FL3: 20-70) and FSC: E02, SSC: 400; FL1: 511, FL2: 400, FL3: 590 (threshold a FL1: 72), respectively.

BD FACScalibur equipment was cleaned with distilled and filtered (0.2 μm) water and an internal reference was performed using beads following the technical recommendation. Prokaryotic samples were stained for 10 min at dark with a DMSO diluted Syber Green I (Molecular Probes) at 10 µm/L final concentration. We added 10-20 μl of beads (depending on sample dilution level) of a solution of yellow-green 0.92 µm Poysciences latex beads as an internal-reference.

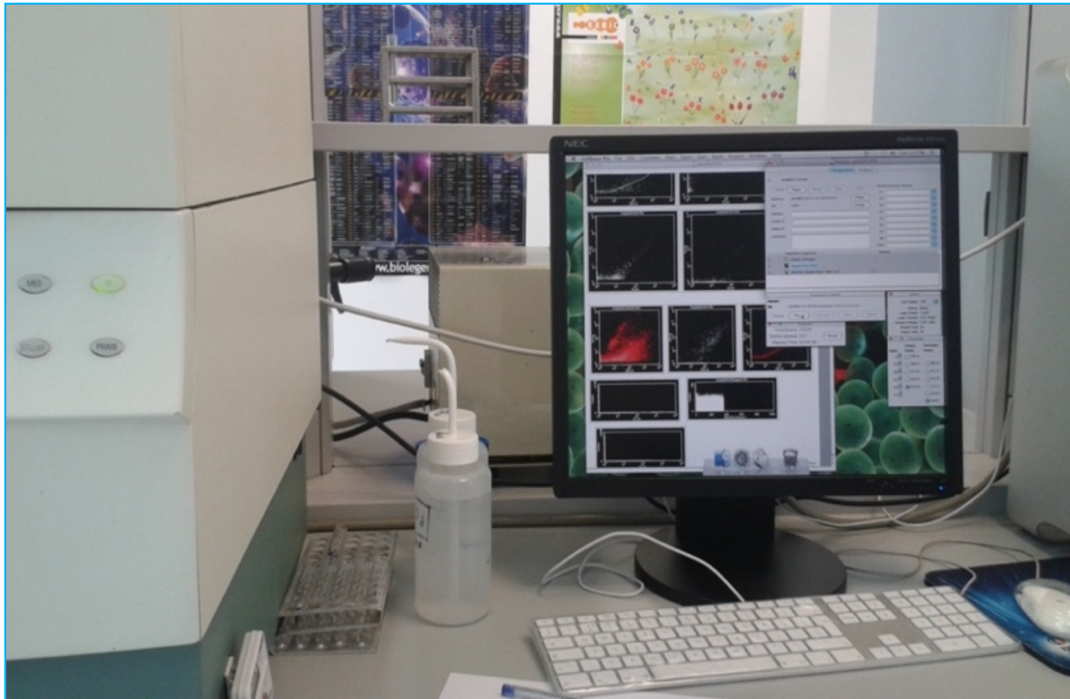


Fig. 2.10. FACScalibur Becton Dickinson cytometer

Samples were acquired in log mode and run at low speed (for 2 minutes at 12 μl min^{-1}) for total prokaryotic abundance and at high speed (for 4 minutes at 60 μl min^{-1}) for cyanobacteria cells until we counted about 10^5 events. The samples were diluted when we obtained events higher than 800 cells/s (to avoid coincidence). During all data processing (with Cell quest software), the noise was eliminated to improve the precision of results. For each day, flow rate (36.63 ± 2.44 μl min^{-1}) and sample dilution was calculated (10^2 or 10^3) for prokaryotes and for cyanobacteria (73.08 ± 4.98 μl min^{-1} and sample dilution (10^2) (equation 4).

$$P = \frac{(E*DR)}{(PD*DFR)} * 1000$$

Where:

P:Prokaryotes (Cell/ml)

DR: Dilution rate

E: FACScalibur Event (Particle/S)

PD: Sample Dilution rate

DFR: Daily Flow Rate of FACScalibur (Low and High speed):

{Weight(A)-Weight(B)/10}*1000

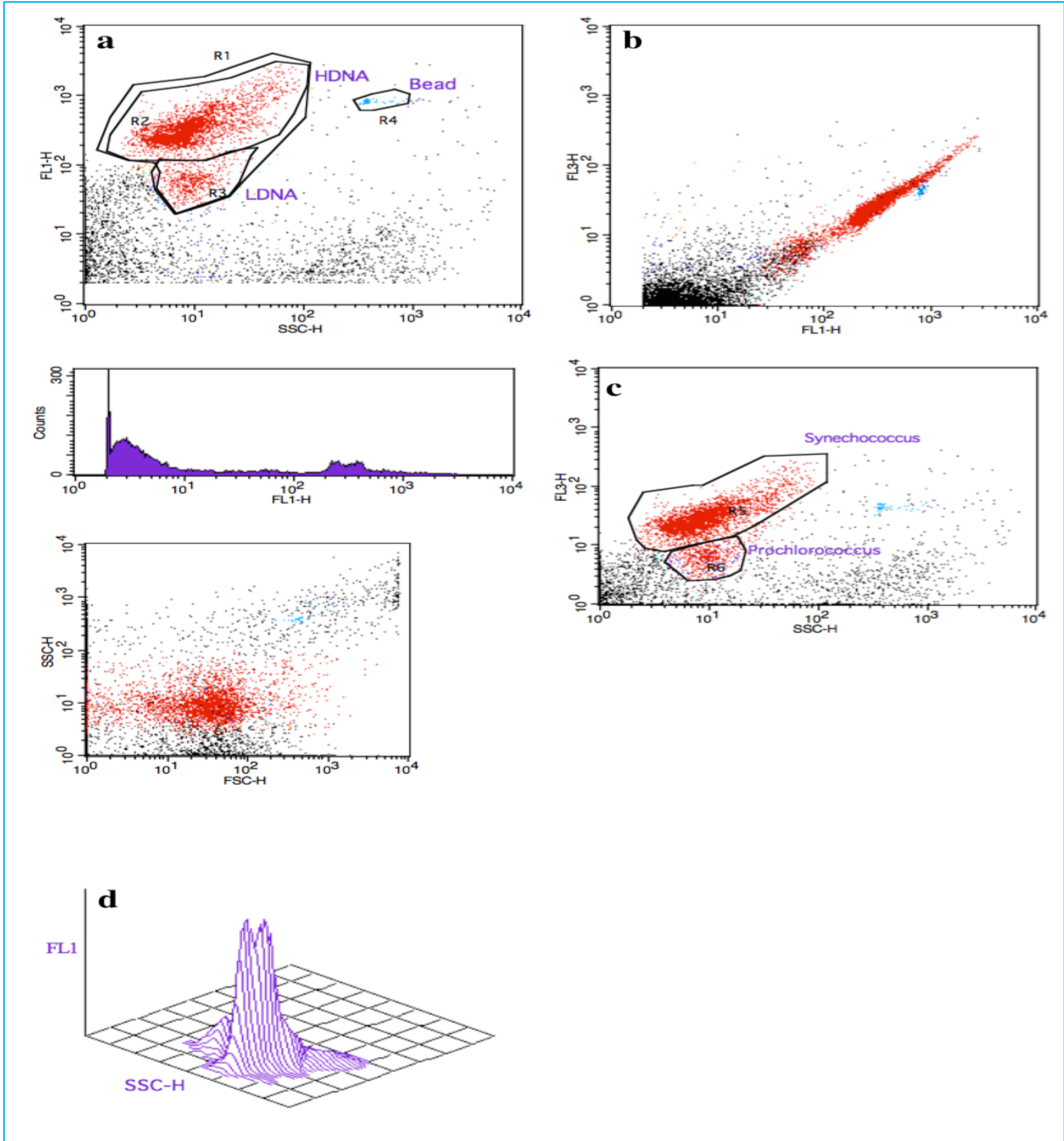


Fig. 2.11. Contour density plots obtained from flow cytometric analysis (using Cell quest program). The representation of 90° light scatter (FL1 in our instrument) vs. (SSC) green fluorescence (a) account for all the bacterial abundance including bacteria with high DNA (HDNA) and bacteria with low DNA (LDNA). (b and c) Cyanobacteria abundance (Prochlorococcus + Synechococcus cells) obtained via plotting FL1 VS. FL3 (in our instrument red fluorescence) Plot (d) showed the three-dimensional plot of FL1 vs. SSC-H 3D plot.

Transparent Exopolymer particles (TEP)

We determined the concentration of exopolymer particles using the basic alcian blue method (Passow & Alldredge, 1995) with minor modifications following Mazuecos *et al*. (2012).

For TEP measurements with the colorimetric method, a standard of alcian blue (0.02%) solutions, patron solutions were prepared. To prepare patron solution, we weighted between 10 to 20 mg of Xanthan Gum in 200 ml of distilled water. This solution was well mixed, passed through a homogenizer three times, kept in refrigerator for half hour and finally, homogenized three more times. To prepare the Alcian blue solution (stock solution), in 50 ml of distilled water 0.5 g of alcian blue and 1.5 ml of acetic acid were added. Then, we prepared a working solution with 10 ml of stock solution and 490 ml of distilled water.

We prepared the calibration curve using two sets of filters: dry filters (to know the precise weight of xanthan gum filtered) and non-dry filters (to determine the absorbance of alcian blue which is bound to xanthan gum). Indeed, alcian blue absorption was calibrated using a solution of the polysaccharide Xanthan Gum. The next procedure was followed for the dry filters. Twenty-five polycarbonate filters (0.4 pore-size and 22 mm diameter) were dried in the oven (60 ºC for 2 hr) and weighted three times. These labeled filters were put again in the oven with same condition and re-weighted. At least 15 filters with the lowest changes in weight were used to make the calibration curve. We used three filters with known weights to filter a specific quantity of Xanthan Gum solution. For example: 0.5, 1, 2, 3, 4 ml of Xanthan Gum solution were filtered by triplicate. After the filtration, the filters were again dried in the oven (60 ºC for 2 hr) and re-weighted (3 times). The difference of the weights (μg) before and after the filtration was used for the calibration curve as the concentration of Xanthan gum equivalent.

The procedure continued with the non-dry filters in a similar way that with the dry filters. Three filters for each concentration were put on the holder of the filtration ramp. Then, we added 0.5, 1, 2, 3, 4 ml of xanthan gum solution and filtered by triplicated. Then, we added 0.5 ml of working solution of Alcian blue; we waited for 30 seconds

approximately and we filtered the alcian blue. Then, the filters were washed with distilled water; were introduced in a tube and immediately in the refrigerator. To

obtain the alcian blue absorbance for each concentration, we added 5 ml of sulfuric acid 80 %, waited for 2 – 3 hours and shook the filters. Finally, we measured the absorbance at 787 nm with disposable polystyrene cuvette (1 cm) in a UV-VIS Perkin Elmer spectrophotometer (Fig.2.12).

Blanks were prepared also by triplicated; washing filters with Mili-Q water and filtered them. Then, we added 0.5 ml of working solution of alcian blue. We added sulfuric acid to make the extraction. Blank was used for final calculation.

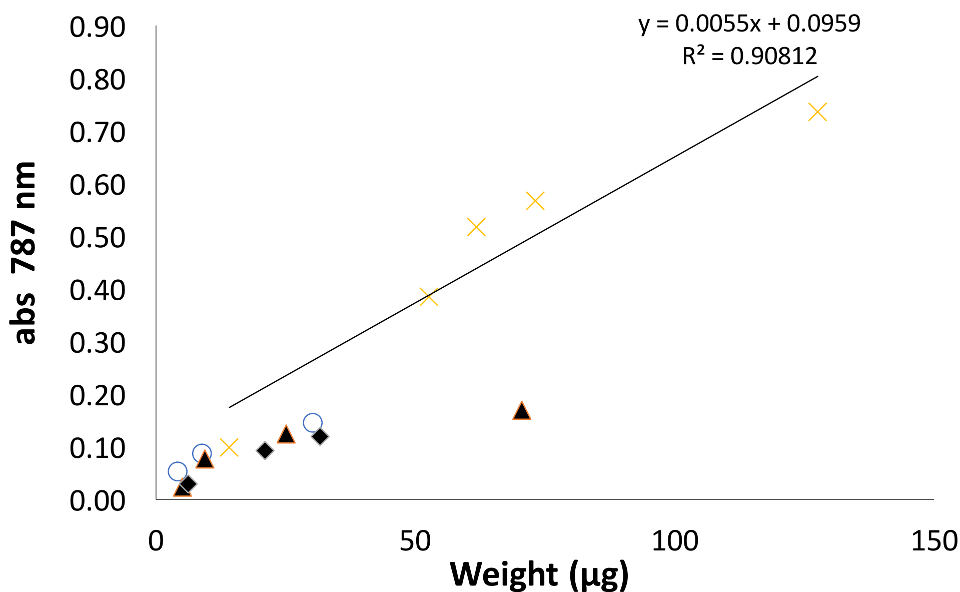


Fig. 2.12. Calibration curve (solid black line) used to relate the absorbance of alcian blue dye at 787 nm with the corresponding weight of Xanthan Gum (μg). Each symbol represents different calibration curves.

Water samples (100-250 ml), previously fixed with formaldehyde (1% final concentration), were filtered using amultiple filtration system and 0.4 µm polycarbonate filters. Then, the filters were dyed with 0.5 ml of working solution of alcian blue and after 30 seconds filtered. The dye was extracted in 80% sulphur acid (5 ml) for three hours and we measured the absorbance at 787 nm (Fig.2.13a-f). The TEP

concentration was expressed in µg of Xanthan Gum (XG) equivalents per liter (µg XG eq L-1). TEP concentration was calculated using the next equations (equation 5 and 6):

$$\text{TEP } (\mu\text{g XG eq L} - 1) = \frac{\text{Absorbance of Sample } * \text{ calibration Factor}}{\text{volume of sample (L)}}$$

$$Calibration\ Factor = \frac{1}{\text{Slope}}$$

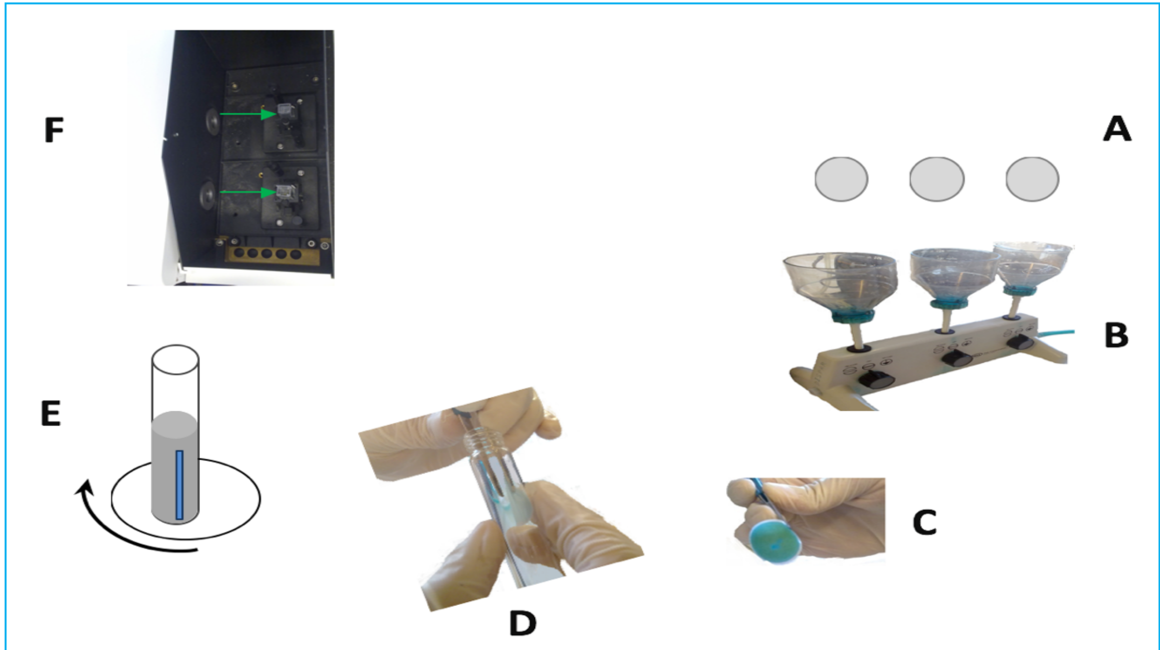


Fig. 2.13. Laboratory procedure to determinate TEP concentration by the colorimetric alcian blue technique. The 0.4 µm polycarbonate filters (a) were placed in the filtration system (b). Then, the retained TEP were stained with alcian blue solution (c) and the filters were frozen (-80 °C). In the laboratory, the unfrozen and stained filters were placed in test tubes and the extraction of the alcian blue dye was performed during 2-3 hour with sulfuric acid (80%)(d). In the next step, we shook the filters several times during extraction (e). Finally, the absorbance of the extracted solution was measured at 787 nm (f). More details in Mazuecos *et al.* (2012)

Chromophoric Dissolved Organic Matter (CDOM)

We took the samples for chromophoric dissolved organic matter (CDOM) from inlet waters, the center of the two tanks and the corresponding water effluents during each sampling day in the long-term time series (biweekly, from July 17th 2013 to August 20th 2014). To avoid sunlight can affect the absorption measurements, we took the samples in amber glass, acid-cleaned bottles and pre-combusted (4h at 500 °C). They were kept in icebox during transportation to the laboratory (about one hour from the tanks). Water samples were filtered at room temperature through pre-combusted Whatman GF/F glass fiber filters with 0.7 nominal pore size (47 mm).

Absorbance spectra of CDOM samples were recorded at wavelengths from 200 nm to 750 nm at 1 nm interval using an UV/VIS Perkin Elmer spectrometer with 10 cm quartz cuvette. The spectrophotometer was connected to a computer with Lambda 25 software. The detection limit of the spectrophotometer (0.001 Absorbance) corresponds to a CDOM absorption coefficient detection limit of 0.02 m^{-1} at wavelength in 200 to 800 nm range. Spectrum corrections due to residual scatering by fine size particle fractions, micro-air bubbles, or colloidal material present in the sample were performed by subtracting the average of the absorption between 600 and 700 nm (Green & Blough, 1994).

CDOM absorption coefficients, $a\lambda$, were calculated using the next equation (7):

$$a_\lambda = 2.303\ \frac{Absorbance\ (\lambda) - Absorbance\ (600-700)}{L}$$

where a_λ is the absorption coefficient in m^{-1} at each λ wavelength, *Absorbance* (λ) is the absorbance at the λ wavelength, *Absorbance (600-700)* is the average absorbance from 600 to 700 nm, 2.303 is the factor that converts from decadic to natural logarithms and (L) is the cuvette path length in m.

Spectral slopes describe the shape decay of absorption coefficient vs. wavelength. Slope were calculated from the linear regression of log-transformed absorption coefficients in the wavelength bands 275-295 nm ($S_{275\text{-}295}$) and 350-400 nm (Helms *et al*., 2008). The spectral slopes for both wavelength calculated as in equation 8.

equation 8:

$$a_\lambda = a_{\lambda\, ref}\, e^{-S(\lambda-\lambda\, ref)}$$

where λ is the selected wavelength in nm, a_λ is the absorption coefficient at λ wavelength in m^{-1}, $a\ \lambda_{ref}$ is the absorption coefficient at a reference wavelength $\lambda_{ref,}$ S is the spectral slope. The spectral slope ratio (S_R) was the calculated as the ratio of the spectral slope from 275 nm to 295 nm ($S_{275-295}$) to the spectral slope from 350 nm to 400 nm (Helms *et al.*, 2008). In addition, we calculated the molar absorption coefficients at 325 nm (a^*_{325}) as the absorption coefficients at 325 nm normalized by the concentration of total organic carbon.

The same procedures to determine CDOM absorption coefficients and spectral slopes were followed for the short-term experiments.

Fluorescent Dissolved Organic Matter (FDOM)

FDOM samples were taken from inlet water and from the center of the tanks during each sampling date. Then, samples were filtered through pre-combusted GF/F glass fiber filter with 0.7µm nominal pore size. To increase FDOM precision, all samples were measured during the sampling days at room temperature. To avoid sunlight interfered with samples, we used dark glass flasks of 250 ml. Before sampling, all the bottles were acid-cleaned (1% HCl) for 24 hours and then washed several times with Milli-Q water. Subsequently, all the flasks were combusted (4 h, 500 °C) to prevent organic contamination. Just before filling the flasks, the bottles were rinsed with seawater and then, kept in icebox during transportation to the University of Granada.

Fluorescence measurements (FDOM) were made with Horiba Jobin Yvon Fluoromax-4 spectrofluorometer equipped with a 1 cm quartz cuvette (4 clear glass sides), Xenon arc lamp and photomultiplier tube as a detector (Fig.2.14). To check the instrument conditions we measured both excitation and emission spectra of sealed Milli-Q cuvette. The excitation check let us know if the lamp intensity (for the pulsed xenon flash tube lamp) has changed over time. It was checked by setting area at excitation and emission range (200-600 and 350 nm, respectively; integration time: 0.1s). Both Ex. Em slits were chosen at 1 nm. For this scan, we accepted the highest peak at 467 nm.

To avoid contamination effects during the measurements, non-latex gloves were used to reduce any contamination on the quartz cuvette. We used special gloves to

avoid cuvette contamination. Cuvettes were rinsed with Milli-Q water several times for each sample batch. Also, to be sure that cuvette was clean, it was checked with equipment (cuvette check for contamination). It was done by setting area at emission and excitation range (270-430 and 240 nm, respectively; integration time: 0.1s). Both Ex. Em. slits were chosen at 5 nm. In our study, Milli-Q water was used as a blank in all samples measurements (Stedmon *et al*., 2003, Lawaetz & Stedmon, 2009).

The emission check (water Raman scan) allows checking the lamp drifting over time. To perform the emission check we set an excitation and emission range of 350 nm and 365-450 nm, respectively and an integration time of 0.1s. Both Ex. Em slits were chosen at 5 nm. To be sure the emission check was well done, we accepted the Raman peak was located at 397±1 nm. During the measurements, at the beginning and the end of samples analysis, we repeated the emission spectrum of the sealed Milli-Q water with the same settings as explained before.

Excitation Emission Matrices (EEMs) Spectroscopy involves a collection of multiple emission spectra at a range of excitation, which are concatenated into a matrix to form a three-dimensional matrix of fluorescence data. To obtain 3D EEMs, both excitation and emission were set for bandwidths to 1 nm. A series of emission scans (300-560 nm, 2 nm increment) were collected over excitation wavelengths ranging from 240 to 450 nm with 10 nm increments) for both samples and blanks (Fig.2.15a).

Fig. 2.14. Horiba Jobin Yvon Fluoromax-4 spectrofluorometer

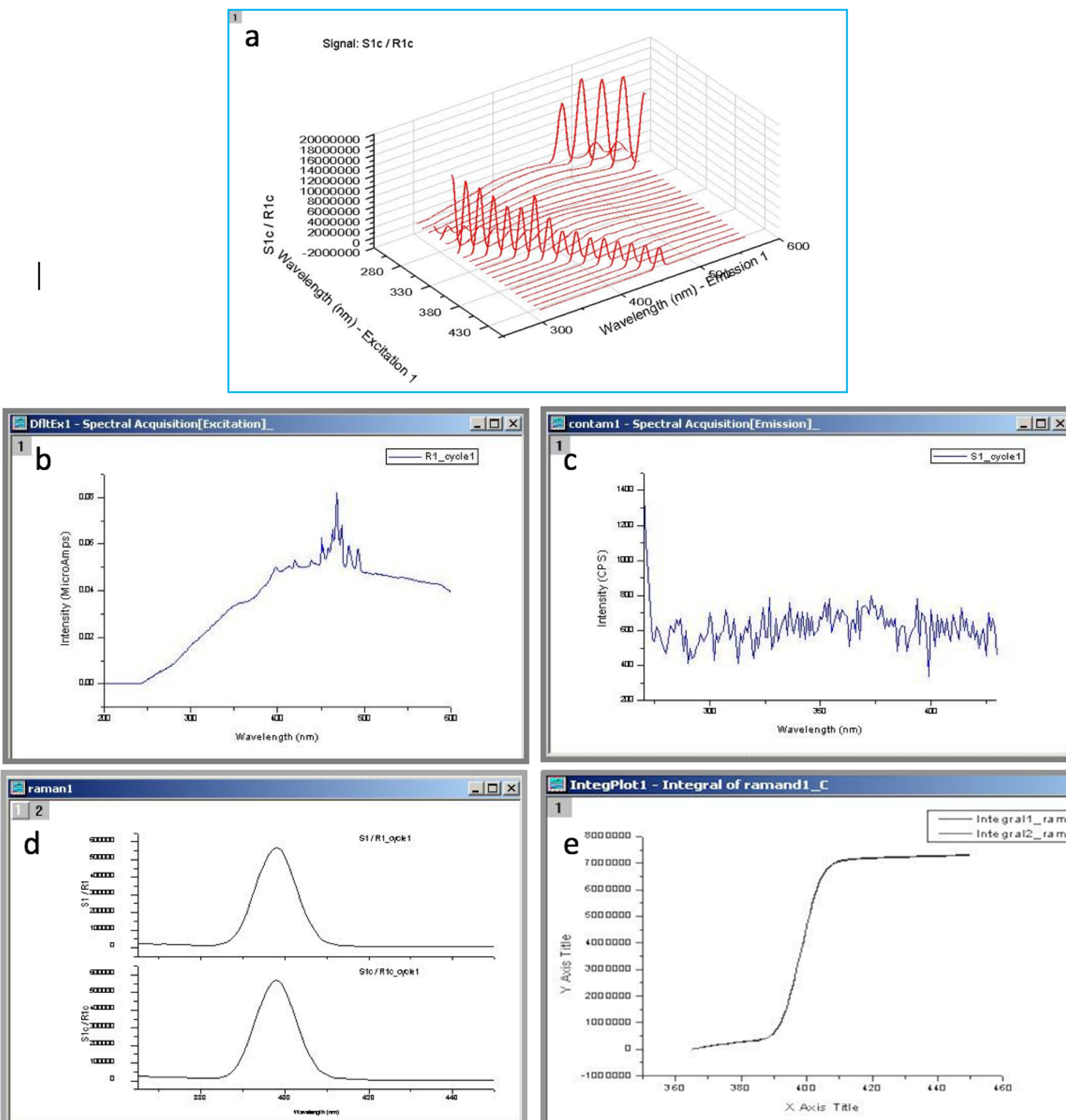


Fig. 2.15. (a)Three-dimensional matrix of fluorescence obtained by Horiba Jobin Yvon Fluoromax-4 spectrofluorometer, b) excitation check, (c)cuvette check, (d and e) water raman scan operation as a daily protocol to check Xenon lamp intensity, cuvette for contamination and lamp drifting over time, respectively. During all sampling analysis, cuvette check scans, were look liked a relatively flat but noisy line with very low intensity (under 2000 CPS). Water raman scan or emission check calculate the integrated area under the Raman Scatter peak for 3D correction, so all data change to Raman Unit to be able to compare final results with other experience.

In our study, the peaks were identified by visual observation on EEMs at maximum fluorescence record by emphasis on Coble peaks (1996, 2007). Where peak A characterized as (terrestrial humic), M (marine fulvic) and C (terrestrial fulvic) belong to humic-like fluorescence group, also peak B, T and N belong to the non-humic (or amino acid-like fluorescence group). Fluorescence maximum of peak A, M and C correspond to Ex. 260/Em. 400-460 nm, Ex. 290-310/Em. 370-410 nm, and Ex. 320-360/Em 420-460 nm, respectively. Normally, the humic-like fluorescence group was detectable at a visible range of spectrum and could be found in all aquatic environments, especially in coastal waters with high terrestrial runoff. The fluorescence maximum of peak B, T and N correspond to Ex. 275/Em. 305 nm, Ex. 275/Em. 340, and Ex. 280/Em. 370, respectively. The fluorescence maximum of the amino acid-like group shows correspondence with the amino acids: tyrosine (peak B), tryptophan (peak T) and phenylalanine (peak N).

FDOM analysis using only the cualitative characterization of EEMs is not sufficiently precise (e.g., several CDOM component can overlap fluorescence). To obtain quantitative values for the fluorescence matrixes, beyond the qualitative peaks, we used parallel factor (PARAFAC) analysis (Stedmon and Bro, 2008). PARAFAC analysis breaks the 3D EEMs output images into individual fluorescent components (Stedmon *et al.*, 2003). In our study, we performed the PARAFAC analysis using MATLAB (Ver 2016b) with DOMFluor N-way toolbox v.3.1 developed by Andersson & Bro (2000). Bro (1997) developed initially the toolbox to PARAFAC modeling (FDOM script) Actually, in this script (toolbox), they provided all requirements for data processing for both EEMs and PARAFAC step by step. PARAFAC output image revealed the final component concentration (intensity) at the fluorescence peak (maximum excitation-emission) position in each sample. The following equation (9) shows PARAFAC model equation.

$$X_{ijk} = \sum_{f=1}^{F} cif\ emjf\ exkf + \varepsilon_{ijk},$$

$i=1,\ldots,I;\ j=1,\ldots,J;\ k=1,\ldots,K$

where x_{ijk} is the fluorescence intensity of sample i at emission wavelength j and excitation wavelength k; cif is the fluorophore intensity, em is the emission spectra; ex is the excitation spectra; ε_{ijk} is the residuals containing noise and other un-modeled variation; i is the sample, F is the combined of present fluorophore in the EEM . In this

equation is supposed that there is no interaction between each fluorophore. By PARAFAC output, we obtained the concentration (c), emission spectra (*em*) and excitation spectra (*ex*) of the underlying fluorophores.

To execute the PARAFAC modeling, a series of actions were made according to Stedmon and Bro (2008). At the beginning, to normalize fluorescence spectra (the area under the Raman peak with the excitation wavelength at 350 nm) of daily Milli-Q EEMs, we corrected by the Raman peak and, then, EEMs fluorescence units were converted into Raman Units (RU). To perform this action, we did Raman normalizing for both Milli-Q each day and then the results were subtracted from recoded results for each sample (Milli-Q -EEMs). So, our final data can be expressed in common units to be compared later with other spectrofluorometers. Hoge *et al.* (1993) described the following formulate to calculate it via a statistical approach (equation 10).

$$\mathrm{Fn}\lambda\,(\mathrm{N.FI.U.}) = \frac{\mathrm{F(S)}\,/\mathrm{R(s)}}{\mathrm{F(r)}/\mathrm{R(r)}}$$

Where (*F)* and (*R)* are the fluorescence and the Raman band of the sample (*s*) and the reference (*r*) compound.

In our study, at wavelengths lower than 260 nm, the excitation intensity shows acute reduction. These events cause low precision of the fluorescence intensity (at these wavelengths). For this reason, we ran a model to have non-negativity constraints application to each dimension (Yamashita & Tanoue, 2003).Therefore, Rayleigh scatter effects were removed from the data by cutting the 1st and 2nd Rayleigh peaks (Rayleigh mask subtraction). Actually, we removed all the first 20 emission wavelengths that are less or equal to excitation wavelengths 260 nm (Stredmon & Bro, 2008). In addition, outlier samples in the data set were identified-cut by running exploratory action (calculating the leverage action) (Zhang *et al.*, 2011).

In the next step, PARAFAC output for this study (components delivered) is interpreted (*Ex*. and *Em*. maximum) based on the existing literature on aquaculture, waste water, water quality monitoring or other related work (Hambly *et al.*, 2015; Nimptsch *et al.*, 2015; Liu *et al.*, 2014; Cohen *et al.*, 2014, Murphy *et al.*, 2014).

Statistical analyses

To compare the time-series of the study variables (i.e. nitrogen, phosphorus, bacteria, transparent exopolymer particles, CDOM optical parameter, and FDOM components) in the inlet waters with the effluents from the tank with holothurians and the tank without holothurians we performed paired t-test (for normally distributed variables) and Wilcoxon matched pairs test (for not-normally distributed variables) using the Statistica software (V8). These statistical analyses ameliorate the problem of temporal pseudo-replication in this type of studies (Millar & Anderson, 2004). In addition, the study variable and potential controlling factors were performed using Statistica software (V8). The relationship between variables was studied by Pearson's correlation analysis.

In the short-term experiments to test the statistical significance of the study variables during presence of holothurians and absence, we performed analysis of variance (ANOVA) comparing the tanks with holothurians (+H) with the tanks without holothurians (-H) using Statistica software (V8).

The PARAFAC analysis was performed with MATLAB (Ver 2012b) with DOMFluor N-way toolbox v.3.1 developed by Anderson & Bro (2000).

References

APHA. 1992. Standard Methods for the Examination of Water and Wastewater. 18th edition. Washington, D.C.: American Public Health Association.

Andersson CA, Bro R. 2000. The *N*-way Toolbox for MATLAB. *Chemometrics Intelligent Laboratory System* **52**:1-4 DOI 10.1016/S0169-7439(00)00071-X.

Bro R. 1997. PARAFAC. Tutorial and applications. *Chemometrics Intelligent Laboratory System* **38**:149–171 DOI 10.1016/S0169-7439(97)00032-4.

Cohen E, Levy GJ, Borisover M. 2014. Fluorescent components of organic matter in wastewater: Efficacy and selectivity of the water treatment. water research **55**:323-334 DOI 10.1016/j.watres.2014.02.040.

Coble PG. 1996. Characterization of marine and terrestrial DOM in seawater using excitation–emission matrix spectroscopy. *Marine Chemistry* **51**:325–346 DOI 10.1016/0304-4203(95)00062-3.

Coble PG. 2007. Marine optical biogeochemistry: the chemistry of ocean color. *Chemical Reviews* **107**:402–418 DOI 10.1021/cr050350.

Gasol JM, del Giorgio PA. 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Scientia Marina* **64**:197-224 DOI 10.3989/scimar.2000.64n2197.

Green SA, Blough NV. 1994. Optical absorption and fluorescence properties of chromophoric dissolved organic matter in natural waters. *Limnology and Oceanography* **39**:1903-1916 DOI 10.4319/lo.1994.39.8.1903.

Hambly AC, Arvin E, Pedersen L-F, Pedersen PB, Seredynska-Sobecka B, Stedmon CA. 2015. Characterizing organic matter in recirculating aquaculture systems with fluorescence EEM spectroscopy. *Water Research* **83**:112-120 DOI 10.1016/j.watres.2015.06.037.

Helms JR, Stubbins A, Ritchie JD, Minor EC, Kieber DJ, Mopper K. 2008. Absorption spectral slopes and slope ratios as indicators of molecular weight, source, and photobleaching of chromophoric dissolved organic matter. *Limnology and Oceanography* **53**:955–969 DOI 10.4319/lo.2008.53.3.0955.

Hoge FE, Swift RN. 1993. The influence of chlorophyll pigment upon upwelling spectral radiances from the North Atlantic Ocean: an active-passive correlation spectroscopy study. *Deep Sea Research Part II: Topical Studies in Oceanography* **40**: 265–277 DOI 10.1016/0967-0645(93)90017-H.

Lawaetz A, Stedmon C. 2009. Fluorescence intensity calibration using the Raman scatter peak of water. *Applied Spectroscopy* **63**:936–940. *Available at*: https://scholar.google.com

Mazuecos IP, Ortega-Retuerta E, Reche I. 2012. Medida de Partículas Exopoliméricas Transparentes (TEP) en agua marina. In: Moreno-Ostos E, ed. Expedición de circunnavegación Malaspina 2010: Cambio global y exploración de la biodiversidad del océano global. *Libro blanco de métodos y técnicas de trabajo oceanográfico*. Spain: 515-520.

Millar RB, Anderson MJ. 2004. Remedies for pseudoreplication. Fisheries Research **70**:397-407 DOI 10.1016/j.fishres.2004.08.016.

Murphy L, Riley JP. 1962. A modified single solution method for the determination of phosphate in natural waters. *Analytical Chimica Acta* **27**: 31-313 DOI 10.1016/S0003- 2670(00)88444-5.

Murphy KR, Stedmon CA, Wenig P, Bro R. 2014. Open Fluoran online spectral library of auto- fluorescence by organic compounds in the environment. *Analytical Methods* **6**:658-661 DOI 10.1039/C3AY41935E.

Nimptsch J, Woelfl S, Osorio S, Valenzuela J, Ebersbach P, Tuempling WV, Palma R, Encina F, Figueroa D, Kamjunke N, Graeber D. 2015. Tracing dissolved organic matter (DOM) from land- based aquaculture systems in North Patagonian streams. *Science of the Total Environment* **537**:129-138 DOI 10.1016/j.scitotenv.2015.07.160.

Passow U, Alldredge AL. 1995. A dye-binding assay for the spectrophotometric measurement of transparent exopolymer particles (TEP). *Limnology and Oceanography* **40**:1326-1335 DOI 10.4319/lo.1995.40.7.1326.

Sharp J & Peltzer ET. 1993. Procedures subgroup report. *Marine Chemistry* **41**:37-49 DOI 10.1016/0304-4203(93)90104-V.

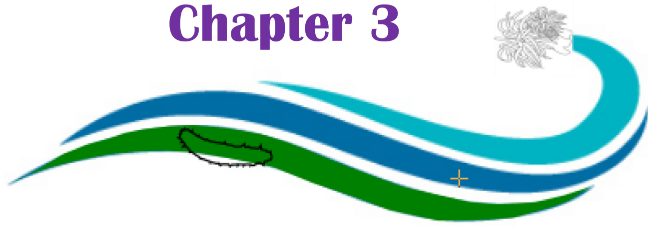
Stedmon CA, Bro R. 2008. Characterizing dissolved organic matter fluorescence with parallel factor analysis: a tutorial. *Limnology and Oceanography Methods* **6**:572–579 DOI 10.4319/lom.2008.6.572.

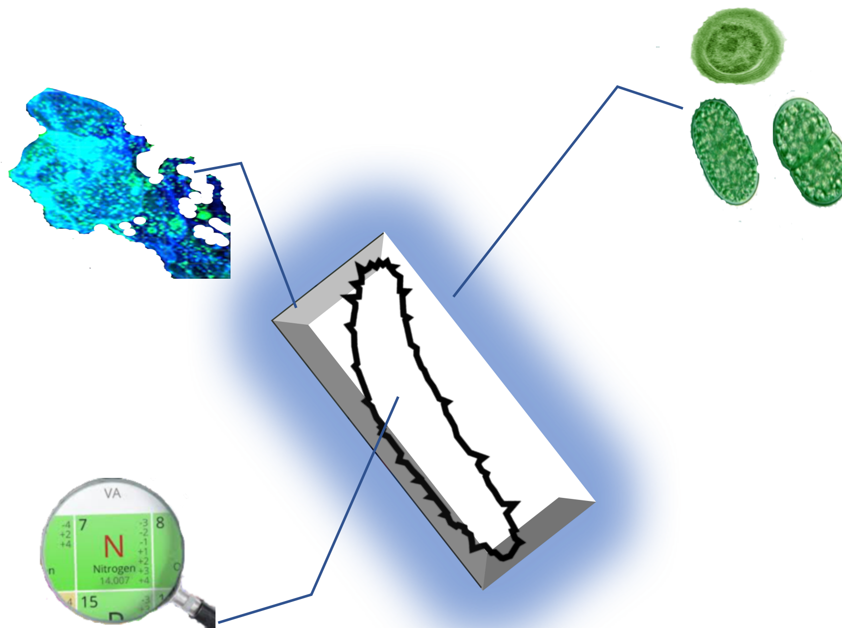
Stedmon CA, Markager S, Bro R. 2003. Tracing dissolved organic matter in aquatic environments using a new approach to fluorescence spectroscopy. *Marine Chemistry* **82**:239-254 DOI 10.1016/S0304-4203(03)00072-0.

Talling JF. 1984. Past and contemporary trends and attitudes in work on primary production. *Journal of Plankton Research* **6**:203–217 DOI 10.1093/plankt/6.2.203.

Zhang Y, Yin Y, Feng L, Zhu G, Shi Z, Liu X. 2011. Characterizing chromophoric dissolved organic matter in Lake Tianmuhu and its catchment basin using excitation-emission matrix fluorescence and parallel factor analysis. *water research* **45**:5110-5122 DOI 10.1016/j.watres.2011.07.014.

Chapter 3





Extractive potential of sea cucumbers to reduce nitrogen, bacteria, and transparent exopolymer particles in aquaculture tanks

Abstract

Traditional aquaculture releases nutrients and organic compounds in its effluents. Polyculture can alleviate this handicap including “extractive species” as sea cucumbers. The specific effects of sea cucumbers on nutrients and organic components have been scantily explored. Here, we monitored during one year the concentration of mineral nutrients, total organic carbon (TOC), particulate organic matter (POM), transparent exopolymer particles (TEP), chlorophyll-a, and bacteria in two big-volume tanks. One tank only contained *Anemonia sulcata*, whereas the other tank also included *Holothuria tubulosa* and *Holothuria forskali*. In addition, we performed three short-term experiments with three replicated tanks with *A. sulcata* plus *H. tubulosa* (+ H treatment) and four replicated tanks that contained only *A. sulcata* (–H treatment). In the time-series, we found that the concentration of ammonium, nitrate, TOC, POM, TEP, and bacterial abundance in the effluent waters from the tank with holothurians was significantly lower than the effluent waters of the tank without holothurians. These experiments confirmed the previous results with reductions statistically significant in nitrates, bacteria and TEP concentration in the treatments with holothurians. Therefore, bacterial proliferation and biofilm formation could be controlled by holothurians minimizing the risk of pathogenic outbreak of bacteria and improving the hygiene of the tanks.

Keywords: multitrophic aquaculture, extractive species, sea cucumbers, nitrates, bacteria, transparent exopolymer particles

Introduction

During last decades, human activities as overfishing, addition of pollutants and climate change are substantially affecting species stocks and diversity in marine ecosystems (Halpern *et al.,* 2008; Purcell *et al.,* 2013). In addition, the exponential growth of human population has boosted the global demand of fish and seafood (FAO, 2009). However, extractive fisheries are more and more limited. In fact, aquaculture currently accounts for more than 40% of human consumption of fish and seafood (Bostock *et al.,* 2010). Therefore, a responsible aquaculture is a global challenge for both marine biologists and food producers (Diana *et al.,* 2013). These last authors have proposed poly-culture and -integrated multitrophic aquaculture (IMTA) as alternative procedures to alleviate, to some extent, the handicaps of traditional aquaculture.

Traditional aquaculture produces wastewater that usually contains high loads of organic and inorganic nutrients, antibiotic and uneaten food pellets (Black, 2001; Read & Fernandes, 2003; Klinger & Naylor, 2012). The influence of this wastewater on the marine environment depends on the production system (extensive vs. intensive/semi-intensive), aquaculture system type (tank, pond, cage), the cultured species, as well as the carrying capacity of the recipient waters. During wastewater discharges, from aquaculture facilities or under the cages, eutrophication and sediment anoxia in coastal waters can happen (Crab *et al.,* 2007). Aquaculture wastewater can be a relevant source of nitrogen stimulating primary producers and increasing the risk of algal blooms or red tides (Ajin *et al.,* 2016). Similarly, oxygen depletion in sediments can cause the release ammonia and sulfide changing the physicochemical properties of water affecting fishes and corals (Kalantzi & Karakassis, 2006). Fecal waste and uneaten foods constitute a fraction of particulate organic matter that settles down in the bottom of the tanks or below the cages in offshore installations affecting bacterial activity and sediment properties. These changes appear to influence also biomass and diversity of macrobenthos (Yokoyama, 2002) and benthic food-webs (Boyd & Massaut, 1999). In the particular case of inshore installations, wastewater effluents from aquaculture tanks usually are treated before being returned to the aquatic ecosystems.

Several procedures to treat aquaculture wastewaters are in practice. To decide which treatment is more appropriate several factors should be considered such as land and water availability, wastewater local regulation and operational expenses.

Wastewater treatments such as Fenton's oxidation (Lee & Shoda, 2008), sequencing batch reactor (Fontenot *et al*., 2007), up-flow anaerobic sludge bed or integrated anaerobic/aerobic biological treatments (Bortone, 2009) have been used but they imply high economical costs and can eventually generate toxic by-products and membrane fouling in comparison with alternative biological treatments. In general, biological treatments are more acceptable by fish producers and policy makers. For instance, Da *et al*. (2015) proposed the reuse of wastewater from Striped Catfish farms in rice crops. Other authors proposed the recovering of phosphorous from wastewaters using the gastropod shell (Oladoja *et al.,* 2015) or aquatic plants (Buhmann & Papenbrock, 2013; Zhang *et al.,* 2014). Diana *et al*. (2013) recommended the integrated multitrophic aquaculture (IMTA) and poly-culture as responsible procedures that can decrease inorganic and organic nutrient loads in the effluents using "extractive species" that also can reduce the costs of wastewater treatments.

IMTA and polycultures, unlike monospecific aquaculture, use trophically complementary species where the excretion, fecal and food wastes from the primary species are nutritional resources for the extractive species (Chopin *et al.,* 2012). Therefore, these aquaculture systems include the primary species (e.g., fish), the extractive species that filter or ingest the suspended or settled down organic matter (e.g., mussels, oysters, holothurians), and species that assimilate inorganic nutrients (e.g., seaweeds) reducing the loads of inorganic nutrients and organic matter in the effluents. Therefore, it is desirable that the future expansion of aquaculture develops these co-culture procedures to remove or, at least, reduce these organic and inorganic loads in the effluents and, simultaneously, provide an extra co-cultured species with additional economical and ecological value.

Sea cucumbers are species with reported extractive capacity for organic carbon (Slater & Carton 2009; Nelson *et al.,* 2012a; Yokoyama, 2013;2015) as well as they are very demanded for human consumption (Purcell *et al*., 2013). Currently, sea cucumber overfishing is declining their stocks particularly in Asia (Nelson *et al*., 2012 a, b; Purcell *et al.,* 2013). Therefore, sea cucumber cultures could, on the one hand, mitigate this overfishing and diversity problem and, on the other hand, improve water quality in poly-culture installations. In addition, the microbiome of different species of sea cucumbers appears to be important source

of new antimicrobial substances (Chludil *et al.,* 2002; Haug *et al.,* 2002; Kumar *et al.,* 2007; Gowda *et al.,* 2008). Therefore, the use of sea cucumbers in poly-culture can have also an interest in pharmaceutical bioprospecting (Bhatnagar & Kim 2010; Valliappan *et al.,* 2014).

In this study, we assess the effects of the presence of holothurians, as extractive species, in aquaculture tanks with *Anemonia sulcata* as the primary species. During one year, we monitored the changes in the concentration of total organic carbon, particulate organic matter, bacteria, chlorophyll-*a*, transparent exopolymer particles and major nutrients (ammonium, nitrate, nitrite, and total phosphorous) in two big-volume (50,000 l) aquaculture tanks that only differed in the presence of holothurians. Afterwards, to corroborate the observations obtained in the time-series, we performed three short-term experiments manipulating the presence of holothurians in small tanks (300 liters). We observed, both in the time-series of the big tanks and in the short-term experiments, that the presence of holothurians reduced significantly the concentration of nitrate, transparent exopolymer particles, and bacteria. Therefore, holothurians appear to have a high environmental value to improve the wastewater in aquaculture installations.

Material and Methods:

Time-series in the big-volume tanks

We monitored for one year two aquaculture tanks at iMareNatural S.L. facilities (http://www.imarenatural.com) in Southern Spain (36°44′38″ N, 3°35′59″ W). Each tank (50,000 liters of capacity) was connected directly with the coastal water by one inlet pipe (inlet waters) and the water from each tank was released by one outlet pipe located in the bottom of the tank (effluent). The seawater was pumped into the tanks at a continuous flow of 1,200 lh^{-1}. Therefore, water residence time in the tanks was ca. 42 hours. In one of the tanks, 811 ± 125 individuals of the primary species, the sea anemone *Anemonia sulcata*, and 93 ± 3 adults of sea cucumbers *Holothuria tubulosa* (≈ 80 %) and *H. forskali* (≈ 20 %) were included (hereafter designated as + *holothurian* tank). In the other tank only 690 ± 87 individuals of the primary specie were included (hereafter designated as – *holothurian* tank). Sea anemones were placed on floating plastic boxes in the surface of the tanks and holothurians were free in the bottom and walls of the tanks. Sea anemones were fed with about 900-1800 g of fresh chopped fish, mainly *Scomber scombrus* (Van-Praët, 1985; Chintiroglou & Koukouras, 1992) twice

per week. *Anemonia sulcata* was selected as the primary species because is a very palatable species, highly demanded for catering in Southern Spain. In addition, the company iMareNatural S.L. is also involved in the study of this species due to its pharmacological potential (http://www.tascmar.eu).

Water samples from each tank were collected biweekly from July 2013 to August 2014. We took the samples from the center of the tanks, transferred to sampling bottles and immediately placed on ice for their transport to the laboratory. Sampling bottles were previously acid-clean and then rinsed several times with seawater. Before sampling, basic parameters as temperature (°C), pH, salinity (psu), total dissolved solids, and conductivity (mS cm^{-1}) were measured in the tanks using a multi-parameter HANNA probe (HI9828 model). Small, precombusted at 500ºC, amber bottles were used for the total organic carbon (TOC) samples. Once in the laboratory (about one hour from the tanks), samples for dissolved nutrients were filtered through Whatman GF/F filters and the filtrates stored at -20 ºC until analysis. Samples for bacterial abundance were fixed with 1% paraformaldehyde and 0.05% glutaraldehyde and then immediately stored at -80 °C.

Nutrient analysis

Analyses of ammonium, nitrite, nitrate, and total phosphorous (TP) were performed by triplicate using standard methods (APHA, 1992). The dissolved fraction of the nutrients was previously filtered using Whatman GF/F filters and total nutrients were analyzed from unfiltered samples. To determine ammonium the phenate method was used (APHA, 1992) but with the Spectroquant® Test Kit (Merck Millipore). Nitrate concentrations were measured following the ultraviolet spectrophotometric method. Briefly, 25 ml-samples were acidified with 0.5 ml of hydrochloric acid (1M), shaked and the absorbance at 220 nm and 275 nm were measured using 10 cm cuvettes in an UV-VIS Perkin-Elmer spectrophotometer connected to a computer equipped with UV-Winlab software. The nitrite concentration was determined spectrophotometrically through the formation of a reddish purple azo dye produced at pH 2.0 to 2.5 by coupling diazotized sulfanilamide with N-(1-naftile)-ethylendiamine dihydrochloride. The concentration of TP was determined spectrophotometrically using the ascorbic acid technique (Murphy & Riley, 1962).

Particulate organic components

Total organic carbon (TOC) concentration was measured by a high-temperature catalytic oxidation as non-purgeable organic carbon using a Shimadzu TOC-V CSN. Samples by triplicate were acidified with hydrochloric acid and purged for 20 min to eliminate the remaining dissolved inorganic carbon. Three to five injections were analyzed for each sample and the blanks (Milli-Q water). Standardization of the instrument was done with potassium hydrogen phthalate (4-point calibration curve).

To determine the concentration of particulate organic matter (POM) between 1.5 and 2.0 l of water from the tanks were filtered through pre-weighed and precombusted (500 °C for 4 h) Whatman GF/F glass fiber filters (0.7 µm nominal pore size). The filters containing all the solids were dried at 60 °C for >24 h and reweighed to determine the total mass (mineral + organic matter). Then, the filters were combusted at 500 °C for 6 h and the organic fraction was burned; the filters were reweighed again to determine the mineral residue. POM was obtained after the subtraction of the mineral residue to the total mass.

The concentration of chlorophyll-*a* was determined after pigment extraction with methanol spectrophotometrically (APHA, 1992). In the laboratory, a volume of two liters of water from the tanks was filtered through Whatman GF/F filters covered with aluminum foil and frozen at -20 ºC until analysis. Pigments were extracted with methanol during 24 h at 2-4 ºC. To obtain the concentration of chlorophyll-*a*, absorbance at 665 nm was measured using a spectrophotometer UV/VIS Perkin Elmer and, if needed, corrected for turbidity using the absorption at 750 nm.

Bacterial abundance was determined in triplicate using flow cytometry (Gasol & del Giorgio 2000) with a FACScalibur Becton Dickinson cytometer equipped with a laser emitting at 488 nm. Samples were stained for 10 min in the dark with a DMSO diluted Syber Green I (Molecular Probes) at 10 µM final concentration. A volume of 10-20 μl of a solution of yellow-green 0.92 µM Polysciences latex beads was added as an internal standard. Bacterial abundance was detected by their signature in bivariate plots of Side scatter (SSC) vs. FL1 (green fluorescence). Samples were acquired in log mode and run at low speed (for 2 minutes at 12 μl min^{-1}) for bacterial abundance until around 10^5 events. Dilution of the samples was performed for events higher than 800 cells s^{-1}. Data were processed using Cell quest software.

Transparent exopolymer particles (TEP), as biofilms precursors (Bar-Zeev *et al.,* 2012), can affect recirculation systems and tank hygiene (Joyce & Utting, 2015). TEP concentration was determined using the basic alcian blue method (Passow & Alldredge, 1995) with minor modifications after Mazuecos *et al*. (2012). Briefly, water samples (100-250 ml), previously fixed with formaldehyde (1% final concentration), were filtered through 0.4 µm polycarbonate filters. Then, the filters were dyed with 0.5 ml of alcian blue (0.02%) and after 30 seconds filtered again. The filters were soaked in 80% sulphuric acid (5 ml) for 3 h and the solution measured at 787 nm in a spectrophotometer. Stained filters without sample were used as blanks. Alcian blue absorption was calibrated using a solution of xanthan gum (XG) that was homogenized using a tissue grinder and measured by weight. Therefore, TEP concentration was expressed in µg XG eq L^{-1}.

Short-term experiments

To corroborate the results obtained in the time-series, we performed three short-term (3 days) experiments manipulating the presence of holothurians. Each experiment was carried out in seven tanks of 300 liters that contained a floating plastic box with 80 individuals of *A. sulcata* per tank and consisted of two treatments: + *holothurians* (+H) and – *holothurians* (-H). At the initial time, in three of the tanks we included 10 individuals of *H. tubulosa* in each tank. These three tanks are the replicates of the *+holothurians* treatment. The other four tanks only contained the 80 individuals of *A. sulcata* and represent the replicates of the *–holothurians* treatment. The experiment 1 was carried out from 6th to 9th October 2017, the experiment 2 from 27th to 30th October 2017, and the experiment 3 from 3rd to 6th November 2017. During the duration of each experiment the anemones were not fed to control the net effect of holothurian activity and avoid interactions with food supply. At the initial and final time we took samples for nitrate, total phosphorus, bacteria and transparent exopolymer particles. To analyze the samples, we followed the same procedures used in the time-series.

Statistical analyses

To compare the time-series of the study variables in the tank with holothurians vs. the tank without holothurians paired t-test (normally distributed variables) and Wilcoxon matched pairs test (not normally distributed variables) were performed using the software Statistica (V8) and R

3.2.2. These statistical analyses ameliorate the problem of temporal pseudoreplication in this type of studies (Millar & Anderson 2004). In the short-term experiments, to test the statistical significance of the presence of holothurians we performed analysis of variance (ANOVA) comparing the tanks with holothurians (+H treatment) with the tanks without holothurians (-H treatment) using Statistica software (V8).

Results

Time-series of the nutrients and particulate matter in the big-volume tanks

During the study period, in the inlet waters, we found pH values that ranged from 7.71 to 8.31, temperature values from 13.58 to 25.58 ºC, salinity from 35.8 to 41.6 psu, conductivity between 52.28 and 61.96 mS $cm^{-1}$and total dissolved solids from18.26 to 30.84 ppt.

Ammonium concentration in the inlet waters ranged from 0.006 to 0.038 µmol-N l^{-1} with values usually below 0.025 µmol-N l^{-1} (Fig. 3.1a, white circles). However, in the effluent of the *-holothurian* tank we detected punctual higher values during fall 2013 and winter 2014 reaching concentrations up to 0.162 µmol-N l^{-1} (Fig. 3.1a, grey triangles). The ammonium concentration in the effluent of the *+holothurian* tank (Fig.3.1a, red squares) was consistently lower than in effluent of the *-holothurian* tank (Fig.3.1a, grey triangles). No relevant changes in the nitrite concentration between the inlet waters (white circles) and the effluents of both tanks (grey triangles and red squares) were observed (Fig.3.1b). The nitrate concentration was usually lower in the effluent of *+holothurian* tank (Fig. 3.1c, red squares) than in the effluent of *-holothurian* tank (Fig.3.1c, grey triangles) and in the inlet waters (Fig.3.1c withe circles). The nitrate concentration in the inlet waters ranged from 3.45 to 26.91µmol-N l^{-1}. The maximum value of nitrate in the inlet waters was observed in the spring of 2014. Total phosphorus (TP) ranged from 0.039 to 1.157 µmol-P l^{-1} in the inlet waters. In fall and spring, total phosphorus (TP) was higher in the inlet waters (Fig.3.1d, white circles) than in the effluents of both tanks (Fig. 3.1d, red squares and grey triangles).

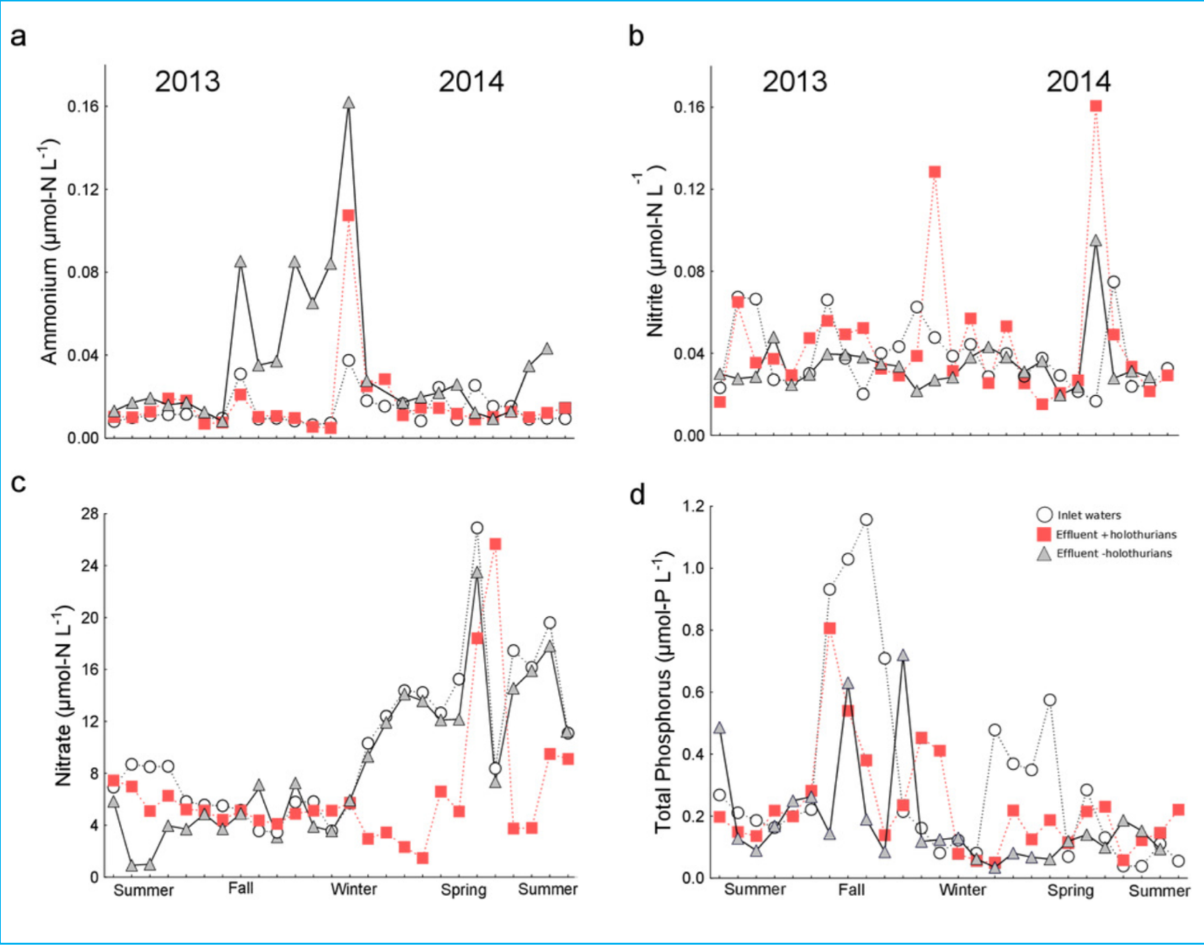


Fig. 3.1. Time-series of nutrient concentration in the big volume tanks. Time series of the concentration of (a) ammonium, (b) nitrite, (c) nitrate, and (d) total phosphorus in the inlet waters (white circles), in the effluent waters of the +*holothurian* tank (red squares) and in the effluent waters of the -*holothurian* tank (grey triangles).

Table 3.1. Results of paired t-tests (for normally distributed variables) and Wilcoxon matched pairs test (for not normally distributed variables) between the inlet waters and the effluents from the + *holothurian* tank and –*holothurian* tank. Bold means statistically significant differences.

	Statistical Analysis	*t or z*	*p*-value
Inlet waters vs. +*holothurian* effluent			
Ammonium	Wilcoxon matched pairs test	0.74	0.4537
Nitrite	Paired t-test	0.58	0.5639
Nitrate	Paired t-test	2.19	0.037
Total phosphorus	Paired t-test	0.64	0.5253
Total organic carbon	Paired t-test	0.03	0.9691
Particulate organic matter	Paired t-test	0.19	0.8443
Transparent exopolymer particles	Paired t-test	1.51	0.1439
Chlorophyll-a	Paired t-test	0.61	0.5455
Bacteria abundance	Paired t-test	1.49	0.1468
Inlet waters vs. – *holothurian* effluent			
Ammonium	Wilcoxon matched pairs test	**3.37**	**0.0008**
Nitrite	Wilcoxon matched pairs test	1.00	0.3130
Nitrate	Paired t-test	**0.60**	**0.0058**
Total phosphorus	Paired t-test	1.93	0.0644
Total organic carbon	Paired t-test	**6.17**	**0.0000**
Particulate organic matter	Paired t-test	**2.05**	**0.0493**
Transparent exopolymer particles	Paired t-test	**5.43**	**0.0000**
Chlorophyll-a	Paired t-test	1.41	0.1737
Bacteria abundance	Paired t-test	**2.97**	**0.0070**

Total organic carbon (TOC) ranged from 0.05 to 0.43 mg C l^{-1} in the inlet waters (Fig. 3.2a, white circles) similar to the values measured in the effluent of +*holothurian* tank from 0.05 to 0.51 mg C l^{-1} (Fig. 3.2a, red squares). However, the values in the effluent of -*holothurian* tank were higher ranging from 0.12 to 0.63 mg C l^{-1} (Fig. 3.2a, grey triangles). The maximum values were reached in all cases during spring of 2014, particularly in the -*holothurian* effluent (Fig.3.2a). In general, the concentration of particulate organic matter (POM) in the effluent of the -*holothurian* tank (Fig.3.2b, grey triangles) was higher than in the inlet waters (Fig.3.2b, white circles) and in the effluent of +*holothurian* tank (Fig.3.2b, red squares). The concentration of transparent exopolymer particles (TEP) ranged from 32 to 270 µg XG eq. l^{-1} in the inlet waters (Fig.3.2c, white circles) and from 22 to 321 µg XG eq. l^{-1} in the effluent of +*holothurian* tank (Fig. 3.2c, red squares) and 35 to 522 µg XG eq. l^{-1} in the effluent of -*holothurian* tank (Fig.3.2c, grey triangles). Chlorophyll-a ranged one order of magnitude in the inlet waters from 0.27 to 2.62 µg l^{-1} (Fig. 3.2d, white circles) and from 0.20 to 2.48 µg l^{-1} in the effluent of +*holothurian* tank (Fig.3.2d, red squares) and from 0.29 to 2.31 µg l^{-1} in the effluent

of *-holothurian* tank (Fig.3.2d, grey triangles). Bacteria abundance ranged from 0.5 to 20.7 $x10^6$ cell ml^{-1} in the inlet waters, (Fig.3.2e, white circles) and from 1.1 to 20.2 $x10^6$ cell ml^{-1} in the effluent of +holothurian tank (Fig.3.2e, red squares) and from 2.3 to 17.6 $x10^6$ cell ml^{-1} in the effluent of *-holothurian* tank (Fig.3.2e, grey triangles).

To assess if the inclusion of holothurians in the tank produces significant changes in the water quality of the tank and its effluent we performed paired t-test or Wilcoxon matched-pairs test (Table 3.1). In Fig.3.3 we pooled all the time-series data in median values, 25-75 % percentiles and the non-outlier values to obtain a more integrated information on the changes in nutrient concentration among, inlet waters, tanks and effluents. We did not obtain significant differences between the inlet and the *+holothurian* tank waters and its corresponding effluent neither for ammonium (Fig.3.3a), nitrite (Fig.3.3b), nitrate (Fig.3.3c), nor TP (Fig.3.3d) (Table 3.1). By contrast, we obtained significant differences between the inlet waters and *-holothurian* effluent waters for ammonium (Fig. 3.3a), nitrate (Fig.3.3c), and TP (Fig.3. 3d) (Table 3.1). It is particularly remarkable the increase of ammonium in the *-holothurian* tank and in its effluent in comparison with the inlet waters and the *+holothurian* tank and its effluent (Fig. 3.3a).

In the Figure.3.4 we pooled data (median, the 25-75% percentile, the non-outliers range, and the outliers) of the organic components such as TOC (Fig. 3.4a), POM (Fig.3.4b), TEP (Fig.3.4c), chlorophyll a (Fig.3.4d), and bacterial abundance (Fig.3.4e). We did not find statistically significant differences between the inlet waters and the *+holothurian* effluent waters for any variables considered in this study (Table 3.1). By contrast, the *-holothurian* effluent waters showed significantly higher concentrations than the inlet waters in TOC (Fig.3.4a), POM (Fig. 3.4b), and TEP (Fig.3.4c) (Table 3.1). It is particularly remarkable the increase of TEP in the *-holothurian* tank and in its effluent waters in comparison with the inlet waters and the *+holothurian* tank and its effluent (Fig.3.4c). We also found significantly higher abundance of bacteria in the *-holothurian* effluent waters than in the inlet waters (Table 3.1, Fig.3.4e).

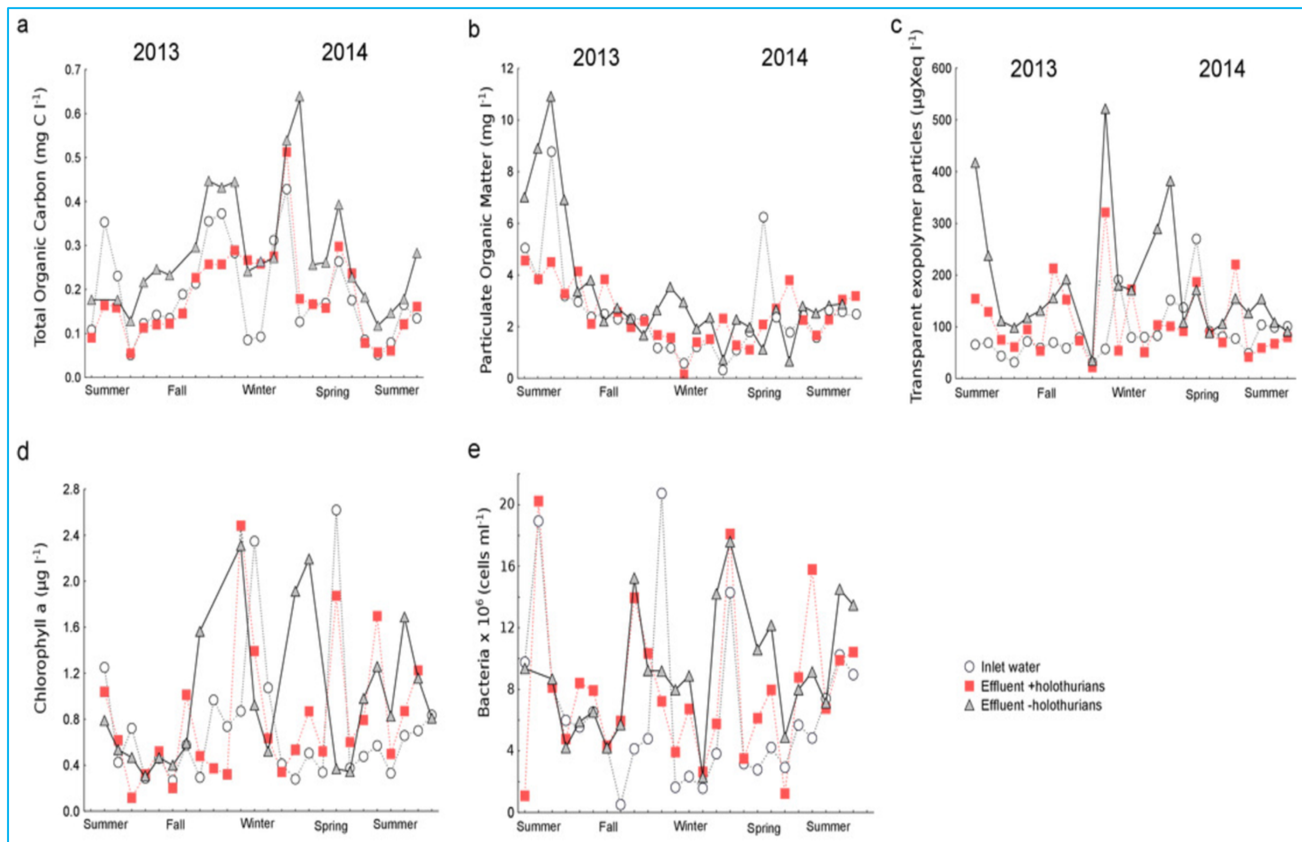


Fig. 3.2. Time-series of particulate organic components in the big tanks. Time series of the concentration (a) total organic carbon, (b) particulate organic matter, (c) transparent exopolymer particles, (d) chlorophyll-*a*, and (e) bacterial abundance in the inlet waters (white circles), in the effluent waters of the +*holothurian* tank (red squares) and in the effluent waters of the -*holothurian* tank (grey triangles).

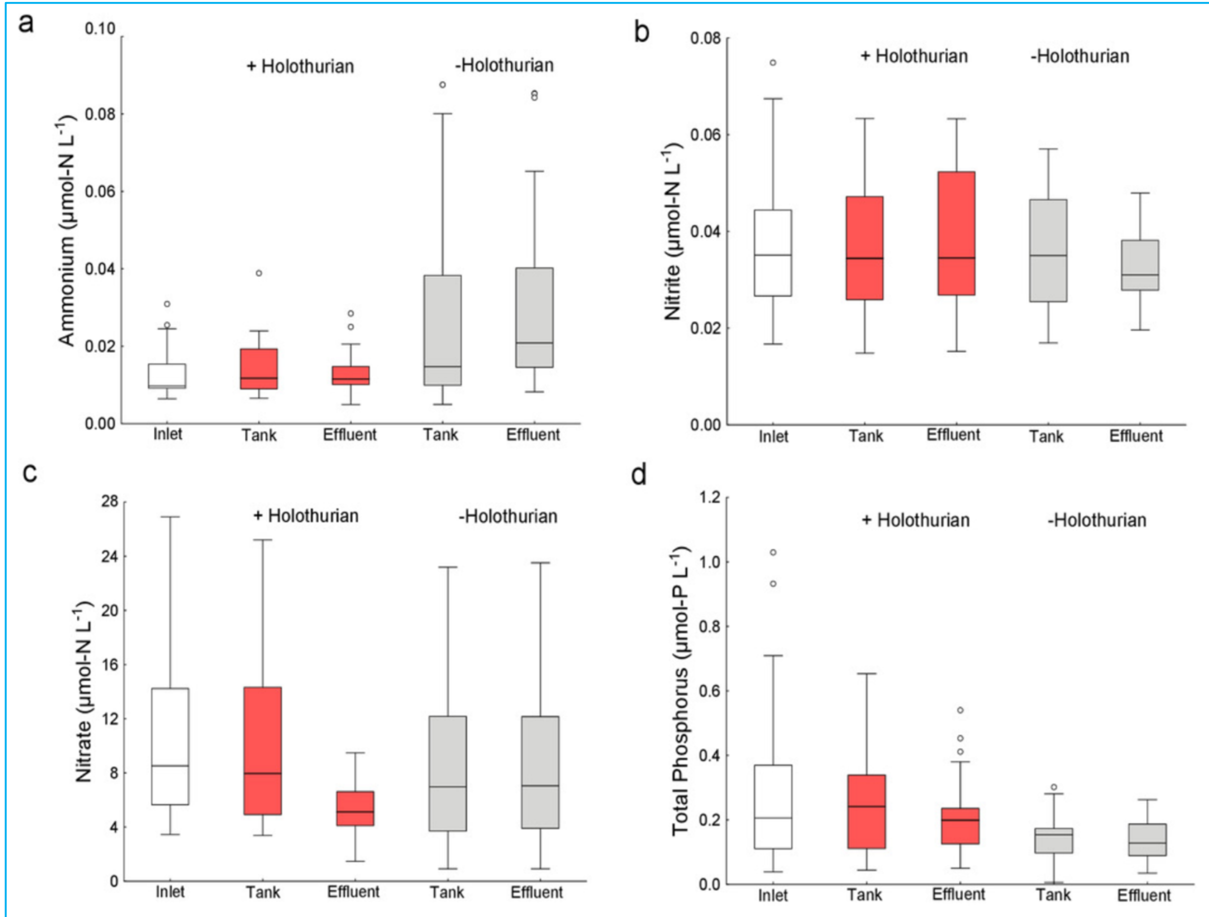


Fig .3.3. Median (line), the 25-75% percentile (box), the non-outliers range (whisker), and outliers (dots) of nutrient concentration in the time-series. Values of (a) ammonium, (b) nitrite, (c) nitrate, and (d) total phosphorous in the inlet waters (white box), in the + *holothurian* tank and effluent (red boxes), and in the - *holothurian* tank and effluent (grey boxes).

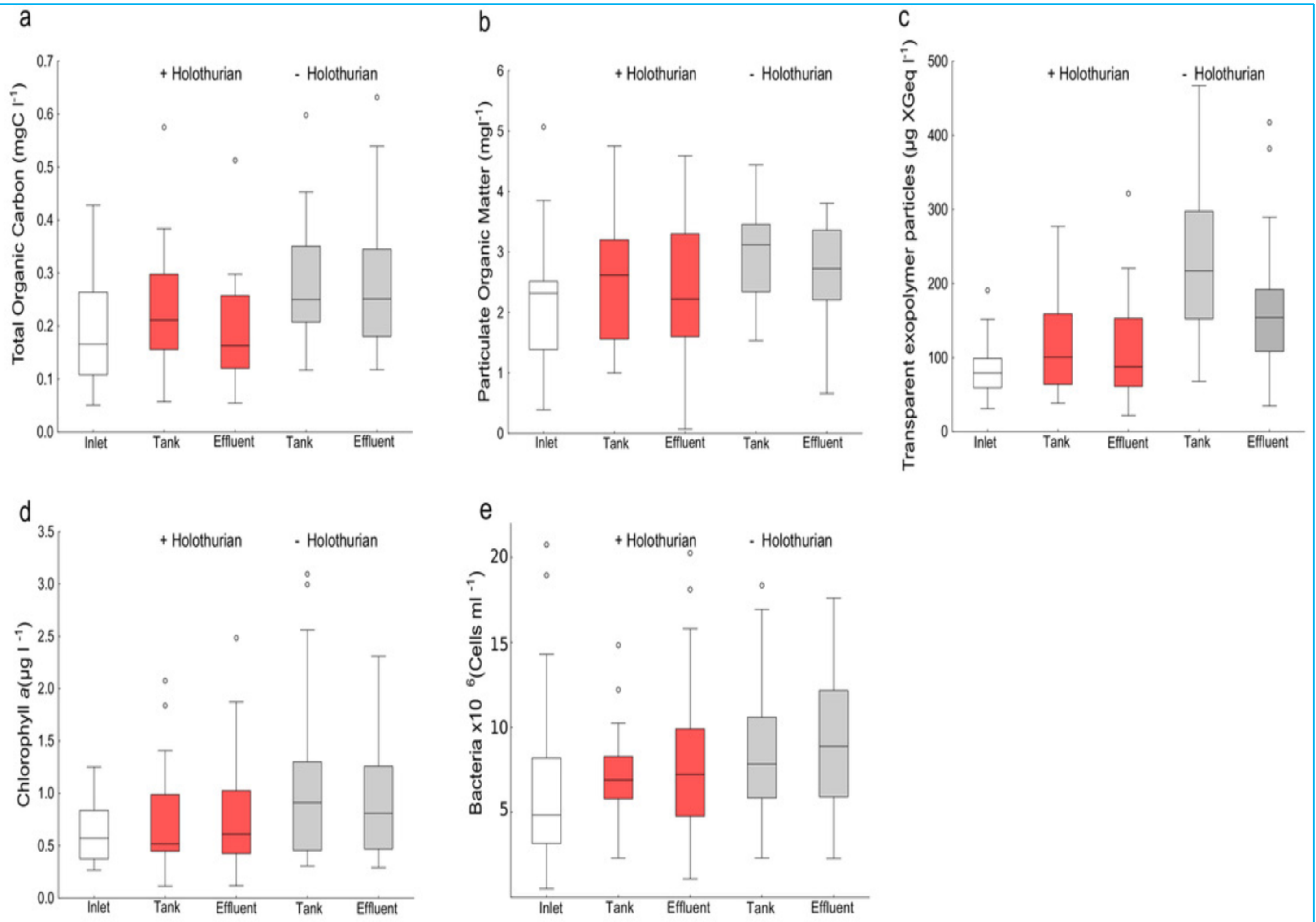


Fig. 3.4. Median (line), the 25-75% percentile (box), the non-outliers range (whisker), and outliers (dots) of particulate organic components in the time-series. Values of the concentration of (a) total organic carbon, (b) particle organic matter, (c) transparent exopolymer particles, (d) chlorophyll-*a* (a), and (e) bacterial abundance in the inlet waters (white box), in the + *holothurian* tank and effluent (red boxes), and in the -*holothurian* tank and effluent (grey boxes).

Short-term experiments

In the experiments, at the initial time, we did not observe significant differences in the concentration of nutrients (nitrate and total phosphorus), TEP nor bacterial abundance between both treatments (+H and –H) indicating that the experiments started with identical conditions (Table 3.2, Fig.3.5 initial time). By contrast, after three days of the inclusion of holothurians, at the final time, we found a statistically significant reduction in the concentration of nitrates (Fig.3.5a), bacterial abundance (Fig.3.5b), and transparent exopolymer particles (Fig.3.5c) in treatment with holothurians (+H) in comparison with the treatment without holothurians (-H) (Table 3.2). This reduction was particularly high for the case of TEP concentration (Fig.3.5c).

	Experiment # 1		Experiment # 2		Experiment # 3	
	F	*p-value*	*F*	*p-value*	*F*	*p-value*
Initial time						
Nitrate	2.71	0.116	3	0.125	384.2	0.0831
Total Phosphorus	0.28	0.604	1.128	0.301	0.227	0.639
Bacteria	3.04	0.097	2.958	0.101	0.550	0.467
TEP	17.84	0.118	8.4	0.134	0.45	0.516
Final time						
Nitrate	**605.93**	**<0.001**	**354.4**	**<0.001**	**174.81**	**<0.001**
Total Phosphorus	1.34	0.261	1.704	0.207	0.770	0.391
Bacteria	**7.06**	**0.015**	**89.366**	**<0.001**	**83.221**	**<0.001**
TEP	**1162.64**	**<0.001**	**5158.59**	**0.00**	**6916.2**	**0.00**

Table 3.2. Results of the analysis of variance (ANOVA) in the three experiments performed to compare the nitrate, total phosphorus, bacterial abundance, transparent exopolymer particles in the treatments with holothurians (+H) vs. the treatments without holothurians (-H) at the initial and the final times. Bold means statistically significant differences.

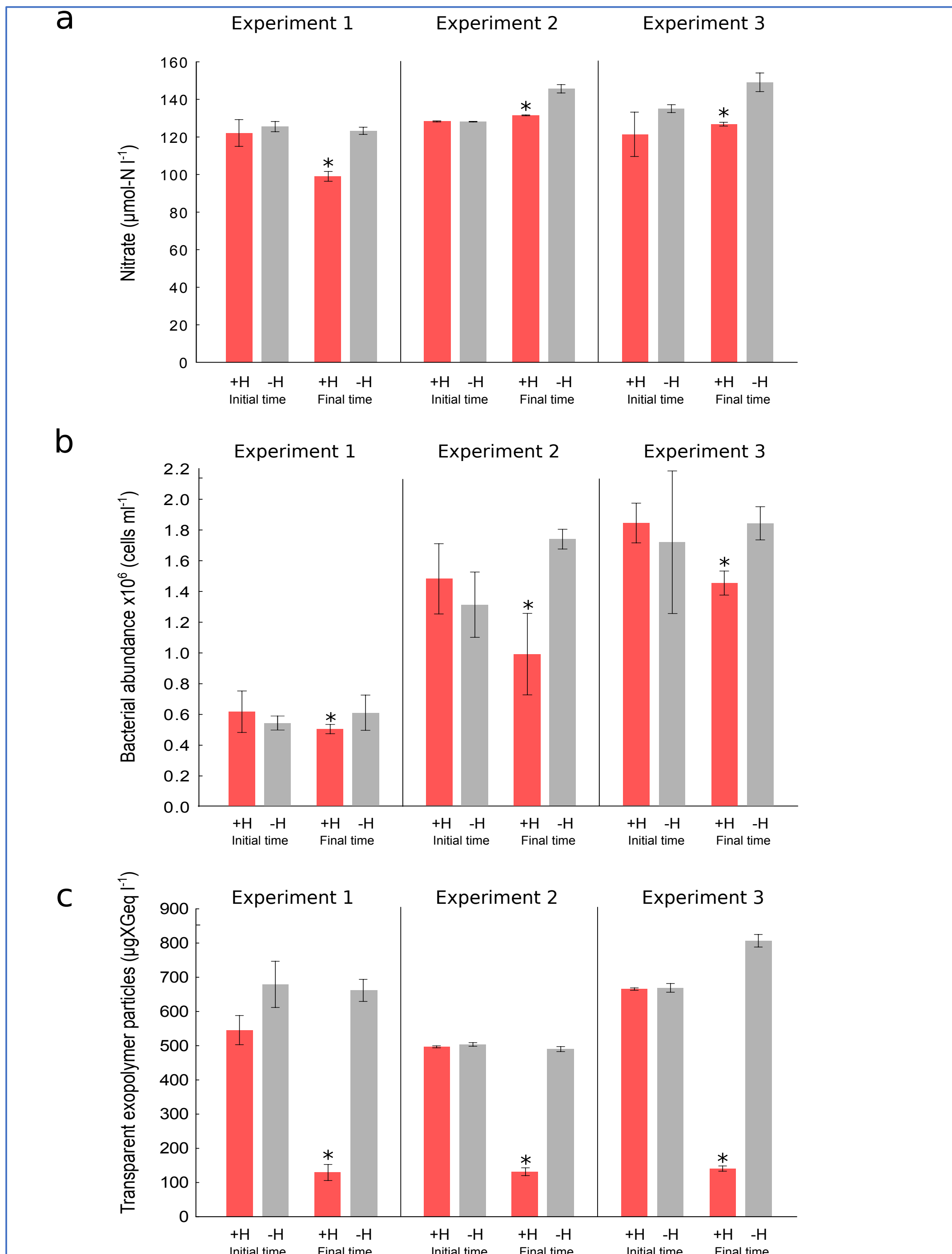


Fig. 3.5. Changes in the concentration of nitrates, bacterial abundance and transparent exopolymer particles in the short-term experiments. Mean (bars) and the standard deviations (whiskers) of the replicates of (a) nitrates, (b) bacterial abundance, and (c) transparent exopolymer particles in the treatments with holothurians (+H) and without holothurians (-H) at the initial and final times. Red bars represent the treatment with holothurians and grey bars represent the treatment without holothurians. Asterisks show the statistically significant differences at the final time of the experiments (more details in Table 3.2).

Discussion

Sea cucumbers are considered highly marketable species both as food and pharmaceutical resource (Silchenko *et al.,* 2005; Farouk, 2007; Gowda *et al.,* 2008; Nelson *et al.,* 2012a,b). Since holothurians are detritus-feeders, they are also used as an extractive species in integrated multitrophic aquaculture (Yokoyama, 2013; Zamora *et al.,* 2016). In fact, here we showed that the inclusion of *Holothuria tubulosa* and *H. forskali* in a tank of sea anemones reduced significantly the concentration of dissolved nitrogen and total organic carbon, particulate organic matter, transparent exopolymer particles, and bacterial abundance. Therefore, this environmental use of holothurians, besides their marketable value, should be also considered in sustainable aquaculture installations.

The effect of holothurians on the concentration of nitrates and ammonium was likely indirect through the interaction bacteria-detritus. We did not observe significant increases of mineral nutrients in the tank with holothurians (Table 3.1, Fig.3.3 red bars), whereas the concentration of ammonium and nitrate was significantly higher in the tank without holothurians in comparison with the tank with holothurians (Table 3.1, Fig.3.3 grey bars). Considering that the concentration of TOC, POM, TEP and the abundance of bacteria were significantly higher in the *–holothurian* tank (Table 3.1, Fig.3.4 grey bars) than in the *+holothurian* tank (Fig.3.4 red bars), we can assume that bacterial mineralization of detritus and organic matter was likely higher in the -*holothurian* tank than in the *+holothurian* tank. This mineralization extra in the –*holothurian* tank can eventually increase the concentration of mineral nutrients such as ammonium and nitrate in the tank. This increment in the concentration of nitrates in the –H treatment was statistically significant in the short-term experiments (Fig.3.5, Table 3.2) indicating that this increase in the mineralization process in the –H treatment can be a plausible explanation.

Unlike on nitrate and ammonium, the effect of holothurian on particulate organic components is direct. Several authors (e.g., Amon & Herndl, 1991; Coulon & Jangoux, 1993) have reported that *H. tubulosa* is a clear deposit feeder with higher concentration of particulate organic matter, total particulate carbohydrates, and bacteria biomass in its foregut than in the surrounding sediment. Most holothurians have a digestive tract specialized for the assimilation of organic matter from sediments (Roberts *et al.,* 2000). Indeed,

they consume sediments particularly enriched in organic matter selecting bacteria, pigments, chlorophyll, and fungi (Paltzat *et al.,* 2008; Navarro *et al.,* 2013; Yokoyama, 2013; 2015), although there are also species with a more limited selective capacity (Slater *et al.,* 2011). This non-selective consumption usually appears in species living in benthic zones on surface sediments with low nutritional quantity (Slater *et al.,* 2011; Zamora & Jeffs, 2011). In our study, we observed a significant decrease in the concentration of TOC, POM, and TEP in the tank that included holothurians in comparison with the tank without holothurians (Fig.3.4, Table 3.1). These results suggest a general consumption of particulate organic matter by *Holothuria tubulosa and H. forskali* reducing the concentration of the pool of particulate organic compounds. This result was particularly significant for the case of transparent exopolymer particles (Table 3.1, Fig.3.4c).

Detritus consumption is not the unique way of sea cucumbers to obtain energy. They can also absorb dissolved organic matter directly (Brothers *et al.,* 2015, Sadeghi-Nassaj *et al.,* 2018), ingest biofilms, and microorganisms (Tamura & Tsuchia 2011; Navarro *et al*. 2013). Biofilms are formed by transparent exopolymer particles (TEP) with strong adhesive forces and they are a key substrate for microbial colonization (Bar-Zeev *et al.,* 2012). Both phytoplankton and bacteria release TEP precursors (Passow, 2002, Ortega-Retuerta *et al.,* 2010, Iuculano *et al.,* 2017). In fact, we observed a significant correlation between TEP and chlorophyll-*a* concentration for inlet waters ($r= 0.73$; $p< 0.05$) and the effluents of both tanks ($r=0.71$ and $r=0.80$, respectively; $P<0.05$). Joyce and Utting (2015) have underlined the dual role of TEP in hatcheries. On the one hand, they can be used as food and, in the other hand; they can attract commensal bacteria and sequester micronutrients and toxins, which can be essential in the hygiene of hatcheries. In our study, we observed a significant reduction in the TEP concentration in the tank containing holothurians both in the time-series and in the experiments (Tables 3.1 and 2, Fig.3.4c and Fig.3.5c), suggesting that TEP were mostly used as a food source by the holothurians. Hence, holothurians could be an excellent regulator of potentially toxic or problematic biofilms. Similarly, Wotton (2005, 2011) observed that the reduction of TEP in the water column is mostly due to marine invertebrate.

The importance of chlorophyll-*a*, as a surrogate of rich organic food, has been highlighted in previous studies (Uthicke &

Karez, 1999; Uthicke, 2001; Hudson *et al.,* 2005). However, we did not find significant differences in the concentration of chlorophyll-a between the tank containing holothurians and the tank without holothurians (Table 3.1, Fig.3.4d). Bacteria are also considered a food source for holothurians (Moriarty, 1982; Moriarty *et al.,* 1985; Amon & Herndl 1991). Unlike chlorophyll-*a*, we found significant differences in the abundance of bacteria between the *–holothurian* effluent and the inlet waters (Table 3.1, Fig.3.4e). The bacterial abundance in the *+holothurian* tank was lower than in the *–holothurian* tank suggesting a net consumption of bacteria by holothurians. In fact, in the short-term experiments, we found statistically significant lower values in the abundance of bacteria in the +H treatment with respect the –H treatment (Table 3.2, Fig. 3.5b). Therefore, holothurians might control bacterial populations in term of days. This bacterial consumption by holothurians could have beneficial consequences to avoid pathogen bacterial outbreaks as for instance *Vibrio* sp., a serious problem in aquaculture (Roux *et al.,* 2015).

Conclusions

Sea cucumbers in aquaculture tanks reduce the load of nitrogen, total organic carbon, particulate organic matter, transparent exopolymer particles and bacteria in tanks and effluents of aquaculture. Therefore, they are a relevant extractive species both for mineral nutrients and particulate organic components. Nitrate and ammonium reduction in aquaculture effluents can be beneficial to reduce eutrophication problems associated with aquaculture installations. It is also particularly remarkable the TEP consumption by holothurian. This process might be relevant in the maintenance of the tank hygiene and in the control of pathogenic bacterial outbreaks. This versatile facet of holothurians confers them a high economical and environmental value in multitrophic or poly-culture aquaculture.

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References

Ajin AM, Silvester R, Alexander D, Nashad M, Abdulla MH. 2016. Characterization of blooming algae and bloom-associated changes in the water quality parameters of traditional pokkali cum prawn fields along the South West coast of India. *Environmental Monitoring and Assessment* **188**:145 DOI 10.1007/s10661-016-5133-6.

Amon RMW, Herndl GJ. 1991. Interrelationship between *Holothuria tubulosa* (*Holothurioida, Echinodermata*) and the sediment microbial community. *Marine Ecology* **12**:163-174.

APHA. 1992. Standard Methods for the Examination of Water and Wastewater. 18th edition. Washington, D.C.: American Public Health Association.

Bar-Zeev E, Berman-Frank I, Girshevitz O, Berman T. 2012. Revised paradigm of aquatic biofilm formation facilitated by microgel transparent exopolymer particles. *Proceedings of the National Academy of Science of the United States of America* **109**: 9119-9124 DOI 10.1073/pnas.1203708109.

Bhatnagar I, Kim SK. 2010. Immense essence of excellence: Marine Microbial Bioactive Compounds. *Marine Drugs* **8**: 2673-2701 DOI 10.3390/md8102673.

Black KD. 2001. Environmental Impacts of Aquaculture. *Aquaculture Research* **28**: 829- 839.

Bortone G. 2009. Integrated anaerobic/aerobic biological treatment for intensives swine production. *Bioresource Technology* **100**: 5424-5430 DOI 10.1016/j.biortech.2008.12.005.

Bostock J, McAndrew B, Richards R, Jauncey K, Telfer T, Lorenzen K, Little D, Ross L, Handisyde N, Gatward I, Corner, R. 2010. Aquaculture: global status and trends. *Philosphical Transactions of the Royal Society* **365**: 2897-2912 DOI 10.1098/rstb.2010.0170.

Boyd CE, Massaut L. 1999. Risks associated with the use of chemicals in pond aquaculture. *Aquacultural Engineering* 20:113-132 DOI 10.1016/S0144-8609(99)00010-2.

Brothers CJ, Lee RW, Nestler JR. 2015. The uptake of dissolved organic material by the sea cucumber *Parastichopus californicus* (Stimpson) and its potential role in visceral regeneration. *Journal of Experimental Marine Biology and Ecology* **469**: 69-75 DOI 10.1016/j.jembe.2015.04.016.

Buhmann AH, Papenbrock J. 2013. Biofiltering of aquaculture effluents by halophytic plants: Basic principles, current uses and future perspectives. *Environmental and Experimental Botany* **92**: 122-133 DOI 10.1016/j.envexpbot.2012.07.005.

Chintiroglou C, Koukouras A. 1992. The feeding habits of three Mediterranean Sea anemone species, *Anemonia viridis* (Forskal), *Actinia equina* (Linnaeus) and *Cereus pedunculatus* (Pennant). *Helgolander Meeresunters* **46**: 53-68 DOI 10.1007/BF02366212.

Chludil HD, Muniain CC, Seldes AM, Maier MS. 2002. Cytotoxic and antifungal triterpene glycosides from the Patagonian Sea cucumber *Hemoiedema spectabilis*. *Journal of Natural Products* **65**: 860-865 DOI:10.1021/np0106236.

Chopin T, Cooper JA, Reid G, Cross S, Moore C. 2012. Open-water integrated multi-trophic aquaculture: environmental biomitigation and economic diversification of fed aquaculture by extractive aquaculture. *Reviews in Aquaculture* **4**: 209-220 DOI 10.1111/j.1753-5131.2012.01074.x.

Coulon P, Jangoux M. 1993. Feeding rate and sediment reworking by the holothuroid *Holothuria tubulosa* (Echinodermata) in a Mediterranean seagrass bed off Ischia Island, Italy. *Marine Ecology Progress Series* **92**: 201-204.

Crab R, Avnimelech Y, Defoirdt T, Bossier P, Verstraete W. 2007. Nitrogen removal techniques in aquaculture for a sustainable production. *Aquaculture* **270**: 1-14 DOI 10.1016/j.aquaculture.2007.05.006.

Da CT, Phuoc LH, Duc HN, Troell M, Berg H . 2015. Use of Wastewater from Striped Catfish (*Pangasianodon hypophthalmus*) Pond Culture for Integrated Rice-Fish vegetable Farming systems in the Mekong Delta, Vietnam. *Agroecology and sustainable food system* **39**: 580-597 DOI:10.1080/21683565.2015.1013241.

Diana JS, Egna HS, Chopin T, Peterson MS, Cao L, Pomeroy R, Verdegem M, Slack WT, Bondad-reantaso MG, Cabello F. 2013. Responsible Aquaculture in 2050: Valuing Local Condition and Human Innovation will be Key to Success. *BioScience* **4**: 255-262 DOI 10.1525/bio.2013.63.4.5.

FAO (Food and Agriculture Organization of the United Nations). 2009. The state of world fisheries and aquaculture 2008. Rome: Food and Agriculture Organization of the United Nations. Available at http://www.fao.org/docrep/011/i0250e/i0250e00.htm.

Farouk A. 2007. New Bacterial Species Isolated from Malaysian Sea Cucumbers with Optimized Secreted Antibacterial Activity. *American Journal of Biochemistry and Biotechnology* **3**:60-65.

Fontenot Q, Bonvillain C, Kilgen M, Boopathy R. 2007. Effects of temperature, salinity, and carbon: nitrogen ratio on sequencing batch reactor treating shrimp aquaculture wastewater. *Bioresource Technology* **98**: 1700-1703 DOI 10.1016/j.biortech.2006.07.031.

Gasol JM, del Giorgio PA. 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Scientia Marina* **64**:197-224 DOI 10.3989/scimar.2000.64n2197.

Gowda NM, Goswami U, Khan MI. 2008. T-antigen binding lectin with antibacterial activity from marine invertebrate, sea cucumber (*Holothuria scabra*): possible involvement in differential recognition of bacteria. *Journal of Invertebrate Pathology* **99**: 141-145 DOI: 10.1016/j.jip.

Halpern BS, Walbridge S, Selkoe KA, Kappel CV, Micheli F, D'Agrosa C, Bruno JF, Casey KS, Ebert C, Fox HE, Fujita R, Heinemann D, Lenihan HS, Madin EMP, Perry MT, Selig ER, Spalding M, Steneck R, Watson R. 2008. A global map of human impact on marine ecosystems. *Science* **319**: 948-952 DOI 10.1126/science.1149345.

Haug, T. Kjuul AK, Styrvold OB, Sandsdalen E, Olsen ØM, Stensvag K. 2002. Antibacterial activity in *Strongylocentrotus droebachiensis* (Echinoidea), *Cucumaria frondosa* (Holothuroidea), and *Asterias rubens* (Asteroidea). *Journal of Invertebrate Pathology* **81**:94-102 DOI 10.1016/S0022-2011(02)00153-2.

Hudson IR, Wigham BD, Solan M, Rosenberg R. 2005. Feeding behaviour of deep-sea dwelling holothurians: Inferences from a laboratory investigation of shallow fjordic species. *Journal of Marine Systems* **57**: 201-218 DOI 10.1016/j.jmarsys.2005.02.004.

Iuculano F, Mazuecos IP, Reche I, Agustí S. 2017. *Prochlorococcus* as a possible source for Transparent Exopolymer Particles (TEP). *Frontiers in Microbiology* **8**:709 DOI 10.3389/fmicb.2017.00709.

Lee H, Shoda M. 2008. Removal of COD and color from livestock wastewater by the Fenton method. *Journal of Hazardous Materials* **153**:1314-1319 DOI 10.1016/j.jhazmat.2007.09.097.

Joyce A, Utting S. 2015. The role of exopolymers in hatcheries: an overlooked factor in hatchery hygiene and feed quality. *Aquaculture* **446**: 122-131 DOI 10.1016/j.aquaculture.2015.04.037.

Kalantzi I, Karakassis I. 2006. Benthic impacts of fish farming: meta-analysis of community and geochemical data. *Marine Pollution Bulletin* **52**: 484-493 DOI 10.1016/j.marpolbul.2005.09.034.

Klinger D, Naylor R. 2012. Searching for solutions in aquaculture: charting a sustainable course. *The Annual Review of Environment and Resources* **37**: 247-276 DOI 10.1146/annurev-environ-021111-161531.

Kumar R, Chaturvedi AK, Shukla PK, Lakshmi V. 2007. Antifungal activity in triterpene glycosides from the sea cucumber *Actinopyga lecanora*. *Bioorganic & Medicinal Chemistry Letters* **17**: 4387-4391 DOI 10.1016/j.bmcl.2006.12.052.

Mazuecos IP, Ortega-Retuerta E, Reche I. 2012. Medida de Partículas Exopoliméricas Transparentes (TEP) en agua marina. In: Moreno-Ostos E, ed. Expedición de circunnavegación Malaspina 2010: Cambio global y exploración de la biodiversidad del océano global. Libro blanco de métodos y técnicas de trabajo oceanográfico. Spain: 515-520.

Millar RB, Anderson MJ. 2004. Remedies for pseudoreplication. *Fisheries Research* **70**: 397-407 DOI 10.1016/j.fishres.2004.08.016.

Moriarty DJW. 1982. Feeding of *Holoturia atra* and *Stichopus chloronotus* on bacteria, organic carbon and organic nitrogen in sediments of the Great Barrier Reef. *Australian Journal of Marine and Freshwater Research* **33**: 255-263 DOI 10.1071/MF9820255.

Moriarty DJW, Pollard PC, Hunt WG, Moriarty CM, Wassenberg TJ. 1985. Productivity of bacteria and microalgae and the effect of grazing by holothurians in sediments on a coral reef flat. *Marine Biology* **85**: 293-300. Available at: https://link.springer.com/article/10.1007(accessed 05 January 1985).

Murphy L, Riley JP. 1962. A modified single solution method for the determination of phosphate in natural waters. *Analytical Chimica Acta* **27**: 31-313 DOI 10.1016/S0003-2670(00)88444-5.

Navarro PG, García-Sanz S, Barrio JM, Tuya F. 2013. Feeding and movement patterns of the sea cucumber *Holothuria sanctori*. *Marine Biology* **160**: 2957-2966 DOI 10.1007/s00227-013-2286-5.

Nelson EJ, MacDonald BA, Robinson SMC. 2012a. The absorption efficiency of the suspension-feeding sea cucumber, *Cucumaria frondosa*, and its potential as an extractive integrated multi-trophic aquaculture (IMTA) species. *Aquaculture* 370-371:19-25 DOI 10.1016/j.aquaculture.2012.09.029.

Nelson EJ, MacDonald BA, Robinson SMC. 2012b. A review of the northern sea cucumber *Cucumaria frondosa* (Junnerus, 1767) as a potential Aquaculture Species. *Reviews in Fisheries Science* **20**: 212-219 DOI 10.1080/10641262.2012.719043.

Oladoja NA, Adelagun ROA, Ahmad AL, Ololade I. 2015. Phosphorus recovery from aquaculture wastewater using thermally treated gastropod shell. *Process Safety and Environmental Protection* **98**: 296-308 DOI 10.1016/j.psep.2015.09.006.

Ortega-Retuerta E, Duarte C M, Reche I .2010. Significance of Bacterial Activity for the distribution and dynamics transparent exopolymer particles in the Mediterranean Sea. *Microbial Ecology* **59**: 808-818 DOI 10.1007/s00248-010-9640-7.

Paltzat DL, Pearce CM, Barnes PA, McKinley RS. 2008. Growth and production of California sea cucumbers co-cultured with suspended Pacific oysters. *Aquaculture* **275**:124-137 DOI 10.1016/j.aquaculture.2007.12.014.

Passow U, Alldredge AL. 1995. A dye-binding assay for the spectrophotometric measurement of transparent exopolymer particles (TEP). *Limnology and Oceanography* **40**:1326-1335.

Passow U. 2002. Production of transparent exopolymer particles (TEP) by phyto- and bacterioplankton. Marine Ecology Progress Series 236: 1-12.

Purcell SW, Mercier A, Conand C, Hamel J-F, Toral-Granda MV, Lovatelli A, Uthicke S. 2013. Sea cucumber fisheries: global analysis of stocks, management measures and drivers of overfishing. *Fish and Fisheries* **14**: 34-59 DOI 10.1111/j.1467-2979.2011.00443.x.

Read P, Fernandes T. 2003. Management of environmental impacts of marine aquaculture in Europe. *Aquaculture* **226**:139-163 DOI 10.1016/S0044-8486(03)00474-5.

Roberts D, Gebruk A, Levin V, Manship BAD. 2000. Feeding and digestive strategies in deposit feeding holothurians. In: Barnes H, Gibson NH, Barnes M, eds. *Oceanography and Marine Biology*. London: Taylor and Francis, 257–310.

Roux FL, Wegner KM, Baker-Austin C, Vezzulli L , Osorio CR , Amaro C , Ritchie JM, Defoirdt T, Destoumieux-Garzón D, Blokesch M, Mazel D, Jacq A, Cava F, Gram L, Wendling CC, Strauch E, Kirschner A, Huehn S. 2015. The emergence of Vibrio pathogens in Europe: ecology, evolution, and pathogenesis (Paris, 11-12th March 2015). *Frontiers in Microbiology* **6**:830 DOI:10.3389/fmicb.2015.00830.

Sadeghi-Nassaj SM, Catalá TS, Álvarez PA, Reche I. 2018. Sea cucumbers reduce chromophoric dissolved organic matter in aquaculture tanks. *PeerJ* **6:** e4344 DOI 10.7717/peerj.4344.

Silchenko AS, Stonik VA, Avilov SA, Kalinin VI, Kalinovsky AI, Zaharenko AM, Smirnov AV, Mollo E, Cimino G. 2005. Holothurins B2, B3, and B4, new triterpene glycosides from Mediterranean Sea cucumbers of the genus *Holothuria*. *Journal of Natural Products* **68**: 564-567 DOI 10.1021/np04963.

Slater MJ, Jeffs AG, Sewell MA. 2011. Organically selective movement and deposit-feeding in juvenile sea cucumber, *Australostichopus mollis* determined in situ and in the laboratory. *Journal of Experimental Marine Biology and Ecology* **409**:315-323 DOI 10.1016/j.jembe.2011.09.010.

Slater MJ, Carton AJ. 2009. Effect of sea cucumber (*Australostichopus mollis*) grazing on coastal sediments impacted by mussel farm deposition. *Marine Pollution Bulletin* **58**:1123-1129 DOI 10.1016/j.marpolbul.2009.04.008.

Tamura Y, Tsuchiya M. 2011. Floating mucus aggregates derived from benthic microorganisms on rocky intertidal reefs: Potential as food sources for benthic animals. *Estuarine, Coastal and Shelf Science* **94**: 199-209 DOI 10.1016/j.ecss.2011.06.012.

Uthicke S. 2001. Interactions between sediment-feeders and micro- algae on coral reefs: grazing losses versus production enhancement. *Marine Ecology Progress Series* **210**: 125-138.

Uthicke S, Karez R.1999. Sediment patch selectivity in tropical sea cucumbers analyzed with multiple choice experiments. *Journal of Experimental Marine Biology and Ecology* **236**:69-87 DOI 10.1016/S0022-0981(98)00190-7.

Valliappan K, Sun W, Li Z. 2014. Marine actinobacteria associated with marine organisms and their potentials in producing pharmaceutical natural products. *Applied Microbiology and Biotechnology* **98**:7365–7377 DOI 10.1007/s00253-014-5954-6.

Van-Praët M. 1985. Nutrition of sea anemones. *Advances in Marine Biology* **22**: 65-99 DOI 10.1016/S0065-2881(08)60050-4.

Wotton RS. 2005. The essential role of exopolymers (EPS) in aquatic systems. In: Barnes H, Gibson RN, Atkinson RJA, Gordon JDM, eds. *Oceanography and Marine Biology*. New York (NY): Taylor and Francis Group (CRC), 57–9.

Wotton RS. 2011. EPS (Extracellular Polymeric Substances), silk, and chitin: vitally important exudates in aquatic ecosystems. *Journal of the North American Benthological Society* **30**: 762-769 DOI 10.1899/10-120.1.

Yokoyama H. 2002. Impact of fish and pearl farming on the benthic environment in Gokash bay; evaluation from seasonal fluctuation of the macrobenthos. *Fisheries Science* **68**:258-268.

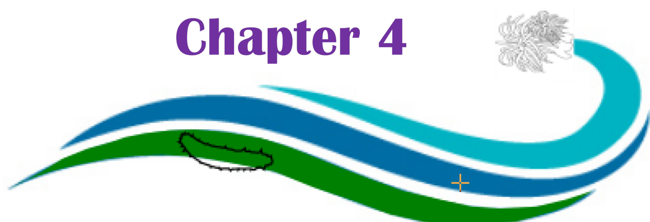
Yokoyama H. 2013. Growth and food source of the sea cucumber *Apostichopus japonicus* cultured below fish cages. Potential for integrated multitrophic aquaculture. *Aquaculture* **372-375**:28–38 DOI 10.1016/j.aquaculture.2012.10.022.

Yokoyama H. 2015. Suspended culture of the sea cucumber *Apostichopus japonicus* below a Pacific oyster raft-potential for integrated multi-trophic aquaculture. *Aquaculture Research* **46**:825-832 DOI 10.1111/are.12234.

Zamora LN, Jeffs A. 2011. Feeding, selection, digestion and absorption of the organic matter from mussel waste by juveniles of the deposit-feeding sea cucumber, *Australostichopu smollis*. *Aquaculture* **317**: 223-228 DOI 10.1016/j.aquaculture.2011.04.011.

Zamora LN, Yuan X, Carton AG, Slater MJ. 2016. Role of deposit-feeding sea cucumbers in integrated multitrophic aquaculture: progress, problems, potential and future challenges. *Reviews in Aquaculture* DOI:10.1111/raq.12147.

Zhang Q, Achal V, Xu Y, Xiang W-N. 2104. Aquaculture Wastewater quality improvement by water Spinach (*Ipomoea aquatica* Forskall) floating bed and ecological benefit assessment in ecological agriculture district. *Aquacultural Engineering* **60**: 48-55 DOI 10.1016/j.aquaeng.2014.04.002.



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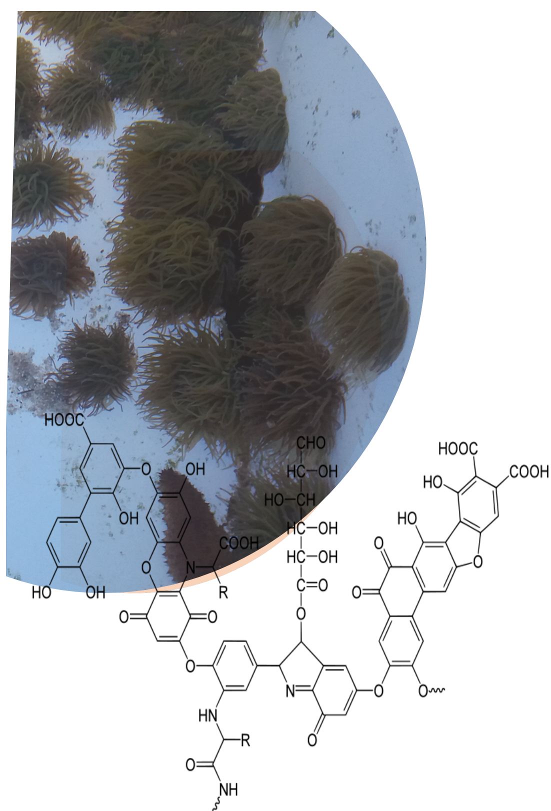

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Sea cucumbers reduce chromophoric dissolved organic matter in aquaculture tanks

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Abstract:

Background. Mono-specific aquaculture effluents contain high concentrations of nutrients and organic matter, which affect negatively the water quality of the recipient ecosystems. A fundamental feature of water quality is its transparency. The fraction of dissolved organic matter that absorbs light is named chromophoric dissolved organic matter (CDOM). A sustainable alternative to mono-specific aquaculture is the multitrophic aquaculture that includes species trophically complementary named "extractive" species that uptake the waste byproducts. Sea cucumbers are recognized as efficient extractive species due to the consumption of particulate organic matter (POM). However, the effects of sea cucumbers on CDOM are still unknown.

Methods. During more than one year, we monitored CDOM in two big-volume tanks with different trophic structure. One of the tanks (–holothurian) only contained around 810 individuals of *Anemonia sulcata*, whereas the other tank (+ holothurian) also included 90 individuals of *Holothuria tubulosa* and *H. forskali*. We routinely analyzed CDOM absorption spectra and determined quantitative (absorption coefficients at 325 nm) and qualitative (spectral slopes) optical parameters in the inlet waters, within the tanks, and in their corresponding effluents. To confirm the time-series results, we also performed three experiments. Each experiment consisted of two treatments: + holothurians (+H) and –holothurians (-H). We set up three + H tanks with 80 individuals of *A. sulcata* and 10 individuals of *H. tubulosa* in each tank and four –H tanks that contained only 80 individuals of *A. sulcata*.

Results. In the time-series, absorption coefficients at 325 nm (a_{325}) and spectral slopes from 275 to 295 nm ($S_{275-295}$) were significantly lower in the effluent of the +holothurian tank (average: 0.33 and 16 μm^{-1}, respectively) than in the effluent of the –holothurian tank (average: 0.69 m^{-1} and 34 μm^{-1}, respectively), the former being similar to those found in the inlet waters (average: 0.32 m^{-1} and 22 μm^{-1}, respectively). This reduction in the absorption of the dissolved organic matter appears to be mediated by the POM consumption by holothurians. The experiments confirmed the results observed in the time-series. The a_{325} and $S_{275-295}$ values were significantly lower in the treatment with holothurians than in the treatment without holothurians indicating a reduction in the concentration of chromophoric organic compounds, particularly of low molecular weight. **Discussion.** Consequently, sea cucumbers appear to improve water transparency in aquaculture tanks. The underlying mechanism of this improvement might be related to the POM consumption by holothurians; which reduces the concentration of CDOM derived from POM disaggregation or to the direct assimilation of dissolved compounds of low molecular weight as chromophoric amino acids.

Introduction

The exponential growth of human population has boosted the global demand of fish and seafood (FAO, 2009). Nevertheless, the extractive fisheries are more and more reduced and the aquaculture is gaining importance accounting for more than 40% of human consumption of fish and seafood (Bostock *et al*., 2010). Mono-specific aquaculture produces wastewater that usually contains high concentrations of organic matter as well as inorganic nutrients, antibiotics and uneaten food pellets (Read & Fernandes, 2003; Klinger & Naylor, 2012). At ecosystem level, the effluents of mineral nutrients associated with the aquaculture activity can produce problems of eutrophication (Ajin *et al*., 2016; Ruiz-Zarzuela *et al*., 2009). On the other hand, the loads of dissolved and particulate organic matter with the effluents can reduce water transparency due to an increase in light backscattering and absorption (Ibarra *et al*., 2012; Del Bel Belluz *et al*., 2016). Therefore, a sustainable aquaculture with effluents of low environmental impact is a global challenge for both scientists and food producers. The polyculture and the integrated multitrophic aquaculture (IMTA) is an alternative practice to alleviate the handicaps of the traditional, mono-specific aquaculture (Diana *et al*., 2013). Unlike mono-specific aquaculture, polyculture and IMTA uses trophically complementary "extractive" species that consume the excretion products, fecal and food wastes of the primary species reducing these loads in the effluents (Chopin *et al*., 2012). Hence, it is desirable that the future expansion of aquaculture promotes this practice to reduce the inputs of organic matter in the environment, at the same time that aquaculture farmers can obtain an economical value from the co-cultured species.

The chromophoric dissolved organic matter (CDOM) is the fraction of the dissolved organic matter (DOM) that absorbs light in the ultraviolet (UV) and, to a lesser extent, in the visible range of the spectrum. Therefore, CDOM is largely responsible for UV and blue light attenuation in marine ecosystems (Bricaud *et al*., 1981; Nelson & Siegel, 2013). Since CDOM absorption overlaps one of the chlorophyll *a* absorption peaks, CDOM can diminish the potential for primary productivity. This fact also affects the algorithms used in remote sensing to determine ocean color and infer primary productivity (Carder *et al*., 1989; Siegel *et al*., 2005; Ortega-Retuerta *et al*., 2010). Remote sensing has been suggested as an excellent tool to monitor at large scale the

impact of offshore aquaculture (Populus *et al*., 1995; Rajitha *et al*., 2007; Saitoh *et al*., 2011). However, the relation between aquaculture waste and CDOM has been scarcely explored (Ibarra *et al.,* 2012; Nimptsch *et al.,* 2015; Del Bel Belluz *et al*., 2016).

Sea cucumbers are highly demanded food for human consumption in some countries (Purcell *et al*., 2013), but they are also important extractive species with a high capacity to consume waste particulate organic matter in sediment deposits (Nelson *et al*., 2012a,b; Yokoyama 2013, 2015). Despite the effects of sea cucumbers on different components of the particulate organic matter has been studied, particularly in open waters under fish cages or mollusk rafts (Slater & Carton, 2009; Slater *et al.,* 2009; Nelson *et al*., 2012a; Zamora & Jeff, 2011; Yokoyama, 2013, 2015; Zhang *et al*., 2014), their influence on the optical properties of the dissolved organic matter still remains unexplored (Zamora *et al*., 2016). The influence of sea cucumbers on the optical properties of the organic matter can be relevant in land-based installations submitted to long-term water recirculation.

In this study, we evaluate the effects of sea cucumbers (*Holothuria tubulosa* and *H. forskali*) on optical properties of the dissolved organic matter in aquaculture tanks with *Anemonia sulcata* as primary species. *A. sulcata* is a very palatable species, highly demanded for catering in Spain with also a great pharmacological interest. During one year, we monitored the changes in DOM optical properties in a big-volume (50,000 liters) tank with holothurians and in another similar tank without them, exploring the main factors that determine CDOM changes. Subsequently, to corroborate the observations obtained in the time-series of the big-volume tanks we performed three short-term experiments manipulating the presence of holothurians in small tanks (300 liters). We observed, both in the time-series of the big tanks and in the short-term experiments, that the presence of holothurians reduced significantly the absorption due to dissolved organic matter increasing, consequently, the water transparency in comparison with the tanks without holothurians. Therefore, holothurians appear to have a high environmental value to improve the water quality in aquaculture installations.

Material and Methods

Time-series in the big-volume tanks

We monitored during more than one year two aquaculture tanks located at the iMare Natural S.L. facilities (http://www.imarenatural.com) in Motril, Spain (36°44′38″ N, 3°35′59″ W). Each

tank of 50,000 liters of capacity was connected directly with the coastal water by one inlet pipe (inlet water) and the water from each tank was released by one outlet pipe located in the bottom of the tank (effluent). The seawater was pumped into the tanks at a continuous flow of 1,200 l h^{-1}. Therefore, water residence time in the tanks was ca. 42 hours and the total annual effluents accounted for 10,512 m^3. In one of the tanks, 811 ± 125 individuals of the primary species, the sea anemone *Anemonia sulcata*, and 93 ± 3 adults of sea cucumbers *Holothuria tubulosa* (≈ 80 %) and *H. forskali* (≈ 20 %) were included (hereafter designated as + *holothurian* tank). In the other tank only 690 ± 87 individuals of the primary specie were included (hereafter designated as – *holothurian* tank). Sea anemones were placed on floating plastic boxes in the surface of the tanks and holothurians were free in the bottom and walls of the tanks. Sea anemones were fed with about 900-1800 g of fresh chopped fish, mainly *Scomber scombrus* (Chintiroglou & Koukouras, 1992) twice per week.

Water samples for different chemical and biological analysis were taken biweekly from July 17th 2013 to August 20th 2014. Each sampling day, we took water samples from the inlet pipe, the center of the two tanks using a telescopic stick with a plastic beaker located in its extreme and from their corresponding effluents. To avoid that light can affect absorption measurements; we immediately took the CDOM samples in pre-combusted (4h at 500 °C), acid-cleaned, amber glass bottles. They were kept in ice during transportation to the laboratory (about one hour from the tanks). Water samples were filtered through pre-combusted Whatman GF/F glass fiber filters of 0.7μm nominal pore size and the <0.7 μm fraction was used for the optical characterization of chromophoric dissolved organic matter.

Chromophoric Dissolved Organic Matter (CDOM)

Absorption spectra of dissolved organic matter provide information on CDOM concentration and other qualitative properties. Absorption coefficients at specific wavelengths (e.g., 325 nm and 443 nm) are used as proxies of CDOM concentration, and the spectral slopes and spectral ratios, which are largely independent of the concentration, are surrogates of CDOM origin, molecular weight and chemical structure (Weishaar *et al*., 2003; Twardowski *et al*., 2004; Helms *et al*., 2008; Nelson & Siegel, 2013; Martínez-Pérez *et al*., 2017).

CDOM absorbance spectra were recorded at wavelengths from 200 nm to

750 nm at 1-nm interval using an UV/VIS Perkin Elmer spectrometer with a 10 cm-quartz cuvette. The spectrophotometer was connected to a computer with Lambda 25 software. The detection limit of the spectrophotometer (0.001Absorbance) corresponds to a CDOM absorption coefficient detection limit of 0.02 m^{-1}. Spectrum corrections due to residual scattering by fine size particle fractions, micro-air bubbles, or colloidal material present in the sample were performed by subtracting the average of the absorption between 600 and 700 nm (Green & Blough, 1994).

CDOM absorption coefficients, a_λ, were calculated using the next equation 1:

$$a_\lambda = 2.303 \frac{Absorbance\ (\lambda) - Absorbance\ (600-700)}{l}$$

Where a_λ is the absorption coefficients in m^{-1} at each λ wavelength, *Absorbance (λ)* is the absorbance at wavelength λ, *Absorbance (600-700)* is the average absorbance from 600 to 700 nm, 2.303 is the factor that converts decadic to natural logarithms, *l* is the cuvette path length in m^{-1}.

Spectral slopes describe the shape decay of absorption coefficients vs. wavelengths. Slopes were calculated from the linear regression of log-transformed absorption coefficients in the wavelength bands 275-295 nm ($S_{275-295}$) and 350-400 nm ($S_{350-400}$) (Helms *et al*., 2008). The spectral slopes for both wavelength ranges were calculated as in equation 2.

$$a_\lambda = a_{\lambda ref}\ e^{-S(\lambda - \lambda_{ref})}$$

Where λ is the selected wavelength in nm, a_λ is the absorption coefficient at λ wavelength in m^{-1}, $a_{\lambda ref}$ is the absorption coefficient at a reference wavelength λ_{ref}, and *S* is the spectral slope. The spectral slope ratio (S_R) was calculated as the ratio of the spectral slope from 275 nm to 295 nm ($S_{275-295}$) to the spectral slope from 350 nm to 400 nm (Helms *et al*., 2008).

CDOM absorption usually decreases exponentially as wavelengths increase. Therefore, the shorter the wavelength, the more sensitive to changes is (Fig.4.1). Despite a low sensitivity (Fig.4.1), the visible wavelength at 443 nm is used in remote sensing studies due to its correspondence with the satellite sensor (Siegel *et al*., 2005; Ortega-Retuerta *et al*., 2010). Therefore, the spectral slope from 275 nm to 295 nm is the most sensitive optical parameter of CDOM changes (Helms *et al*., 2008), but this parameter is not quantitative. CDOM quantity was measured as absorption coefficient at 325 nm (a_{325}), since this wavelength is the most common in the literature (Nelson & Siegel

2013; Catalá *et al*., 2015) and has a higher sensitivity than 443 nm (Fig.4.1).

Basic parameters as temperature (°C), pH, salinity (psu), total dissolved solids (TDS), and conductivity (mScm^{-1}) were measured in the tanks using a multi-parameter HANNA probe (HI9828 model). Total organic carbon (TOC) concentration was measured as non-purgeable organic carbon after a high-temperature catalytic oxidation using a Shimadzu TOC-V CSN. Samples, by triplicate, were acidified with hydrochloric acid and purged for 20 min to eliminate the remaining dissolved inorganic carbon. Three to five injections were analyzed for each sample. Standardization of the instrument was done with potassium hydrogen phthalate. Particulate organic matter (POM) was obtained filtering between 1.5 and 2.0 of water through pre-weighed and pre-combusted (500°C for 4 h) Whatman GF/F glass fiber filters. The filters containing all the solids were dried at 60°C for >24 h and reweighed to determine the total mass (mineral + organic matter). Then, the organic fraction was burned by combusting the filters at 500°C for 6 h; finally, the filters were reweighed again to determine the mineral residue. POM was obtained after the subtraction of the mineral residue to the total mass. The concentration of chlorophyll-*a* was determined spectrophotometrically after pigment extraction with methanol (APHA, 1992). Bacteria abundance was determined in triplicate using flow cytometry (Gasol & del Giorgio, 2000) with a FACScalibur Becton Dickinson cytometer equipped with a laser emitting at 488 nm. Data were processed using Cell quest software.

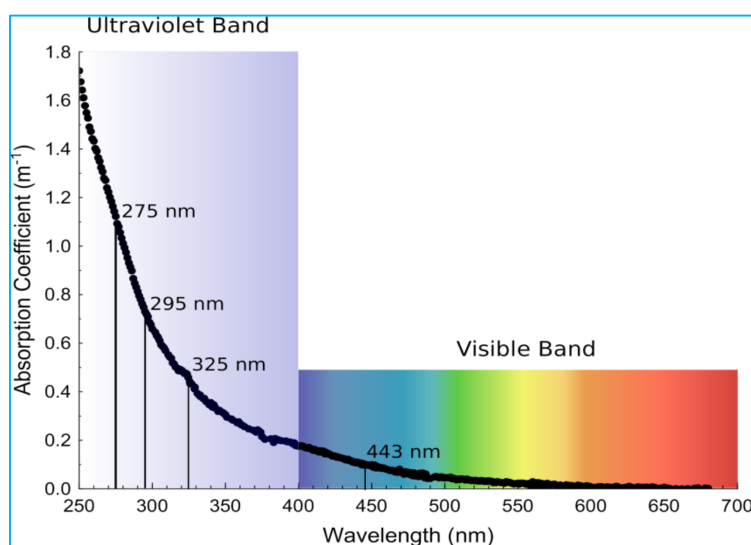

Fig.4.1. Exponential decay of absorption coefficients as wavelengths increase from the ultraviolet to the visible band. Example of a decay curve of the chromophoric dissolved organic matter in a sample taken on January 30th of 2014 from the big-volume tank that contains holothurians. The wavelengths selected for the calculation of the optical parameters used in this study are marked on the decay curve.

Short-term experiments

To test the statistical significance of the results obtained in the time-series, we performed three short-term (3 days) experiments. Each experiment was carried out in seven tanks of 300 liters that contained a floating plastic box with 80 individuals of *A. sulcata* per tank and consisted of two treatments: + *holothurians* (+H) and – *holothurians* (-H). At the initial time, in three of the tanks we included 10 individuals of *H. tubulosa* in each tank(Fig.2.3a). These three tanks are the replicates of the +*holothurians* treatment. The other four tanks only contained the 80 individuals of *A. sulcata* and represent the replicates of the –*holothurians* treatment. The experiment 1 was carried out from 6th to 9th October 2017, the experiment 2 from 27th to 30th October 2017, and the experiment 3 from 3rd to 6th November 2017. During the duration of each experiment the anemones were not fed to control the net effect of holothurian activity. At the initial and final time, we took samples for the optical characterization of dissolved organic matter. To analyze the samples, we followed the same procedures used in the time-series.

Statistical analysis

To compare the time-series of the CDOM optical parameters in the inlet water with the effluents from the tank with holothurians and the tank without holothurians we performed paired t-test (for normally distributed variables) and Wilcoxon matched pairs test (for not-normally distributed variables) using the Statistica software (V8). These statistical analyses ameliorate the problem of temporal pseudoreplication in this type of studies (Millar & Anderson, 2004). Correlations between CDOM optical parameters and potential controlling factors were performed using Statistica software (V8). In the short-term experiments to test the statistical significance of the presence of holothurians on the CDOM optical parameters we performed analysis of variance (ANOVA) comparing the tanks with holothurians (+H) with the tanks without holothurians (-H) using Statistica software (V8).

Results and discussion

During the study period, the pH in the inlet waters ranged from 7.71 to 8.31, the temperature from 13.58 ºC to 25.58 ºC, the salinity from 35.8 to 41.6 psu, the conductivity between 52.28 and 61.96 mS cm^{-1} and total dissolved solids from 18.26 to 30.84 ppt. These basic parameters were similar in the tanks and in their corresponding effluents (See Appendix II, supplementary Tables 1-6).

Time-series of the optical parameters in the big-volume tanks

In the inlet waters, the a_{325} values ranged from 0.06 to 0.83 m^{-1} (See Appendix II, supplementary Tables 1) and in the effluents of +*holothurian* and–*holothurian* tanks from 0.06 to 0.79 m^{-1} and from 0.37 to 1.27 m^{-1}, respectively (See Appendix II, supplementary Tables 1 and 2, respectively). The absorption coefficients of the inlet waters (i.e. coastal waters of Western Mediterranean Sea) were similar to those ones found in other coastal waters (Catalá *et al.,* 2013; Nima *et al*., 2016) or in the open Mediterranean Sea (Bracchini *et al*., 2010; Organelli *et al*., 2014). Systematically, throughout the time-series, the effluent of the–*holothurian* tank showed higher a_{325} values (grey triangles in Fig.4.2a) than the effluent of the + *holothurian* tank (red squares in Fig. 4.2a). These last values were similar to the a_{325} values in the inlet waters (white circles). Spectral slopes and spectral slope ratios are qualitative parameters, which are independent of the CDOM concentration. The higher the spectral slope, the smaller the DOM molecular weight is (Helms *et al*., 2008). The slope in the band from 275 nm to 295 nm ($S_{275-295}$) is an optical parameter particularly sensitive to environmental changes as solar radiation or salinity (Helms *et al.,* 2008; 2013; Catalá *et al.,* 2013). In the inlet water, the values of $S_{275-295}$ ranged from 10 to 38 μm^{-1} (See Appendix II, supplementary Tables 1) and in the effluents of +*holothurian* and –*holothurian* tanks from 6 to 28 μm^{-1} and from 13 to 40 μm^{-1}, respectively (See Appendix II, supplementary Tables 1 and 2, respectively). In the inlet water, the values were similar to those reported for coastal and estuary waters, usually characterized with lower slopes (~ 15-25 μm^{-1}) than the values for the open ocean (~ 25-50 μm^{-1}) (Helms *et al*., 2008; 2013; Catalá *et al*., 2015). Like the a_{325} values, the $S_{275-295}$ values in the effluent waters of the –*holothurian* tank (grey triangles in Fig.4.2b) showed consistently higher values than the inlet water (white circles in Fig.4.2b) and in the effluents of the +*holothurian* tank (red squares in Fig.4.2b). In the inlet waters, the

spectral slope ratios (S_R) ranged from 0.6 to 2.6 (See Appendix II, supplementary Tables 1) and in the effluents of +*holothurian* and –*holothurian* tanks from 0.5 to 3.1 and from 0.4 to 3.9, respectively (See Appendix II, supplementary Tables 2 and 3 , respectively). The S_R values in –*holothurian* effluents (grey triangles in Fig.4.2c) showed consistently higher values than the inlet water (white circles in Fig.4.2c) and +*holothurian* effluent (red squares in Fig.4.2c).

Table 4.1. Results of paired t -test and Wilcoxon matched pairs test between the inlet water and the effluent of the tank with holothurians and the effluent of the tank without holothurians. Results of paired t -test (for normally distributed variables) and Wilcoxon matched pairs test (for not normally distributed variables) between the inlet water and the effluent of the tank with holothurians (+holothurians) and the effluent of the tank without holothurians (holothurian) for the CDOM optical properties considered in this study. Bold means that there is a significant difference.

	Statistical analysis	***t* or *z***	***p*-value**
Inlet water vs. +*holothurian* effluent			
Absorption coefficients (a_{325})	Paired t-test	19.67	**0.0000**
Spectral slope ($S_{275-295}$)	Paired t-test	4.86	**0.0000**
Spectral slope ratio (S_R)	Wilcoxon	2.35	**0.0188**
Inlet water vs. –*holothurian* effluent			
Absorption coefficients (a_{325})	Paired t-test	24.91	**0.0000**
Spectral slope ($S_{275-295}$)	Wilcoxon	3.98	**0.0000**
Spectral slope ratio (S_R)	Paired t-test	6.18	**0.0000**
+*holothurian* vs. –*holothurian* effluents			
Absorption coefficients (a_{325})	Paired t-test	11.45	**0.0000**
Spectral slope ($S_{275-295}$)	Wilcoxon	3.82	**0.0001**
Spectral slope ratio (S_R)	Wilcoxon test	3.78	**0.0001**

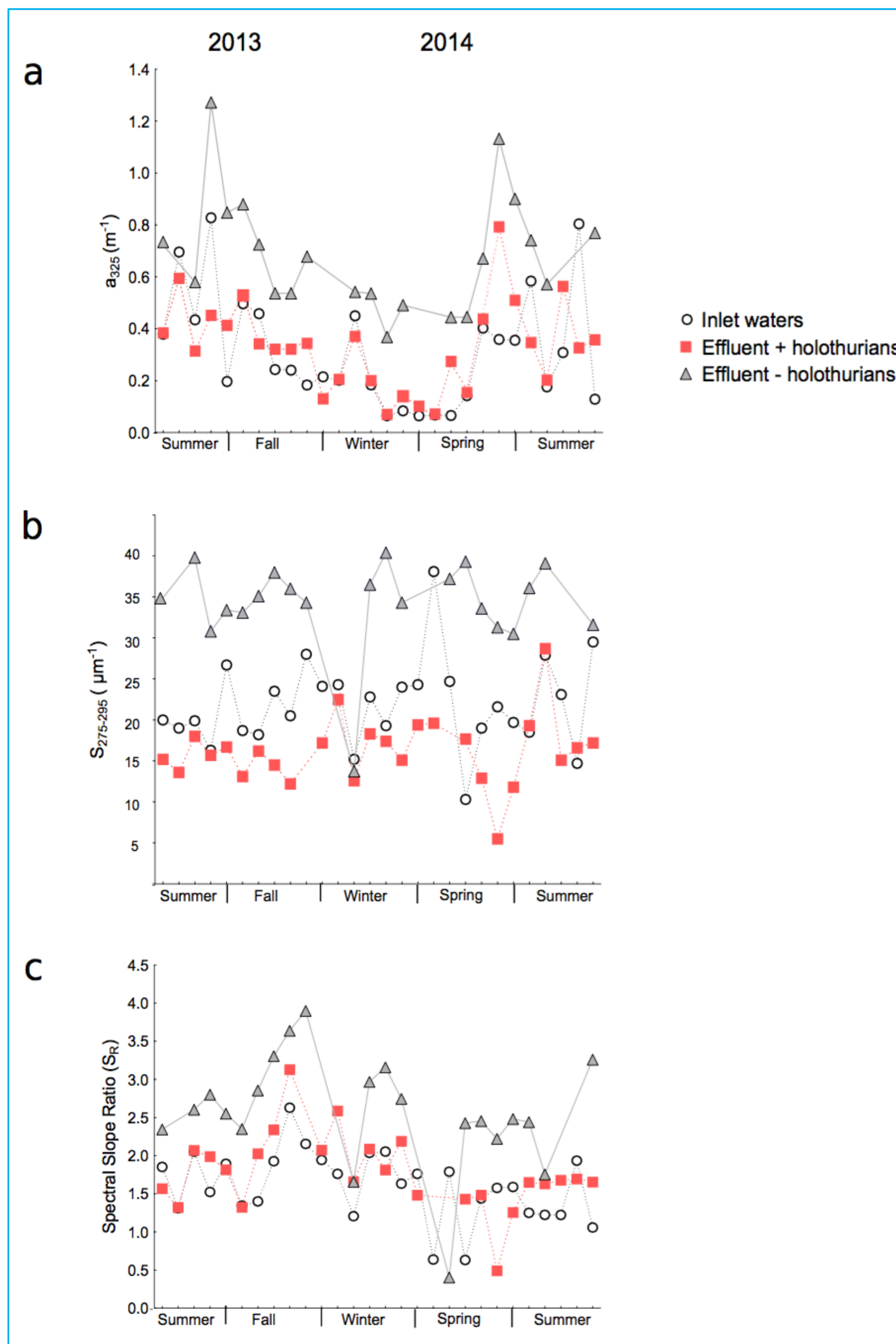

Fig.4.2. Time-series of the optical parameters of chromophoric dissolved organic matter in the big-volume tanks. Values of (A) absorption coefficients at 325 nm (a_{325}), (B) spectral slopes from 275 to 295 nm (S_{275295}), and (C) spectral slope ratios (S_R) in the inlet water (white circles), in the effluent water of the +holothurian tank (red squares) and in the effluent water of the –holothurian tank (grey triangles).

To assess if the presence of holothurians can modify significantly CDOM we performed paired t-test or Wilcoxon matched pair tests pooling all the time-series data (Table 4.1). In the Figure 4.3 we show all the time-series data pooled in median values, 25-75 % percentiles and non-outliers values. The a_{325} values in the *–holothurian* tank and effluent (Fig. 4.3a, grey boxes) were significantly higher that the values in the *+holothurian* tank and effluent and the inlet water (Table 4.1). A similar effect was found for the spectral slope (S_{275-29}) (Fig.4.3b, grey boxes), and the spectral ratios (S_R) (Fig.4.3c, grey boxes). Indeed, we observed higher CDOM concentration with higher spectral slopes (surrogate of smaller molecular size, Helms *et al.,* 2008) in the tank without holothurians than in the tank with holothurians. Therefore, holothurians appear to reduce significantly CDOM concentration, particularly of compounds with comparatively lower molecular weight making the spectral slopes smaller. On the other hand, the differences between the inlet water and the *+holothurian* tank and effluent water, although significant, were less relevant (Fig. 4.3, Table 4.1).

These results suggest that effluent of the monoculture of A. sulcata increases CDOM in comparison with the inlet water, which could affect the recipient coastal waters. These higher CDOM values in the –holothurian tank in comparison with the values in +holothurian tank could be related to: (1) a higher abundance of bacteria and their metabolic by-products that produce CDOM or (2) a higher concentration of particulate organic matter (derived from uneaten food, detritus and microbial cells) which disaggregation in dissolved compounds also produce CDOM. In both cases, an increment in CDOM concentration is expected in absence of holothurians. Several studies have shown that bacteria and phytoplankton can produce CDOM as metabolic by-products (Nelson *et al.*, 1998, 2004; Ortega-Retuerta *et al.,* 2009; Romera-Castillo *et al.,* 2010; Catalá *et al.,* 2015; 2016). However, we did not find significant relationships between the a_{325} values and the concentration of chlorophyll-*a* (+holothurian r^2= 0.009, p = 0.513; -holothurian r^2= 0.002 p= 0.804) or the abundance of bacteria (+holothurian r^2= 0.014, p = 0.403; -holothurian r^2= 0.068 p= 0.095) (Fig. 4.4a). Therefore, phytoplankton and bacterial carbon processing appear to have a minor importance in these tanks. In contrast, we found significant and positive relationships between the concentration of particulate organic matter (POM) and the a_{325} values in

the +*holothurian* tank and effluent water (red squares; $r^2 = 0.41$, p=0.002; regression line $a_{325} = 0.20 + 0.079$ POM) and the a_{325} values in the –*holothurian* tank and effluent water (grey triangles; $r^2=0.20$, p=0.006; regression line $a_{325} = 0.42 + 0.102$ POM) (Fig. 4.4b). Therefore, POM concentration in the tanks appears to be the main driver of CDOM changes. POM disaggregation into dissolved components is a common process in coastal waters (He *et al.,* 2016), particularly under sunny conditions (Shank *et al*., 2011; Pisani *et al.,* 2011). Holothurians consume several components of POM as phytoplankton cells, bacteria, uneaten food, animal feces, and transparent exopolymer particles (Hudson *et al*., 2005; Slater *et al*., 2009; Navarro *et al*., 2013; Yokoyama, 2013; Wotton, 2011). Since holothurians reduce POM concentration by consumption in these tanks (Sadeghi-Nassaj *et al.,* in prep.), we think that POM disaggregation into DOM in the tank with holothurians was significantly lower than in the tank without holothurians where a relevant fraction of POM might have been converted into CDOM. In addition, recently Brothers *et al*. (2015) have demonstrated a direct uptake of free amino acids in several tissues as the respiratory trees, epidermis, and oral tentacles of a sea cucumber species (*Parastichopus californicus*) during the visceral regeneration. It is well known that amino acids such as the tyrosine and tryptophan are able to absorb light in the ultraviolet band (e.g., Catalá *et al.,* 2013). Therefore, a direct and selective assimilation of free amino acids by holothurians could also explain a reduction in the a_{325} values and in the spectral slopes.

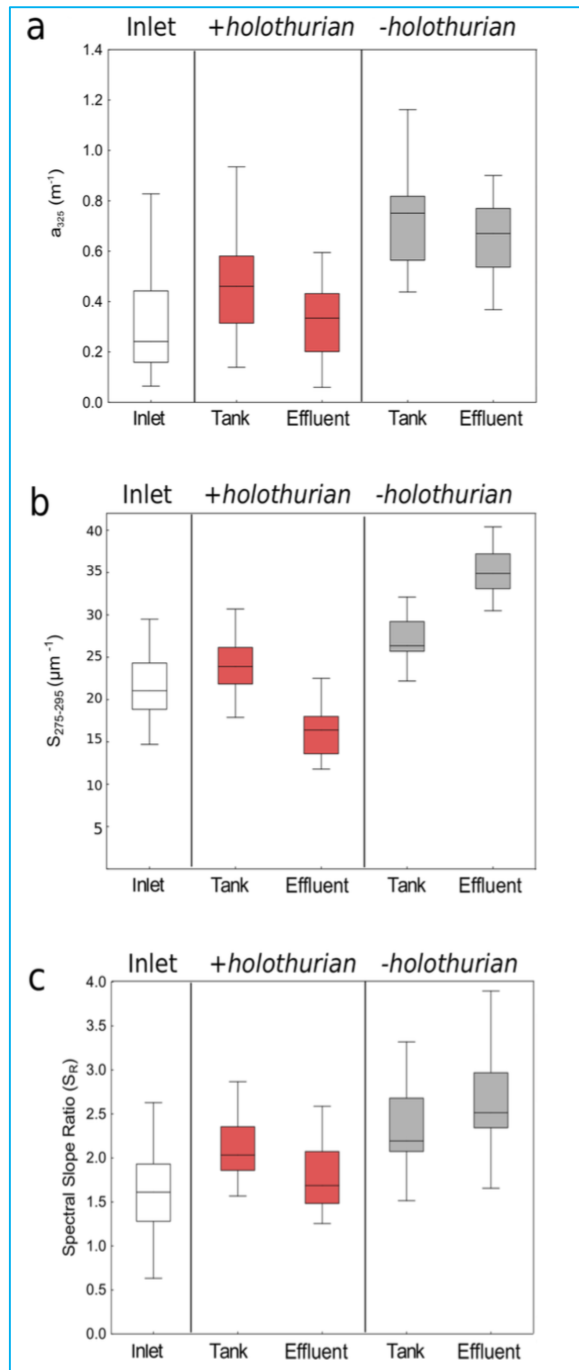

Fig.4.3. Median values (line), the 25–75% percentiles (box), and the non-outlier ranges (whisker) of the optical parameters of chromophoric dissolved organic matter pooling all time-series data. Values of (A) absorption coefficients at 325 nm (a_{325}), (B) spectral slopes from 275 to 295 nm (S_{275295}) and (C) spectral slope ratios (S_R) in the inlet water (white box), in the +holothurian effluent and tank (red boxes) and in the –holothurian effluent and tank (grey boxes).

Short-term experiments

To corroborate the results obtained in the time-series, we performed three short-term (3 days) experiments in smaller tanks manipulating the presence of holothurians. At the initial time, we did not observe significant differences in the optical parameters between both treatments (+H and –H) indicating the experiments started with identical conditions with the exception of the presence of holothurians (Table 4.2). However, at the final time after three days, the presence of holothurians (+H treatment) significantly reduced the values of the CDOM absorption coefficients at 325 nm (a_{325}) in 2 out of 3 experiments (Fig. 4.5a red bars, Table 4.2) and in 1 out of 3 experiments at 443 nm (a_{443}) (Fig. 4.5b red bars, Table 4.2). In the three experiments, at the final time, the presence of holothurians (+H treatment) reduced significantly the spectral slopes ($S_{275\text{-}295}$) (Fig. 4.5c red bars, Table 4.2). This variability in the statistical significance for the different optical parameters is related to the inherent higher sensitivity of CDOM as wavelengths are shorter (see Fig.4.1).

Table 4.2. Results of the analysis of variance (ANOVA) in the three experiments performed to compare the absorption coefficients at 325 nm (a_{325}), the absorption coefficients at 443 nm (a_{443}), and the spectral slopes from 275 nm to 295 nm (S_{275295}) in the treatments with holothurians (+H) vs. the treatments without holothurians (H) at initial and final times. Bold means that there is a significant difference between treatments.

	Experiment # 1		Experiment # 2		Experiment # 3	
	F	***p*-value**	***F***	***p*-value**	***F***	***p*-value**
Initial time						
a_{325}	5.56	0.065	0.16	0.702	0.24	0.644
a_{443}	0.74	0.428	0.55	0.493	0.08	0.783
$S_{275-295}$	1.43	0.286	0.04	0.858	2.08	0.211
Final time						
a_{325}	**849.74**	**<0.001**	**60.99**	**<0.001**	3.92	0.105
a_{443}	**1980.15**	**<0.001**	1.70	0.249	3.14	0.137
$S_{275-295}$	**571.43**	**<0.001**	**506.69**	**<0.001**	**7.89**	**0.038**

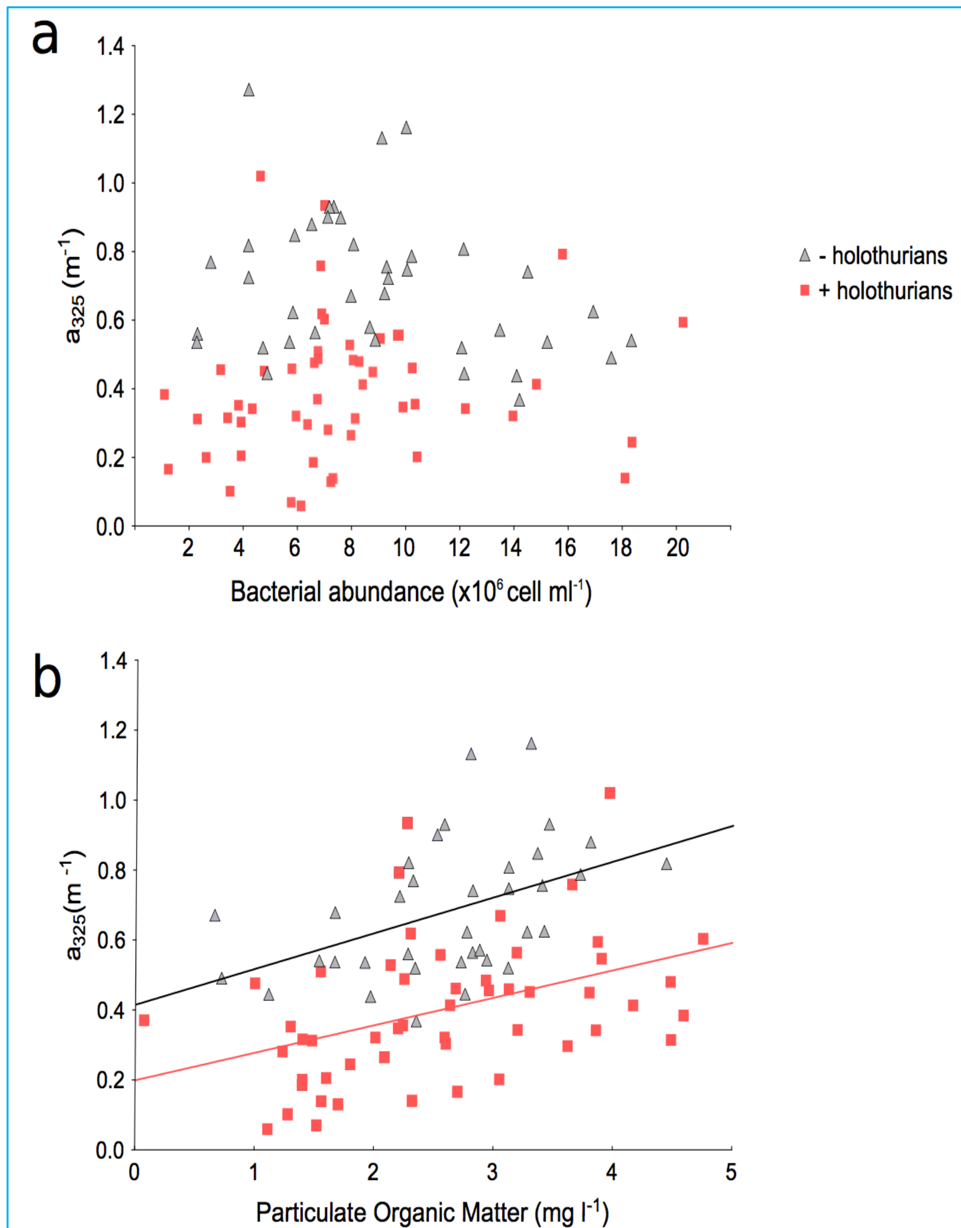

Fig.4.4. Scatterplots of absorption coefficients at 325 nm (a_{325}) vs. bacterial abundance (A) and particulate organic matter (B) of the time-series data. Red squares are the values for the +holothurian (effluent and tank) waters and grey triangles are the values for the –holothurian (effluent and tank) waters. Correlation lines are shown only when are statistically significant ($p < 0.05$).

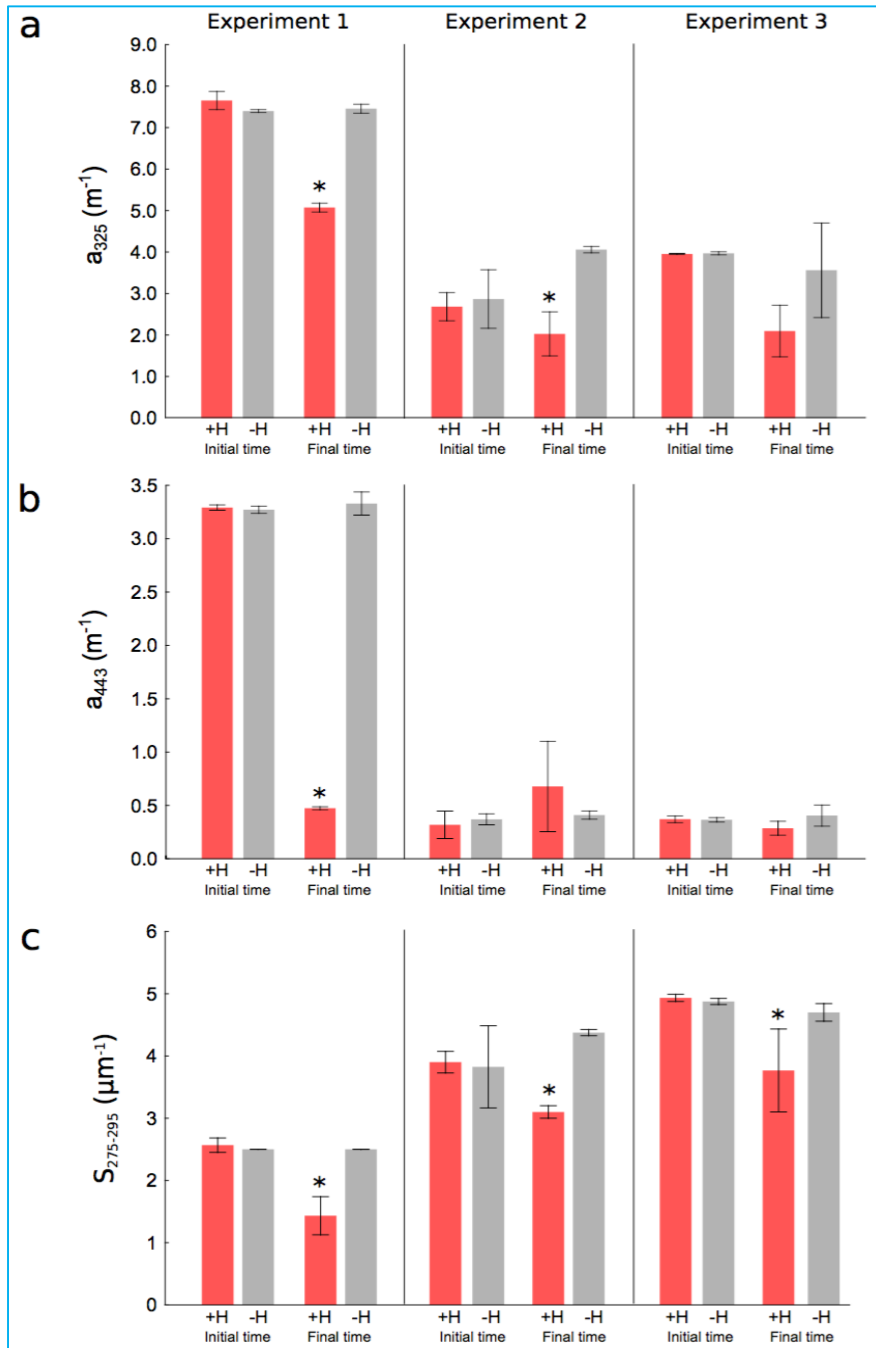

Fig.4.5. Changes in optical parameters of chromophoric dissolved organic matter in the three experiments. Mean (bars) and the standard deviations (whiskers) of the replicates of (A) absorption coefficients at 325 nm (a325), (B) absorption coefficients at 443 nm (a443), and (C) spectral slopes from 275 to 295 nm (S275295) in the treatments with holothurians (+H) and without holothurians (H) at the initial and final times. Red bars represent the treatment with holothurians and grey bars represent the treatment without holothurians. Asterisks show the statistically significant results at the final time of the experiments (more details in Table 4.2).

Conclusions

Overall, we found that the presence of holothurians in aquaculture tanks reduces significantly the concentration of CDOM, particularly of compounds with relatively lower molecular size. A plausible mechanism for this reduction is the consumption of particulate organic matter by holothurians reducing its disaggregation into chromophoric dissolved compounds. Complementary, holothurians could also consume directly dissolved compounds as chromophoric amino acids. This fact can affect positively water transparency. Indeed, CDOM optical parameters in the tank with holothurians in the time-series were quite similar to the inlet water both quantitatively (similar absorption coefficients at 325 nm) and qualitatively (similar spectral slopes and ratios) avoiding the increment of color in the waste effluents observed in the tank without holothurians. In offshore aquaculture installations, the presence of holothurians could also affect positively water transparency by reducing light absorption and scattering (Ibarra, Cembella & Grant, 2012; Del Bel Belluz *et al.*, 2016) as particulate organic matter settle down and they are placed below fish cages. Monitoring CDOM optical properties in aquaculture installations is an easy and inexpensive procedure very sensitive to the changes caused by the extractive species helping in the control of aquaculture waste. Therefore, the use of CDOM probes, for long-term monitoring, or remote sensing for large spatial scales, is a promising research area for the development of a sustainable aquaculture. All data for CDOM optical parameters and ancillary variables are shown in the Appendix II, supplementary Tables 1-6.

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References

Ajin AM, Silvester R, Alexander D, Nashad M, Abdulla MH. 2016. Characterization of blooming algae and bloom-associated changes in the water quality parameters of traditional *pokkali cum prawn* fields along the South West coast of India. *Environmental Monitoring and Assessment* **188**: Article 145 DOI 10.1007/s10661-016-5133-6.

American Public Health Association (APHA). 1992. Standard methods for the exami- nation of water and wastewater. Eighteenth Edition. Washington, D.C.: American Public Health Association. Bostock J, McAndrew B, Richards R, Jauncey K, Telfer T, Lorenzen K, Little D, Ross L, Handisyde N, Gatward I, Corner R. 2010. Aquaculture: global status and trends. *Philosophical Transactions of the Royal Society B* **365**:2897–2912 DOI 10.1098/rstb.2010.0170.

Bracchini L, Tognazzi A, Dattilo AM, Decembrini F, Rossi C, Loiselle SA. 2010. Sensitivity analysis of CDOM spectral slope in artificial and natural samples: an application in the central eastern Mediterranean Basin. *Aquatic Sciences* **72**:485–498 DOI 10.1007/s00027-010-0150-y.

Bricaud A, Morel A, Prieur L. 1981. Absorption by dissolved organic matter of the sea (yellow substance) in the UV and visible domains. *Limnology and Oceanography* **26**:43–53 DOI 10.4319/lo.1981.26.1.0043.

Brothers CJ, Lee RW, Nestler JR. 2015. The uptake of dissolved organic material by the sea cucumber *Parastichopus californicus* (Stimpson) and its potential role in visceral regeneration. Journal of *Experimental Marine Biology and Ecology* **469**:69–75 DOI 10.1016/j.jembe.2015.04.016.

Carder KL, Steward RG, Harvey GR, Ortner PB. 1989. Marine humic and fulvic acids: their effects on remote sensing of ocean chlorophyll. *Limnology and Oceanography* **34**:68–81 DOI 10.4319/lo.1989.34.1.0068.

Catalá TS, Mladenov N, Echevarría F, Reche I. 2013. Positive trends between salinity and chromophoric and fluorescent dissolved organic matter in a seasonally inverse estuary. *Estuarine, Coastal and Shelf Science* **133**:206–216 DOI 10.1016/j.ecss.2013.08.030.

Catalá TS, Reche I, Álvarez M, Khatiwala S, Guallart EF, Benítez-Barrios VM, Fuentes- Lema A, Romera-Castillo C, Nieto-Cid M, Pelejero C, Fraile-Nuez E, Ortega- Retuerta E, Marrasé C, Álvarez Salgado XA. 2015. Water mass age and aging driving chromophoric dissolved organic matter in the dark global ocean. *Global Biogeochemical Cycles* 29:917–934 DOI 10.1002/2014GB005048.

Catalá TS, Reche I, Ramón CL, López-Sanz A, Álvarez M, Calvo E, Álvarez Salgado XA. 2016. Chromophoric signatures of microbial by-products in the dark ocean. *Geophysical Research Letters* **43**:7639–7648 DOI 10.1002/2016GL069878.

Chintiroglou C, Koukouras A. 1992. The feeding habits of three Mediterranean Sea anemone species, *Anemonia viridis* (Forskal), *Actinia equina* (Linnaeus) and *Cereus pedunculatus* (Pennant). *Helgoländer Meeresuntersuchungen* **46**:53–68 DOI 10.1007/BF02366212.

Chopin T, Cooper JA, Reid G, Cross S, Moore C. 2012. Open-water integrated multitrophic aquaculture: environmental biomitigation and economic diversification of fed aquaculture by extractive aquaculture. *Reviews in Aquaculture* **4**:209–220 DOI 10.1111/j.1753-5131.2012.01074.x.

Del Bel Belluz J, Costa M, Reid G, Cross S. 2016. Bio-optical variability at a Vancouver Island aquaculture site. *Limnology and Oceanography* **61**:1686–1704 DOI 10.1002/lno.10332.

Diana JS, Egna HS, Chopin T, Peterson MS, Cao L, Pomeroy R, Verdegem M, Slack WT, Bondad-Reantaso MG, Cabello F. 2013. Responsible aquaculture in 2050: valuing local condition and human innovation will be key to success. *Bioscience* **63**:255–262 DOI 10.1525/bio.2013.63.4.5.

Food and Agriculture Organization of the United Nations (FAO). 2009. The state of world fisheries and aquaculture 2008. Rome: Food and Agriculture Organization of the United Nations.

Gasol JM, Del Giorgio PA. 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Scientia Marina* **64**:197–224 DOI 10.3989/scimar.2000.64n2197.

Green SA, Blough NV. 1994. Optical absorption and fluorescence properties of chromophoric dissolved organic matter in natural waters. *Limnology and Oceanography* **39**:1903–1916 DOI 10.4319/lo.1994.39.8.1903.

He W, Chen M, Schlautman MA, Hur J. 2016. Dynamic exchanges between DOM and POM pools in coastal and inland aquatic ecosystems: a review. *Science of the Total Environment* **551–552**:415–428 DOI 10.1016/j.scitotenv.2016.02.031.

Helms JR, Stubbins A, Perdue EM, Green NW, Chen H, Mopper K. 2013. Photochemical bleaching of oceanic dissolved organic matter and its effect on absorption spectral slope and fluorescence. *Marine Chemistry* **155**:81–91 DOI 10.1016/j.marchem.2013.05.015.

Helms JR, Stubbins A, Ritchie JD, Minor EC, Kieber DJ, Mopper K. 2008. Absorption spectral slopes and slope ratios as indicators of molecular weight, source, and photo-bleaching of chromophoric dissolved organic matter. *Limnology and Oceanography* **53**:955–969 DOI 10.4319/lo.2008.53.3.0955.

Hudson IR, Wigham BD, Solan M, Rosenberg R. 2005. Feeding behavior of deep-sea dwelling holothurians: inferences from a laboratory investigation of shallow fjords species. *Journal of Marine Systems* **57**:201–218 DOI 10.1016/j.jmarsys.2005.02.004.

Ibarra DA, Cembella A, Grant J. 2012. Attenuation of sunlight measured from moored radiometers to assess depletion of suspended particles caused by bivalve aquaculture. Limnology and Oceanography: *Methods* **10**:1051–1069 DOI 10.4319/lom.2012.10.1051.

Klinger D, Naylor R. 2012. Searching for solutions in aquaculture: charting a sustainable course. *Annual Review of Environment and Resources* **37**:247–276 DOI 10.1146/annurev-environ-021111-161531.

Martínez-Pérez AM, Nieto-Cid M, Osterholz H, Catalá TS, Reche I, Dittmar T, Álvarez Salgado XA. 2017. Linking optical and molecular signatures of dissolved organic matter in the Mediterranean Sea. *Scientific Reports* **7**:3436 DOI 10.1038/s41598-017-03735-4.

Millar RB, Anderson MJ. 2004. Remedies for pseudo-replication. *Fisheries Research* **70**:397–407 DOI 10.1016/j.fishres.2004.08.016.

Navarro PG, García-Sanz S, Barrio JM, Tuya F. 2013. Feeding and movement pat- terns of the sea cucumber *Holothuria sanctori*. *Marine Biology* **160**:2957–2966 DOI 10.1007/s00227-013-2286-5.

Nelson EJ, MacDonald BA, Robinson SMC. 2012a. The absorption efficiency of the suspension-feeding sea cucumber, *Cucumaria frondosa,* and its potential as an extractive integrated multi-trophic aquaculture (IMTA) species. *Aquaculture* **370– 371**:19–25 DOI 10.1016/j.aquaculture.2012.09.029.

Nelson EJ, MacDonald BA, Robinson SMC. 2012b. A review of the Northern Sea Cucumber *Cucumaria frondosa* (Gunnerus, 1767) as a potential aquaculture species. *Reviews in Fisheries Science* **20**:212–219 DOI 10.1080/10641262.2012.719043.

Nelson NB, Carlson CA, Steinberg DK. 2004. Production of chromophoric dissolved organic matter by Sargasso Sea microbes. *Marine Chemistry* **89**:273–287 DOI 10.1016/j.marchem.2004.02.017.

Nelson NB, Siegel DA. 2013. The global distribution and dynamics of chromophoric dissolved organic matter. *Annual Review of Marine Science* **5**:447–476 DOI 10.1146/annurev-marine-120710-100751.

Nelson NB, Siegel DA, Michaels AF. 1998. Seasonal dynamics of colored dissolved material in the Sargasso Sea. Deep Sea Research Part I: Deep Sea Research Part I: *Oceanographic Research Papers* **45**:931–957 DOI 10.1016/S0967-0637(97)00106-4.

Nima C, Frette E, Hamre B, Erga SR, Chen YC, Zhao L, Sørensen K, Norli M, Stamnes K, Stamnes JJ. 2016. Absorption properties of high-latitude Norwegian coastal water: the impact of CDOM and particulate matter. *Estuarine, Coastal and Shelf Science* **178**:158–167 DOI 10.1016/j.ecss.2016.05.012.

Nimptsch J, Woelfl S, Osorio S, Valenzuela J, Ebersbach P, Tuempling WV, Palma R, Encina F, Figueroa D, Kamjunke N, Graeber D. 2015. Tracing dissolved organic matter (DOM) from land-based aquaculture systems in North Patagonian streams. *Science of the Total Environment* **537**:129–138 DOI 10.1016/j.scitotenv.2015.07.160.

Organelli E, Bricaud A, Antoine D, Matsuoka A. 2014. Seasonal dynamics of light absorption by chromophoric dissolved organic matter (CDOM) in the NW Mediterranean Sea (BOUSSOLE site). *Deep Sea Research Part I: Oceanographic Research Papers* **91**:72–85 DOI 10.1016/j.dsr.2014.05.003.

Ortega-Retuerta E, Frazer TK, Duarte CM, Ruiz-Halpern S, Tovar-Sánchez A, Arrieta JM, Reche I. 2009. Biogeneration of chromophoric dissolved organic matter by bacteria and krill in the Southern Ocean. *Limnology and Oceanography* **54**:1941–1950 DOI 10.4319/lo.2009.54.6.1941.

Ortega-Retuerta E, Siegel DA, Nelson NB, Duarte CM, Reche I. 2010. Observations of chromophoric dissolved and detrital organic matter distribution using remote sensing in the Southern Ocean: validation, dynamics and regulation. *Journal of Marine Systems* **82**:295–303 DOI 10.1016/j.jmarsys.2010.06.004.

Pisani O, Yamashita Y, Jaffé R. 2011. Photo-dissolution of flocculent, detrital material in aquatic environments: contributions to the dissolved organic matter pool. *Water Research* **45**:3836–3844 DOI 10.1016/j.watres.2011.04.035.

Populus J, Hastuti W, Martin J-LM, Guelorget O, Sumartono B, Wibowo A. 1995. Remote sensing as a tool for diagnosis of water quality in Indonesian seas. *Ocean & Coastal Management* **27**:197–215 DOI 10.1016/0964-5691(95)00033-X.

Purcell SW, Mercier A, Conand C, Hamel J-F, Toral-Granda MV, Lovatelli A, Uthicke S. 2013. Sea cucumber fisheries: global analysis of stocks, management measures and drivers of overfishing. *Fish and Fisheries* **14**:34–59 DOI 10.1111/j.1467-2979.2011.00443.x.

Rajitha K, Mukherjee CK, Vinu Chandran R. 2007. Applications of remote sensing and GIS for sustainable management of shrimp culture in India. *Aquacultural Engineering* **36**:1–17 DOI 10.1016/j.aquaeng.2006.05.003.

Read P, Fernandes T. 2003. Management of environmental impacts of marine aquaculture in Europe. *Aquaculture* **226**:139–163 DOI 10.1016/S0044-8486(03)00474-5.

Romera-Castillo C, Sarmento H, Alvarez-Salgado XA, Gasol JM, Marrase C. 2010. Production of chromophoric dissolved organic matter by marine phytoplankton. *Limnology and Oceanography* **55**:446–454 DOI 10.4319/lo.2010.55.1.0446.

Ruiz-Zarzuela I, Halaihel N, Balcázar J, Ortega C, Vendrell D, Pérez T, Alonso J, De Blas I. 2009. Effect of fish farming on the water quality of rivers in northeast Spain. *Water Science and Technology* **60**:663–671 DOI 10.2166/wst.2009.435.

Saitoh S-I, Mugo R, Radiarta IN, Asaga S, Takahashi F, Hirawake T, Ishikawa Y, Awaji T, In T, Shima S. 2011. Some operational uses of satellite remote sensing and marine GIS for sustainable fisheries and aquaculture. *ICES Journal of Marine Science* **68**:687–695 DOI 10.1093/icesjms/fsq190.

Shank GC, Evans A, Yamashita Y, Jaffé R. 2011. Solar radiation-enhanced dissolution of particulate organic matter from coastal marine sediments. *Limnology and Oceanography* **56**:577–588 DOI 10.4319/lo.2011.56.2.0577.

Siegel DA, Maritorena S, Nelson NB, Behrenfeld MJ, McClain CR. 2005. Colored dissolved organic matter and its influence on the satellite-based characterization of the ocean biosphere. *Geophysical Research Letter* **32**: Article L20605 DOI 10.1029/2005GL024310.

Slater MJ, Carton AG. 2009. Effect of sea cucumber (*Australostichopus mollis*) grazing on coastal sediments impacted by mussel farm deposition. *Marine Pollution Bulletin* **58**:1123–1129 DOI 10.1016/j.marpolbul.2009.04.008.

Slater MJ, Jeffs AG, Carton AG. 2009. The use of the waste from green-lipped mussels as a food source for juvenile sea cucumber, *Australostichopus mollis*. *Aquaculture* **292**:219–224 DOI 10.1016/j.aquaculture.2009.04.027.

Twardowski MS, Boss E, Sullivan JM, Donaghay PL. 2004. Modeling the spectral shape of absorption by chromophoric dissolved organic matter. *Marine Chemistry* **89**:69–88 DOI 10.1016/j.marchem.2004.02.008.

Weishaar JL, Aiken GR, Bergamaschi BA, Fram MS, Fujii R, Mopper K. 2003. Evaluation of specific ultraviolet absorbance as an indicator of the chemical composition and reactivity of dissolved organic carbon. *Environmental Science Technology* **37**:4702–4708 DOI 10.1021/es030360x.

Wotton RS. 2011. EPS (Extracellular Polymeric Substances), silk, and chitin: vitally important exudates in aquatic ecosystems. *Journal of the North American Benthological Society* **30**:762–769 DOI 10.1899/10-120.1.

Yokoyama H. 2013. Growth and food source of the sea cucumber *Apostichopus japonicus* cultured below fish cage. Potential for integrated multitrophic aquaculture. *Aquaculture* **372**–375:28–38 DOI 10.1016/j.aquaculture.2012.10.022.

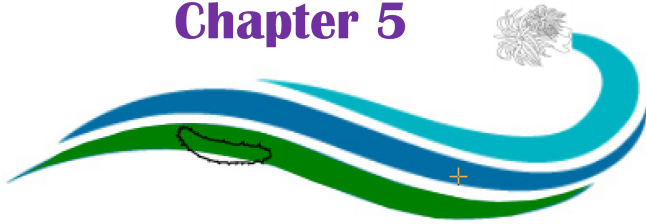
Yokoyama H. 2015. Suspended culture of the sea cucumber *Apostichopus japonicus* below a Pacific oyster raft potential for integrated multi-trophic aquaculture. *Aquaculture Research* **46**:825–832 DOI 10.1111/are.12234.

Zamora LN, Jeffs AG. 2011. Feeding, selection, digestion and absorption of the organic matter from mussel waste by juveniles of the deposit-feeding sea cucumber, *Australostichopus mollis*. *Aquaculture* **317**:223–228 DOI 10.1016/j.aquaculture.2011.04.011.

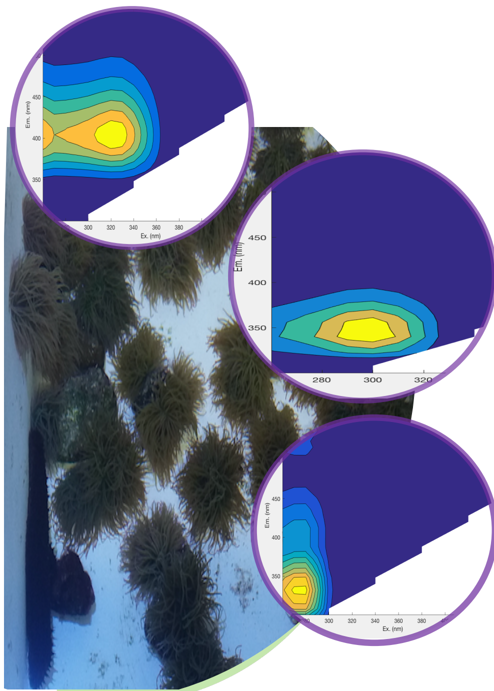
Zamora LN, Yuan X, Carton AG, Slater MJ. 2016. Role of deposit-feeding sea cu- cumbers in integrated multitrophic aquaculture: progress, problems, potential and future challenges. **Reviews in Aquaculture 0**:1-18 DOI 10.1111/raq.12147.

Zhang L, Gao Y, Zhang T, Yang H, Xu Q, Sun L, Yu Z. 2014. A new system for bottom co-culture of the scallop, *Patinopecten yessoensis*, with the sea cucumber, *Apostichopus japonicus*, and the sea urchin, *Anthocidaris crassispina*, in shallow water in China. *Aquaculture International* **22**:1403-1415 DOI 10.1007/s10499-014-9755-9.

Chapter 5



FDOM



Fluorescence spectroscopy to trace dissolved organic matter processed by sea cucumbers in mutitrophic aquaculture tanks

Abstract

Sea cucumbers are able to recycle large fractions of particulate organic matter through their feeding activities on detritus deposits when they are co-cultured with other species. However, there is little information about how they process this organic matter and its effects on dissolved organic matter (DOM) optical properties. Fluorescence spectroscopy (excitation and emission matrixes, EEMs) and parallel factor (PARAFAC) analysis are very powerful procedures to characterize DOM components in more refractory and humic-like compounds vs. more labile and amino acid-like compounds. In this work, we monitored during more than one year the changes in fluorescence DOM (FDOM) in two big-volume tanks with different trophic structure. One of the tanks (-*holothurian*) only contained *Anemonia sulcata*, whereas the other tank (+*holothurian*) also included individuals of *Holothuria tubulosa* and *Holothuria forskali*. PARAFAC analysis gave a six components model with four (C1, C2, C3 and C4) humic-like components and two (C5 and C6) amino acid-like components. We observed than holothurians were able to reduce significantly both humic-like and amino acid-like components, likely due to their great efficiency removing particulate organic matter. To confirm the time-series results, we also performed three experiments. Each experiment consisted of two treatments: with holothurians (+H) and without holothurians (-H). We set up three +H tanks with 80 individuals of *A. sulcata* and 10 individuals of *H. tubulosa* in each tank and four -H tanks that contained only 80 individuals of *A. sulcata*. In these experiments we confirmed that holothurian were able to reduce two of the humic-like components (C2 and C4) and the two amino acid-like components. We suggest that symbiotic bacteria in the tentacle epidermis and below the cuticle in holothurians could directly assimilate amino acids and explain the remarkable significant reduction of the two amino acid-like components that we observed in both the time-series and particularly the short-term experiments.

Introduction:

Currently, aquaculture accounts for half of the human demand of fish and seafood (FAO, 2009). Traditional aquaculture produces wastewaters that contain important quantities of particulate and dissolved organic matter (DOM) affecting water quality of the recipient ecosystems (Chávez-Crooker & Obreque-Contreras, 2010; Nimptsch *et al.,* 2015; Wang *et al.,* 2017). Multitrophic aquaculture can reduce these loads of organic matter including extractive species that process this organic matter (Chopin *et al.,* 2012; Diana *et al.,* 2013). Extractive species filter, ingest or rework suspended or settled particulate organic matter (e.g., mussels, oysters, and holothurians) reducing their loads in the effluents. Particularly, sea cucumbers are species with great extractive capacity of organic matter (Slater & Carton 2009; Nelson *et al*., 2012; Yokoyama, 2013, 2015). More recently, Brothers *et al*. (2015) have demonstrated a direct uptake of free dissolved amino acids in several tissues as the respiratory trees, epidermis, and oral tentacles during the visceral regeneration of a sea cucumber species (*Parastichopus californicus*). This direct assimilation of dissolved amino acids by sea cucumbers has been related to the presence of subcuticular bacteria (Roberts *et al.,* 1991; Lawrence *et al.,* 2010). On the other hand, Sadeghi-Nassaj *et al*. (2018) (chapter 4) also found a reduction in the quantity of chromophoric dissolved organic matter (CDOM). This CDOM reduction was attributed, for these last authors, to the relevant POM consumption by sea cucumbers. This POM consumption indirectly reduces CDOM concentration derived from POM disaggregation. Alternatively, a direct assimilation of dissolved compounds of low molecular weight, such as chromophoric amino acids, by subcuticular bacteria might be also possible.

Particulate and dissolved organic matter in aquaculture installations is monitored using several spectroscopic techniques. Water transparency (absorption and scattering) has been monitored with submergible radiometers in cages (Ibarra *et al.,* 2012); Del Bel Belluz *et al.,* 2016). DOM effluents and biodegradability in aquaculture has been studied with fluorescence spectroscopy and parallel

factor analysis (Hambly *et al.,* 2015; Nimptsch *et al.,* 2015; Wang *et al.,* 2017). Therefore, fluorescence spectroscopy provides comprehensive information on the nature of DOM pool without a specific chemical characterization. Fluorescent compounds include aromatic amino acids, lignin phenols, and humic substances characterized by their specific fluorescence properties (Coble, 1996). Amino acid- and humic-like compounds have very different fluorescent signatures and represent mainly bioavailable DOM and refractory DOM, respectively. Therefore, this approach allows a detailed inspection of different DOM components.

The intensity of the fluorescent dissolved organic matter (FDOM) is measured using three-dimensional matrixes of excitation (240-500 nm) and emission (300-600 nm) (EEMs) with a spectrofluorometer instrument. That is, an EEMs is a collection of multiple emission spectra in a range of excitation wavelengths, which are concatenated into a three-dimensional matrix (Andersen & Bro, 2003; Ohno & Bro, 2006; Borisover *et al.*, 2009; Kowalczuk *et al.*, 2005; Coble *et al.*, 1990; 1996, Zepp *et al.*, 2004). The location of the excitation-emission peaks varies with the composition of DOM. Unlike absorption spectrum with a clear absorption decay as wavelength increases without apparent peaks (Chapter 4, Fig. 4.1), excitation spectra show one or more discrete peaks, commonly around 250 and 350 nm. EEMs spectra provide a comprehensive map (fingerprint) of fluorescence properties of DOM and it is used to discriminate different classes of fluorophore based on their excitation emission maxima. EEMs effectively allow us to discriminate of CDOM composition. Fluorescence spectroscopy is more sensitive than absorption spectroscopy and provides more detailed information about relative DOM composition.

Due to overlapping of CDOM component in many natural environments, EEMs description and peak observation is not quantitatively enough (Coble, 1996). Parallel factor (PARAFAC) analysis of fluorescence matrixes provides a quantitative approach to fluorophores discrimination and currently is widely used by the scientific community (Stedmon *et al.*, 2003). PARAFAC as supplementary multivariate technique for EEMs, provide comprehensive information related to relative quantity-quality and dynamic of CDOM fluorescent components (Bro, 1997; Stedmon *et al.*, 2003; Stedmon & Bro, 2008;

Hall *et al*., 2005; Murphy *et al*., 2006). Numerous studies have used both EEMs and PARAFAC to characterize CDOM in natural aquatic ecosystems (i.e. Stedmon *et al*., 2003; Yamashita *et al*., 2008, 2010; Zhang *et al*., 2011; Stubbins *et al*., 2014; Catalá *et al*., 2015;), but just a few studies on wastewater monitoring (Li *et al*., 2014; Liu *et al*., 2014; Cohen *et al*., 2014). Among these last studies, only two of them were related to aquaculture wastewater (Nimptsch *et al*., 2015; Hambly *et al*., 2015).

Therefore the main objectives of this study were: 1) to characterize FDOM in aquaculture systems with and without the presence of extractive species of organic matter such as the sea cucumbers *Holothuria tubulosa* and *H. forskali*; 2) to determine the influence of sea cucumbers on humic and amino acid- like components; and 3) to determine if amino acid-like components are directly up-taken by sea cucumbers and, then, support with a different technique the assimilation of amino acids by symbiont bacteria in sea cucumbers.

Materials and Method

Time-series in the big-volume tanks

Aquaculture tanks and sampling

We monitored during more than one year two aquaculture tanks located at the iMare Natural S.L. facilities in Motril, Spain ((http://www.imarenatural.com). Each tank of 50,000 liters of capacity was connected directly with the coastal water by one inlet pipe (inlet water) and the water from each tank was released by one outlet pipe located in the bottom of the tank (effluent). The seawater was pumped into the tanks at a continuous flow of 1,200 l h^{-1}. Therefore, water residence time in the tanks was ca. 42 hours and the total annual effluents accounted for 10, 512 m^3. In one of the tanks, 811 ± 125 individuals of the primary species, the sea anemone *Anemonia sulcata*, and 93 ± 3 adults of sea cucumbers *Holothuria tubulosa* (≈ 80 %) and *H. forskali* (≈ 20 %) were included (hereafter designated as + *holothurian* tank). In the other tank only 690 ± 87 individuals of the primary specie were included (hereafter designated as – *holothurian* tank). Sea anemones were placed on floating plastic boxes in the surface of the tanks and holothurians were free in the bottom and walls of the tanks. Sea anemones were fed with about 900-1800 g of fresh chopped fish, mainly *Scomber scombrus* (Chintiroglou & Koukouras, 1992) twice per week.

FDOM sampling

Water samples from each tank were sampled biweekly about noon from July 2013 to August 2014. FDOM samples were taken from inlet water and from the center of the tanks during each sampling date. Then, samples were filtered through pre-combusted GF/F glass fiber filter with 0.7μm nominal pore size. To increase FDOM precision, all fluorescence measurements were done the same day (maximum 2 hours) after sampling at room temperature. To avoid light interference with samples, we only used dark glass flasks of 250 ml. Before sampling, all the bottles were acid-cleaned (1% HCl) for 24 hours and then washed several times with distillated water. Subsequently, all the flasks were combusted (4 h, 500 °C) to prevent any organic contamination. Just before filling the flasks, they were rinsed with seawater and after sampling were kept in an icebox during transportation to the laboratory. Basic parameters such as temperature (°C), pH, salinity (psu), total dissolved solids, and conductivity ($mScm^{-1}$) were routinely measured using a multi parameter HANNA probe (HI9828 model).

Spectrofluorometer checks and practical consideration

DOM fluorescence measurements (EEMs collection) were made with a Jobin Yvon Horiba Fluoromax-4 spectrofluorometer equipped with a 1 cm quartz cuvette (4 transparent glass sides), a Xenon arc lamp and a photomultiplier tube as detector. Xenon lamps are the best choice when a variety of excitation wavelengths are required. To avoid any contamination the spectrofluorescence cuvette was rinsed with deionized and Milli-Q water several times for each sample. We used polietilene plastic gloves instead latex gloves to minimize organic contamination. We routinely performed a series of instrument checks. First we made a check for excitation, this check let us to know if the lamp intensity has changed over time. It was check by setting area at excitation range from 200 to 600 nm and emission at 350 nm (integration time: 0.1 s). For both excitation and emission slits were chosen at 1 nm. Once the scan was complete, the highest peak should be at 467 nm. To warranty that cuvette was clean we performed a cuvette check for contamination. We set emission range from 270 nm to 430 nm and an excitation at 240 nm with integration time of 0.1s. For both Ex. Em slits were 5 nm. If the cuvette contamination check showed a peak we

repeated the process until we get a flat line. In the case the cuvette was contaminated, we cleaned with diluted HCL. Normally, we performed water Raman scan (WRS for emissions) to check potential lamp drifting daily, the computer program (Fluor Essence Ver. 2.5) specified by setting an excitation wavelength (350 nm) and an emission range (365-450 nm) with an integration time of 0.1 s. For both excitation and emission slits were 5 nm. We also performed checks for lamp decay in the fluorescence region of the amino acids using a sealed cuvette of p-tephenyl and in the fluorescence region of the humic acids using a sealed cuvette of tetraphenyl butadiene. Then, setting the ranges of excitation, emission, the slit interval and the integration time configured the fluorescence measurement of the samples.

Three-dimensional fluorescence (EEMs)

To obtain the 3D EEMs, both excitation and emission were set for bandwidths to 1 nm. A series of emission spectra from 300 nm to 560 nm at 2 nm increments were collected over excitation wavelengths ranging from 240 to 450 nm with 10 nm increments.

We obtained a total of 130 EEMs of water samples from the inlet (seawater), +holothurian tank, -holothurian tank and corresponding effluents. The fluorescence peaks were identified based on Coble´s nomenclature (Coble, 1996; 2007). Briefly, peak A (Ex. 260 nm Em. 400-460 nm) is characterized as terrestrial humic acids, peak M (Ex. 290-310 nm Em. 370-410 nm) is characterized as marine fulvic acids and peak C (Ex. 320-360 nm Em. 420-460 nm) is characterized as terrestrial fulvic acids belonging all to the humic-like fluorescence group. Peaks B (tyrosine like Ex. 275 Em. 305 nm), and T (tryptophan like Ex. 275 Em. 340) and N (phenylalanine like Ex. 280 Em. 370) belong to the amino acid-like fluorescence group.

PARAFAC modeling

PARAFAC breaks 3D EEMs output images into individual fluorescent components providing fluorescence intensities for each fluorophore (Stedmon *et al*., 2003 Stedmon & Bro, 2008). PARAFAC analysis was performed by using MATLAB (V 2016b) with DOMFluor N-way toolbox v.3.1 developed by Andersson and Bro (2000). PARAFAC output revealed the final component intensity (concentration) at the fluorescence peak (maximum excitation-emission) position in

each sample. We performed a series of actions for each array according to Stedmon and Bro (2008) using Matlab, R2016b software. We organized data, subtracted blanks, and normalized Raman area. EEMs in fluorescence units were converted into Raman Units (RU) by normalizing by the area under the Raman peak (excitation wavelength at 350 nm) of daily Milli-Q EEM. To perform this action, we did Raman normalizing for Milli-Q EEM each day and the results were subtracted for each sample (Samples-Milli-Q EEMs).

We generated the EEMs cutting the 1st and 2nd Rayleigh peaks (Raylegh masking subtraction action). We removed Rayleigh scatter effects from the data by cutting all the emission wavelength that are plotted at wavelengths lower or equal to excitation +20 nm and bigger or equal to 260 (Stedmon & Bro, 2008). Outliers were detected by running exploratory action (calculating the leverage action) (Zhang *et al*., 2011). Six samples were considered as outliers.

Short-term experiments

To test the statistical signature of the results obtained in the time-series, we performed three short-term experiments. Each experiment was carried out in the seven tanks of 300 liters that contained 80 individuals of *A. sulcata* per tank, lasted 3 days (ca.72 h) and considered of two treatments: + Holothurians (+H) and – Holothurian (-H). At the initial time, in three of tanks (+H tanks) 10 individual of H. *tubulosa* were transplanted in each tank. The other four tanks (-H tanks) contained only 80 individuals of *A. sulcate*. The experiment 1 was carried out from 6th to 9th October 2017, the experiment 2 from 27th to 30th October 2017, and the experiment 3 from 3th to 6th November 2017. To analyze the samples we followed the same procedures used in the time- series.

Statistical analyses

To compare the time-series of the FDOM components in the inlet water with the effluents from the tank with holothurians and the tank without holothurians we performed paired t-test (for normally distributed variables) and Wilcoxon matched pairs test (for not-normally distributed variables) using the Statistica software (V8). These statistical analyses ameliorate the problem of temporal pseudo-replication in this type of studies (Millar & Anderson, 2004). In the short-term experiments to test the statistical significance of the presence of

holothurians on the FDOM parameters we performed analysis of variance (ANOVA) comparing the tanks with holothurians (+H) with the tanks without holothurians (-H) using Statistica software (V8).

Results and discussion

PARAFAC components

During this study, we collected 172 EEMs, but just 162 were considered for the PARAFAC analysis that discarded 10 outliers. PARAFAC analysis identified 6 components (Figure 5.1.). The components 1 (C1), 2 (C2), and 3 (C3) (Figure 5.1a, b, c) were consistent with humic-like compounds and corresponded with peaks C, A+C and A according to Coble´s nomenclature (Coble 1996; 2007) (Table 5.1). These peaks are ubiquitous and have been reported in previous studies in freshwater and marine ecosystems, urban sewage and drinking water (i.e. Coble, 2007; Yamashita *et al.,* 2008; Shutova *et al.,* 2014; Mostofa *et al.,* 2010; Stubbins *et al.*, 2014; Catalá *et al.*, 2015; Baker, 2001) (Table 5.1.). The component 4 (C4), with an emission maximum at 508 nm (Figure 5.1d), has not been reported previously to the best of our knowledge. This component might have a specific origin in the aquaculture system, however it was not found in the few previous works in aquaculture systems (Hambly *et al.*, 2015; Nimptsch *et al.,* 2015; Wang *et al.,* 2017). The components 5 (C5) and 6 (C6) (Figure 5.1e, f) were consistent with amino-like compounds and corresponded with peaks T (tryptophan like), and B (tyrosine like) according to Coble´s nomenclature (Coble, 1996; 2007) (Table 5.1). These peaks are also ubiquitous in aquatic ecosystems and have been reported in previous studies (i.e. Kowalczuk *et al*., 2009; Liu *et al.,* 2014; Stedmon & Markager, 2005; Hambly *et al.,* 2015; Nimptsch *et al*., 2015).

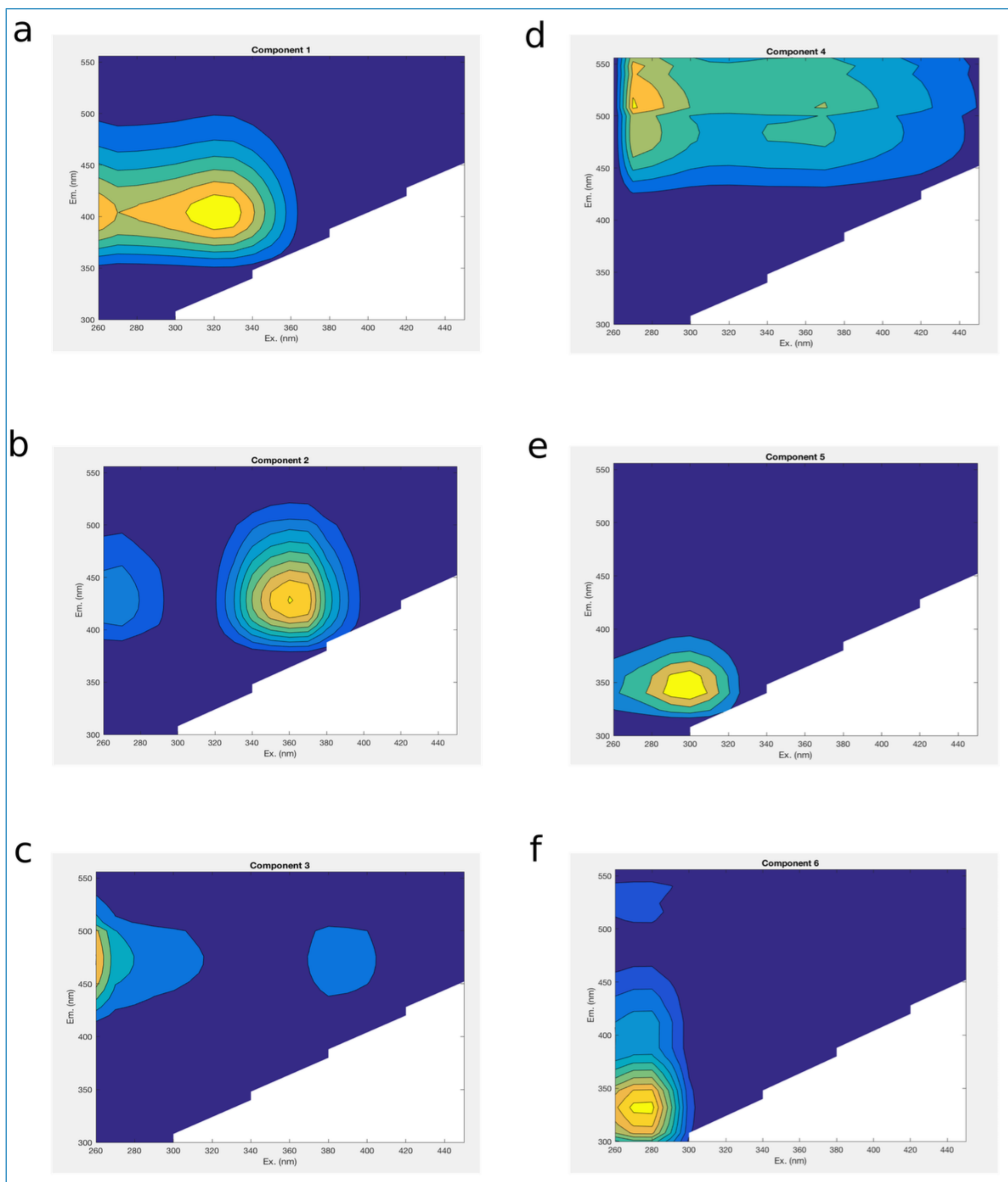


Fig. 5.1. Surface or contour plots of each components created by PARAFAC modeling (a-f). The counter plots present the spectral shapes of the Ex. and Em. of the components. Component 1(a), Component 2(b), Component 3(c), Component 4(d), Component 5(e) and Component 6(f). The unit of the fluorescence intensity is calibrated and normalized in RU (see table 5.1 for more detail).

Table 5.1. Fluorescence components obtained in this study with PARAFAC analysis (C1, C2, C3, C4, C5, and C6) and their equivalences in the EEMs peaks using the nomenclature of Coble et al. (1996). We reported also different sources from the literature where these components have been found previously. Aquaculture sources is marked with the * symbol.

Components	Excitation maximum (nm)	Emission maximum (nm)	Coble *et al.* (1996)	Description	Sources
C1	260 (320-330)	396-412	C	Humic-like	Urban sewage [a]
C2	270 (350-370)	420-436	A+C	Humic-like	Microbial degradation of Terrigenous (anthropogenic & degraded humic) [k], Terrestrial decomposition of plant or soil organic) [i]
C3	260	468-484	A	Humic-like	Marine humic waters [g, k]
C4	270	508	Undefined	----	No literature
C5	290-300	340	T	Tryptophan like amino acid like	Fish feces and food remains *[h], Feeding on aquaculture system*[c], Autochthonous [j, d], Marine & terrigenous (biological degradation) [b], anthropogenic activities [e], Marine blast water [f], Urban sewage [g, a]
C6	280	332	B	Tyrosine like amino acid like	Fish feces and food remains also Feeding on aquaculture system *[c], Autochthonous [l], Microbial delivered [i]

[a]Baker. (2001), [b]Chari *et al.* (2013), [c]Humbly *et al.* (2015), [d]Kowalczuk *et al.* (2009), [e]Liu *et al.* (2014), [f]Murphy *et al.* (2006), [g]Mustafa *et al.* (2010), [h]Nimptsch *et al.* (2015), [i]Shutova *et al.* (2014), [j]Stedmon & Markager (2005), [k]Yamashita *et al.* (2008), [l]Yang *et al.* (2016).

Time-series of the PARAFAC components in the big-volume tanks

The time-series of the six components (C1, C2, C3, C4, C5, and C6) for inlet waters and effluent waters of + *holothurian* tank and *-holothurian* tank is shown in Figure 5.2. In the inlet waters, C1 fluorescence intensity ranged from 2.6 $x10^{-3}$ to 29.1 $x10^{-3}$ RU (Fig. 5.2a, white circles) with the maximum values of this component recorded during the summers. In the effluent of the *-holothurian* tank, C1 fluorescence intensity ranged from 9.8 $x10^{-3}$ to 28.4 $x10^{-3}$ RU (Fig. 5.2a, grey triangles), which are frequently higher than the effluent of the + *holothurian* tank. In the effluent of the *+holothurian* tank, C1 fluorescence intensity ranged from 4.8 $x10^{-3}$ to 22.5 $x10^{-3}$

RU (Fig. 5.2a, red squares). In the inlet waters, C2 fluorescence intensity ranged from 2.2 $x10^{-3}$ to 37.9 $x10^{-3}$ RU(Fig. 5.2b, white circles) with the maximum values also recorded during the summers like C1. In the effluent of the *-holothurian* tank, C2 fluorescence intensity ranged from 3.1 $x10^{-3}$ to 39.5 $x10^{-3}$ RU (Fig. 5.2b, grey triangles). In the effluent of the *+holothurian* tank, C2 fluorescence intensity ranged from 0.9 $x10^{-3}$ to 24.0 $x10^{-3}$ RU (Fig. 5.2b, red squares); which was usually lower than the intensity in the inlet waters and in the *–holothurian* tank. In the inlet waters, C3 fluorescence intensity ranged from 10.3 $x10^{-3}$ to 65.2 $x10^{-3}$ RU (Fig. 5.2c, white circles). In the effluent of the *-holothurian* tank, C3 fluorescence intensity ranged from 12.2 $x10^{-3}$ to 37.2 $x10^{-3}$ RU (Fig. 5.2c, grey triangles). In the effluent of the *+holothurian* tank, C3 fluorescence intensity ranged from 12.4 $x10^{-3}$ to 60.2 $x10^{-3}$ RU (Fig. 5.2c, red squares). All the values of C3 fluorescence intensity were very uniform in the inlet waters and effluents as well as during the time series except in the last sampling days. In the inlet waters, C4 fluorescence intensity ranged from 5.4 $x10^{-3}$ to 17.7 $x10^{-3}$ RU (Fig. 5.2d, white circles). In the effluent of the *-holothurian* tank, C4 fluorescence intensity ranged from 6.3 $x10^{-3}$ to 14.3 $x10^{-3}$ RU (Fig. 5.2d, grey triangles). In the effluent of the *+holothurian* tank, C4 fluorescence intensity ranged from 2.4 $x10^{-3}$ to 10.6 $x10^{-3}$ RU (Fig. 5.2d, red squares). These last values were usually lower than the values obtained in the *-holothurian* tank. In the inlet waters, C5 fluorescence intensity ranged from 3.3 $x10^{-3}$ to 26.8 $x10^{-3}$ RU(Fig. 5.2e, white circles). In the effluent of the *-holothurian* tank, C5 fluorescence intensity ranged from 5.2 $x10^{-3}$ to 14.9 $x10^{-3}$ RU (Fig. 5.2e, grey triangles). In the effluent of the *+holothurian* tank, C5 fluorescence intensity ranged from 1.1 $x10^{-3}$ to 11.4 $x10^{-3}$ RU (Fig. 5.2e, red squares). These last values were usually lower than the values obtained in the effluent of the *-holothurian* tank and in the inlet waters. In the inlet waters, C6 fluorescence intensity ranged from 5.1$x10^{-3}$ to 30.2 $x10^{-3}$ RU (Fig. 5.2f, white circles). In the effluent of the *-holothurian* tank, C5 fluorescence intensity ranged from 15.2 $x10^{-3}$ to 79.1 $x10^{-3}$ RU (Fig. 5.2f, grey triangles). In the effluent of the *+holothurian* tank, C6 fluorescence intensity ranged from 3.7 $x10^{-3}$ to 19.7 $x10^{-3}$ RU (Fig. 5.2f, red squares). These last values were usually lower than the values obtained in the effluent of *-holothurian* tank and in the inlet waters.

To determine if the incorporation of

holothurians in the tank produces significant changes in the different fluorescent components of dissolved organic matter, we performed paired t-test (for normally distributed variables) or Wilcoxon matched-pairs test (for non normally distributed variables) (Table 5.2). We also pooled all the time-series data in median values, 25-75 % percentile of the non-outlier or extreme values (Fig. 5.3).

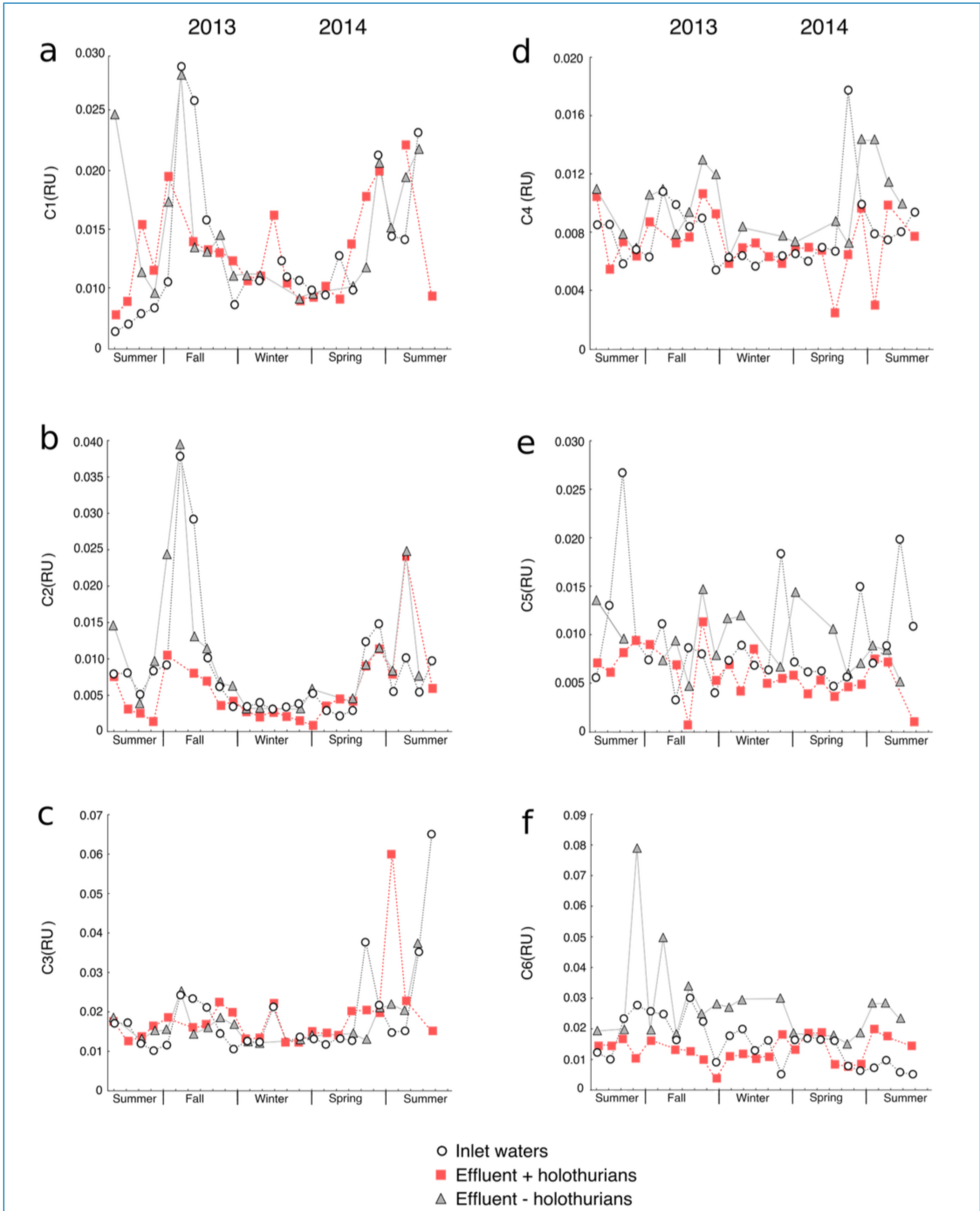


Fig.5.2. Seasonal dynamics of component 1 (a), component 2 (b), component 3 (c), component 4 (d), component 5 (e), component 6 (f) in the inlet waters (white circles), and in the effluent of the +holothurian tank (red squares) and -holothurian tank (grey triangles).

Table 5.2. Results of paired t-test (for normally distributed variables) and Wilcoxon matched pairs test (for not normally distributed variables) between inlet waters and effluents for the different components obtained with PARAFAC. Bold means statistically significant differences.

Statistical Analysis		*t or z*	*p*-value
Inlet waters vs. +holothurian effluent			
C1	Paired t-test	42.19	**0.0000**
C2	Paired t-test	2.45	**0.0223**
C3	Wilcoxon matched pairs test	1.11	0.2669
C4	Wilcoxon matched pairs test	4.19	**0.0000**
C5	Paired t-test	48.92	**0.0000**
C6	Paired t-test	1.28	0.2135
Inlet waters vs. –holothurian effluent			
C1	Paired t-test	1.63	0.1191
C2	Paired t-test	1.26	0.2228
C3	Wilcoxon matched pairs test	1.2	0.2273
C4	Wilcoxon matched pairs test	2.49	**0.0125**
C5	Wilcoxon matched pairs test	0.92	0.3546
C6	Wilcoxon matched pairs test	3.34	**0.0008**
+ holothurian effluent vs. -holothurian effluent			
C1	Paired t-test	62.75	**0.0000**
C2	Paired t-test	25.7	**0.0000**
C3	Wilcoxon matched pairs test	3	**0.0026**
C4	Paired t-test	3.35	**0.0040**
C5	Wilcoxon matched pairs test	3.62	**0.0002**
C6	Wilcoxon matched pairs test	3.62	0.0002

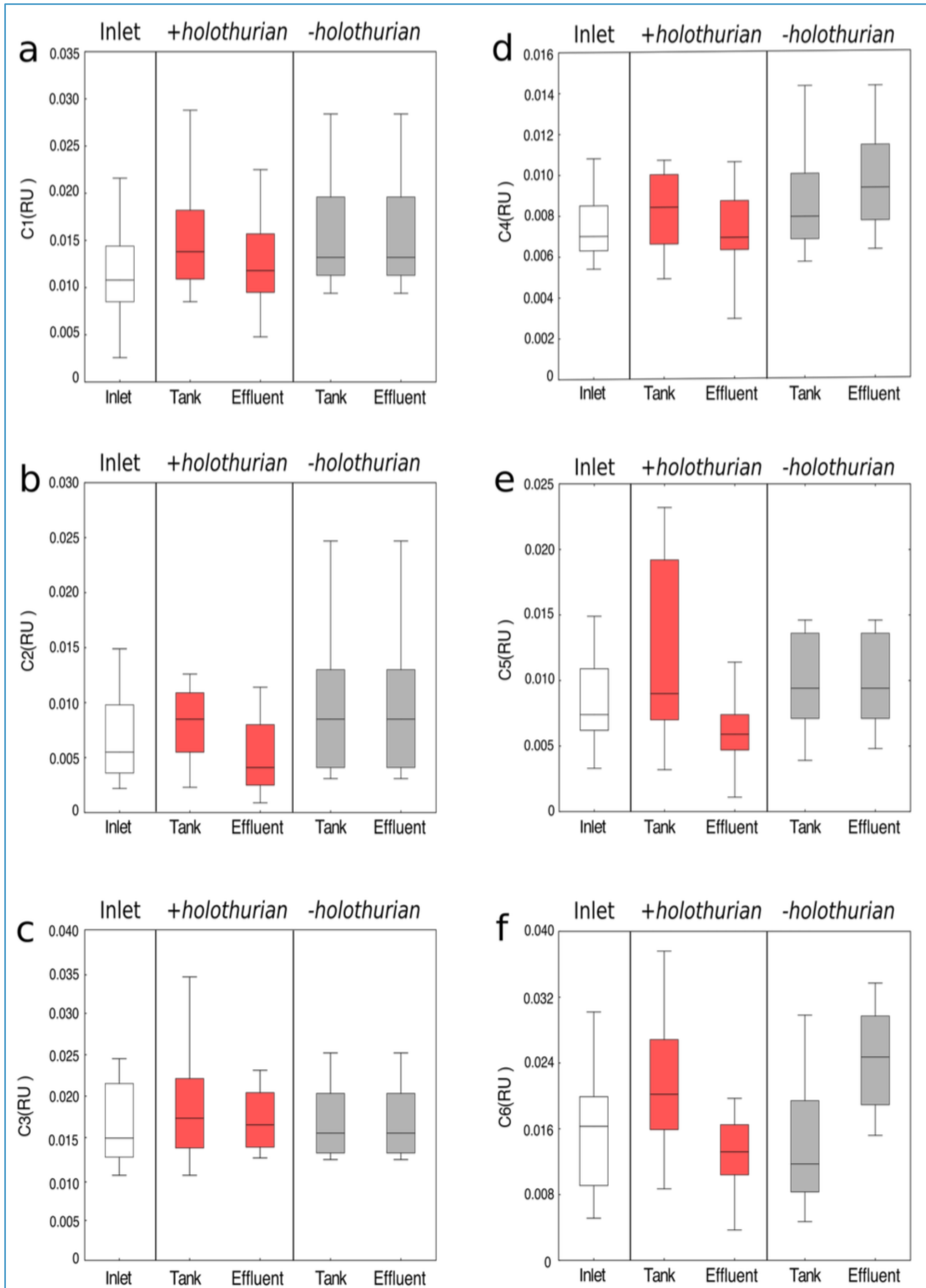


Fig.5.3. Median (line), the 25-75% percentile (box), and the non-outliers range(whisker), of the FDOM components of dissolved organic matter pooling all-time series data. Values of (a) component 1 (C1), (b) component 2 (C2), (c) component 3 (C3), (d) component 4 (C4), (e) component 5 (C5), (f) component 6 (C6) in the inlet water (white box), in the +holothurian effluent and tank (red boxes) and in the –holothurian effluent and tank (grey boxes).

In three out of four humic- like components (C1, C2, and C4) we found significant differences between the inlet waters and the effluent of the tank containing holothurians *(+ holothurian* tank) (Table 5.2). We found a significant reduction of components C1, C2, and C4 in the effluents in the +*holothurian* tank (Fig. 5a, b and d) in comparison with the inlet waters as well as with the effluent from the tank without holothurians (Table 5.2). We did not observe significant differences between the inlet waters and the +*holothurian* tank and its corresponding effluent for C3 (Fig. 5.3c) (Table 5.2). In relation to the amino acid-like components (C5 and C6), we found a significant reduction in the component C5 (Table 5.2) in the effluent of +*holothurian* tank in comparison with the inlet waters. We did not find statistically significant differences between inlet waters and the effluent of the -holothurian tank for most components, except C4 and C6 (Table 5.2); which were higher in the –*holothurian* effluent (Fig. 5d,f).

The fluorescence intensity of the six components was significantly different between the effluents with and without holothurians (Table 5.2). These results suggest a strong influence of the presence of holothurians both for the humic-like components as well as the amino acids like components. The presence of holothurians in the tank significantly reduced all the fluorescent components. There are just a few studies that characterize aquaculture FDOM (Hambly *et al*., 2015; Nimptsch *et al*., 2015; Wang *et al*., 2017). Both Hambly *et al*. (2015) and Nimptsch *et al*. (2015) reported that traditional aquaculture of rain trout and salmon, respectively, produced effluent waters with loads of fluorescence components. These authors attribute the increases of amino acid-like components in aquaculture effluents to the protein content in fish food. The only work that includes multitrophic experiments was Wang *et al*. (2017). They found that in aquaculture mesocosms including both seaweeds and bivalves higher concentrations of both humic-like components and amino acid-like components were produced in comparison with control or just seaweeds or bivalves. We found just the opposite results. In the multitrophic tank (including holothurians as extractive species) there was a reduction in fluorescence components in comparison with the tank including only sea anemones.

Short-term experiment

To corroborate the results observed in the big-volume tanks, we performed three short-term (3 days) experiments without adding fish food for sea anemones to minimize this potential source of fluorescence components. In these experiments we obtained 42 EEMs, but only 40 were included in PARAFAC analysis. Two samples were removed as outliers.

At the initial time of the experiments, we did not find statistically significant differences between the treatment with holothurians (+H) and the treatments without holothurians (-H) indicating the experiments started with identical conditions (Table 5.3, Fig. 5.4). After three days, at the final time, we found significant differences between treatments for the humic- like components C2, C4 (just in one experiment) and for the amino acid-like components C5 and C6 in the three experiments (Table 5.3).

Table 5.3. Results of the analysis of variance (ANOVA) in the three experiments performed to compare FDOM components (C1-C6) in the treatments with holothurians (+H) vs. the treatments without holothurians (-H) at the initial and the final times. Bold means that there is a significant difference between treatments.

INICIAL TIME						
	Experiment # 1		Experiment # 2		Experiment # 3	
	F	*p-value*	*F*	*p-value*	*F*	*p-value*
C1	21.3	0.077	0.45	0.532	10.5	0.081
C2	2.14	0.216	1.76	0.241	1.1	0.352
C3	7.43	0.057	3.01	0.143	2.81	0.168
C4	3.6	0.131	0.41	0.550	0.22	0.663
C5	10.0	0. 104	0.06	0.821	1.30	0.316
C6	3.034	0.156	1.13	0.334	0.18	0.686
FINAL TIME						
	Experiment # 1		Experiment # 2		Experiment # 3	
	F	*p-value*	*F*	*p-value*	*F*	*p-value*
C1	1.31	0.315	0.08	0.794	0	0.872
C2	**77.07**	**0.000**	**57.2**	**0.000**	**7067**	**0.000**
C3	0.49	0.522	0.02	0.894	0.57	0.488
C4	**78.7**	**0.000**	3.1	0.138	0.47	0.528
C5	**1222**	**0.000**	**7.7**	**0.038**	**353.9**	**0.000**
C6	**135**	**0.000**	**98.2**	**0.000**	**1407**	**0.000**

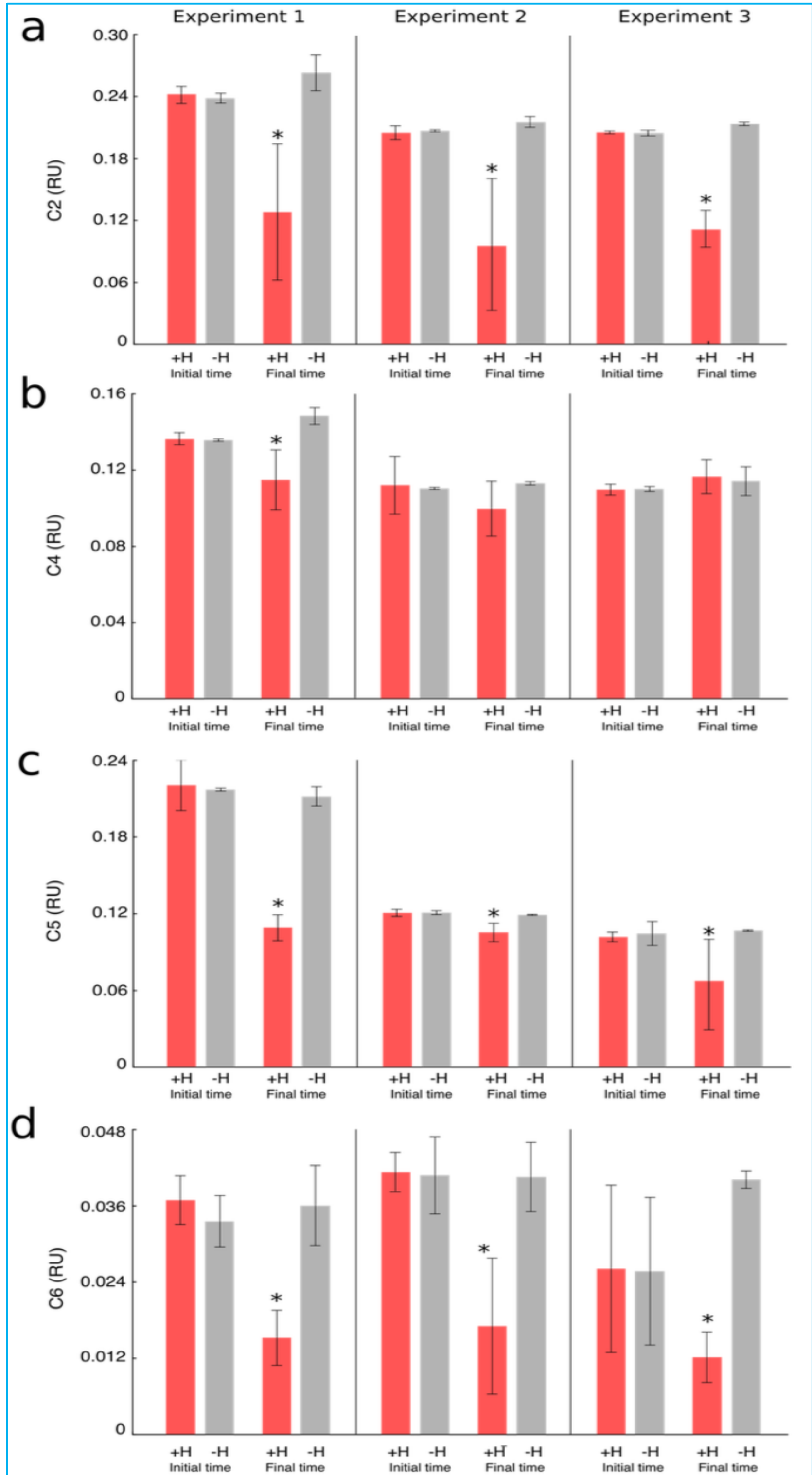


Fig.5.4. Changes in components of fluorescence dissolved organic matter in the three experiments. Mean (bars) and the standard deviations (whiskers) of the replicates of (a) C2, (b) C4, (c) C5 and (d) C6 in the treatments with holothurians (+H) and without holothurians (-H) at the initial and final times. Red bars represent the treatment with holothurians and grey bars represent the treatment without holothurians. Asterisks show the statistically significant results at the final time of the experiments (more details in table 5.3).

The presence of holothurians (+H treatment) significantly reduced the values of the C2 at the final time in the three experiments (Fig. 5.4a, red bars) and just in one of the experiments for the component C4 (Fig. 5.4b, red bars). This result contrasts with the results obtained by Wang *et al.* (2017) using bivalves as extractive species. Perhaps the influence of extractive species on humic like components differs with the feeding system. That is, bivalves filter suspended POM whereas holothurians feed detritus, bacteria and organic deposits. The potential of sea cucumbers to extract POM, bacteria, exopolymeric particles and rework organic sediments is well known (Slater & Carton, 2007; 2009; Slater *et al.*, 2009; Chapter 3). The reduction of the humic-like C2 and C4 components by holothurians can have also implications for water transparency as we analyzed in Chapter 4.

The presence of holothurians (+H treatment) significantly reduced at the final time the values of the amino acid-like components C5 (tryptophan-like) and C6 (tyrosine-like) in the three experiments (Fig. 5.4c and d, red bars). These results again contrast with the results obtained by Wang *et al.* (2017) with bivalves, which in multitrophic mesocosms with seaweeds increased the fluorescence intensity of the amino acid-like components. Two non-exclusive explanations can be argued. As we mentioned before for the humic-like components differences between filtration vs. deposit feeders can affect these contrasting results. In addition, we think that the layer of bacteria in the tentacle epidermis and below the cuticle in holothurians that has been found in holothurians (Roberts *et al.,* 1991; Lawrence *et al.,* 2010) can directly assimilate amino acids as Brothers *et al.* (2015) demonstrated. Therefore, holothurians seem to reduce most of the fluorescence components of dissolved organic matter as it can be appreciated in Fig. 5.5; where fluorescence intensity in the tank without holothurian (Fig. 5.5a) clearly was higher than the fluorescence intensity in the tank with holothurian (Fig. 5.5.b).

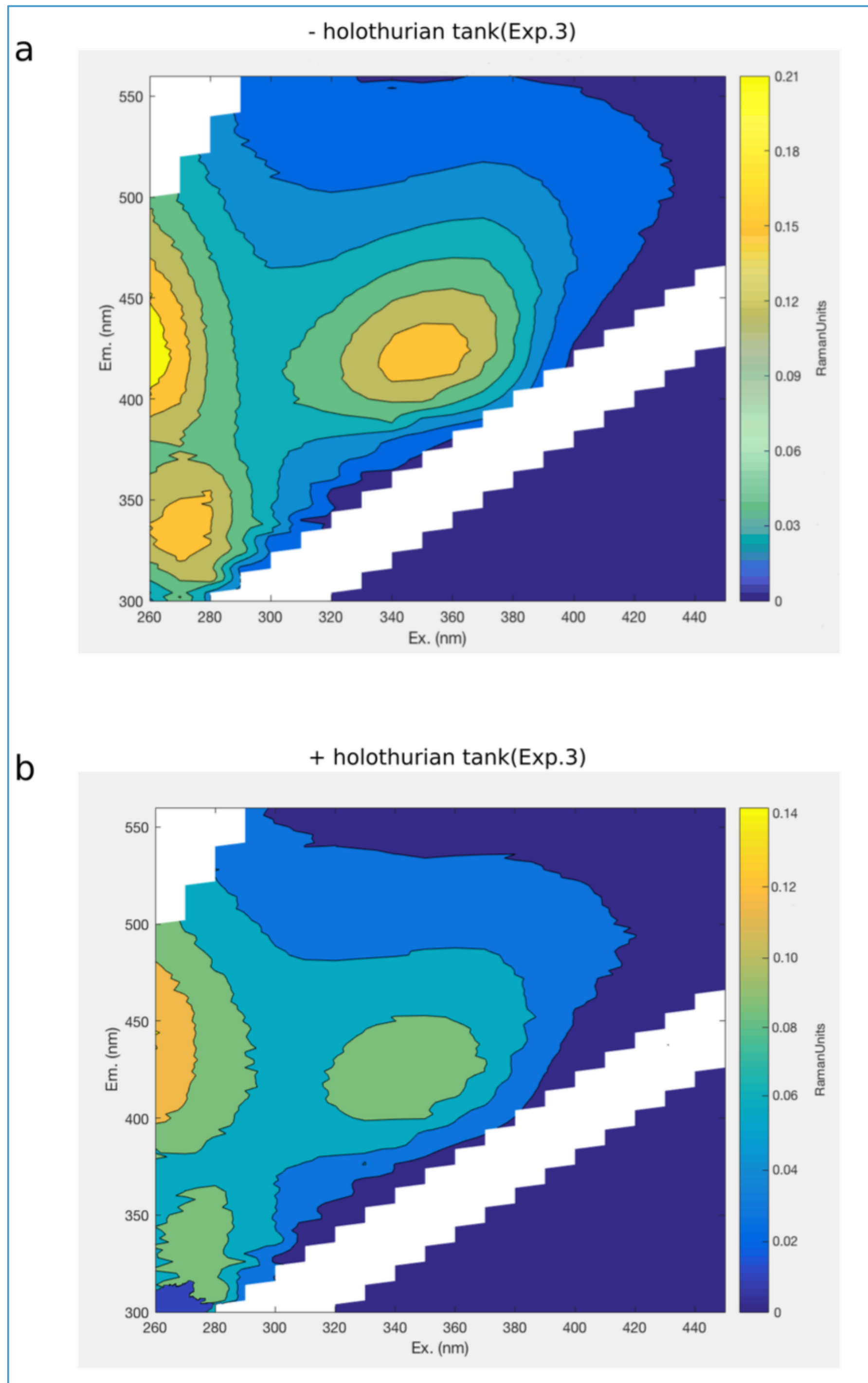


Fig.5.5. Fluorescence intensity in the tank without holothurian (a) and in the tank with holothurian(b) in one of the three short-term experiments.

Reference

Anderson CA, Bro R. 2000. The N-way Toolbox for MATLAB. Chemometrics Intelligent Laboratory System **52**:1-4 DOI: 10.1016/S0169-7439(00)00071-X.

Baker A. 2001. Fluorescence Excitation-Emission Matrix Characterization of some sewage -impacted rivers. *Environmental Science and Technology* **35**: 948-953 DOI 10.1021/es000177t.

Borisover M, Laor Y, Parparov A, Bukhanovsky N, Lado M. 2009. Spatial and seasonal patterns of fluorescent organic matter in Lake Kinneret (Sea of Galilee) and its catchment basin. *Water Research* **43**: 3104-3116 DOI 10.1016/j.watres.2009.04.039.

Bro R. 1997. PARAFAC. Tutorial and applications. *Chemometrics Intelligent Laboratory System* **38**:149–171 DOI 10.1016/S0169-7439(97)00032-4.

Brothers CJ, Lee RW, Nestler JR. 2015. The uptake of dissolved organic material by the sea cucumber *Parastichopus californicus* (Stimpson) and its potential role in visceral regeneration. Journal of Experimental Marine Biology and Ecology **469**:69–75 DOI 10.1016/j.jembe.2015.04.016.

Catala ´TS, Reche I, Fuentes-Lema A, Romera-Castillo C, Nieto-Cid M, Ortega-Retuerta E, CalvoE, A ´lvarez M, Marrase ´C, Stedmon CA, Anto ´n A ´lvarez-Salgado X. 2015. Turnover time of fluorescent dissolved organic matter in the dark global ocean. *Nature Communication* **6**:5986 DOI 10.1038/ncomms6986.

Chávez-Crooker P, Obreque-Contreras J.2010. Bioremediation of aquaculture wastes. *Current Opinion in Biotechnology* **21**:313–317 DOI 10.1016/j.copbio.2010.04.001.

Chintiroglou C, Koukouras A. 1992. The feeding habits of three Mediterranean Sea anemone species, *Anemonia viridis* (Forskal), *Actinia equina* (Linnaeus) and *Cereus pedunculatus* (Pennant). *Helgolander Meeresunters* **46**: 53-68 DOI 10.1007/BF02366212.

Chopin T, Cooper JA, Reid G, Cross S, Moore C. 2012. Open-water integrated multi-trophic aquaculture: environmental biomitigation and economic diversification of fed aquaculture by extractive aquaculture. *Reviews in Aquaculture* **4**: 209-220 DOI 10.1111/j.1753-5131.2012.01074.x.

Coble PG, Green SA, Blough NV, Gagosian RB. 1990. Characterization of dissolved organic matter in the Black Sea by fluorescence spectroscopy. *Nature* **348**: 432–435 DOI 10.1038/348432a0.

Coble PG. 1996. Characterization of marine and terrestrial DOM in seawater using excitation–emission matrix spectroscopy. *Marine Chemistry* **51**:325–346 DOI 10.1016/0304-4203(95)00062-3.

Coble PG. 2007. Marine optical biogeochemistry: the chemistry of ocean color. *Chemical Reviews* **107**:402–418 DOI 10.1021/cr050350+.

Cohen E, Levy GJ, Borisover M. 2014. Fluorescent components of organic matter in wastewater: Efficacy and selectivity of the water treatment. *water research* **55**:323-334 DOI 10.1016/j.watres.2014.02.040.

Del Bel Belluz J, Costa M, Reid G, Cross S. 2016. Bio-optical variability at a Vancouver Island aquaculture site. *Limnology and Oceanography* **61**:1686–1704 DOI 10.1002/lno.10332.

Diana JS, Egna HS, Chopin T, Peterson MS, Cao L, Pomeroy R, Verdegem M, Slack WT, Bondad-reantaso MG, Cabello F. 2013. Responsible Aquaculture in 2050: Valuing Local Condition and Human Innovation will be Key to Success. *BioScience* **4**: 255-262 DOI 10.1525/bio.2013.63.4.5.

Food and Agriculture Organization of the United Nations (FAO). 2009. The state of world fisheries and aquaculture 2008. Rome: Food and Agriculture Organization of the United Nations.

Hall GJ, Clow KE, Kenny JE. 2005. Estuarial finger-printing through multidimensional fluorescence and multivariate analysis. *Environmental Science & Technology* **39**:7560-7567 DOI 10.1021/es0503074.

Hambly AC, Arvin E, Pedersen L-F, Pedersen PB, Seredynska-Sobecka B, Stedmon CA. 2015. Characterizing organic matter in recirculating aquaculture systems with fluorescence EEM spectroscopy. *Water Research* **83**:112-120 DOI 10.1016/j.watres.2015.06.037.

Ibarra DA, Cembella A, Grant J. 2012. Attenuation of sunlight measured from moored radiometers to assess depletion of suspended particles caused by bivalve aquaculture. Limnology and Oceanography: Methods **10**:1051–1069 DOI 10.4319/lom.2012.10.1051.

Kowalczuk P, Durako MJ, Young H, Kahn AE, Cooper WJ, Gonsior M. 2009. Characterization of dissolved organic matter fluorescence in the South Atlantic Bight with use of PARAFAC model: Inter annual variability. *Marine Chemistry* **113**:182–196 DOI 10.1016/j.marchem.2009.01.015.

Kowalczuk P, Ston-Egiert J, Cooper WJ, Whitehead RF, Durako MJ. 2005. Characterization of chromophoric dissolved organic matter (CDOM) in the Baltic Sea by excitation emission matrix fluorescence spectroscopy. *Marine Chemistry* **96**:273–292 DOI 10.1016/j.marchem.2005.03.002.

Lawrence, S. A., O'Toole, R., Taylor, M. W., & Davy, S. K. 2010. Subcuticular bacteria associated with two common New Zealand Echinoderms: characterization using 16S rRNA sequence analysis and fluorescence in situ hybridization. *The Biological Bulletin* **218**: 95-104.

Li WT, Chen SY, Xu ZX, Li Y, Shuang CD, Li AM. 2014. Characterization of Dissolved Organic Matter in Municipal Wastewater Using Fluorescence PARAFAC Analysis and Chromatography Multi-Excitation/Emission Scan: A Comparative Study. *Environmental Science & Technology* **48**:2603–2609 DOI 10.1021/es404624q.

Liu X, Zhang Y, Shi K, Zhu G, Xu H, Zhu M. 2014. Absorption and fluorescence properties of chromophoric dissolved organic matter: implications for the monitoring of water quality in a large subtropical reservoir. *Environmental Science and Pollution Research* **21**:14078–14090. *Available at:* https://link.springer.com.

Millar RB, Anderson MJ. 2004. Remedies for pseudo-replication. *Fisheries Research* **70**:397–407 DOI 10.1016/j.fishres.2004.08.016.

Mostofa KMG, Wu F, Liu C-Q, Fang WL, Yuan J, Ying WL, Wen L, Yi M. 2010. Characterization of Nanming River (southwestern China) sewerage-impacted pollution using an excitation- emission matrix and PARAFAC. *Limnology* **11**: 217–231 DOI 10.1007/s10201-009-0306-4.

Murphy, KR, Ruiz GM, Dunsmuir WTM, Waite TD. 2006. Optimized parameters for rapid fluorescence-based verification of ballast water exchange by ships. *Environmental Science & Technology* **40**:2357-2362 DOI 10.1021/es0519381.

Nelson EJ, MacDonald BA, Robinson SMC. 2012b. A review of the northern sea cucumber *Cucumaria frondosa* (Junnerus, 1767) as a potential Aquaculture Species. *Reviews in Fisheries Science* **20**: 212-219 DOI 10.1080/10641262.2012.719043.

Nimptsch J, Woelfl S, Osorio S, Valenzuela J, Ebersbach P, Tuempling WV, Palma R, Encina F, Figueroa D, Kamjunke N, Graeber D. 2015. Tracing dissolved organic matter (DOM) from land-based aquaculture systems in North Patagonian streams. *Science of the Total Environment* **537**:129–138 DOI 10.1016/j.scitotenv.2015.07.160.

Ohno T, Bro R. 2006. Dissolved organic matter characterization using multiway spectral decomposition of fluorescence landscapes. *Soil Science Society of America Journal Abstract - Soil Chemistry* **70**:2028-2037 DOI 10.2136/sssaj2006.0005.

Roberts, D., D. S. M. Billett, G. McCartney, and G. E. Hayes. 1991. Procaryotes on the tentacles of deep-sea holothurians: a novel form of dietary supplementation. *Limnology and Oceanography*. **36**: 1447–1452 DOI 10.4319/lo.1991.36.7.1447.

Sadeghi-Nassaj SM, Catalá TS, Álvarez PA, Reche I. 2018. Sea cucumbers reduce chromophoric dissolved organic matter in aquaculture tanks. *PeerJ* **6:** e4344 DOI 10.7717/peerj.4344.

Shutova Y, Baker A, Bridgeman J, Henderson RK. 2014. Spectroscopic characterization of dissolved organic matter changes in drinking water treatment: from PARAFAC analysis to online monitoring wavelengths. *Water Research* **54**:159-169 DOI 10.1016/j.watres.2014.01.053.

Slater MJ, Carton AG. 2007. Survivorship and growth of the sea cucumber *Australostichopus mollis* (Hutton 1872) in polyculture trials with green- lipped mussel farms. *Aquaculture* **272**: 389–398 DOI 10.1016/j.aquaculture.2007.07.230.

Slater MJ, Carton, AG. 2009. Effect of sea cucumber (*Australostichopus mollis*) grazing on coastal sediments impacted by mussel farm deposition. *Marine Pollution Bulletin* **58**:1123-1129 DOI 10.1016/j.marpolbul.2009.04.008.

Slater MJ, Jeffs AG, Carton AG. 2009. The use of the waste from green-lipped mussels as a food source for juvenile sea cucumber, *Australostichopus mollis*. *Aquaculture* **292**:219–224 DOI 10.1016/j.aquaculture.2009.04.027.

Stedmon CA, Bro R. 2008. Characterizing dissolved organic matter fluorescence with parallel factor analysis: a tutorial. *Limnology and Oceanography Methods* **6**:572–579 DOI 10.4319/lom.2008.6.572.

Stedmon CA, Markager S, Bro R. 2003. Tracing dissolved organic matter in aquatic environments using a new approach to fluorescence spectroscopy. *Marine Chemistry* **82**:239-254 DOI 10.1016/S0304-4203(03)00072-0.

Stedmon CA, Markager S. 2005. Tracing the production and degradation of autochthonous fractions of dissolved organic matter by fluorescence analysis. *Limnology and Oceanography* **50**:1415–1426 DOI 10.4319/lo.2005.50.5.1415.

Stubbins A, Lapierre J-F, Berggren M, Prairie YT, Dittmar T, del Giorgio PA. 2014. What's in an EEM? Molecular Signatures Associated with Dissolved Organic Fluorescence in Boreal Canada. *Environment Science Technology* **48**:10598–10606 DOI 10.1021/es502086e.

Wang X, Wu Y, Jiang Z, Ma Q, Zhang J, Liu S. 2017. Quantifying Aquaculture-derived Dissolved Organic Matter in the Mesocosms of Sanggou Bay Using Excitation-emission Matrix Spectra and Parallel Factor Analysis. *Journal of the world aquaculture society* **48**:909-926 DOI: 10.1111/jwas.12409.

Yamashita Y, Jaffe R, Maie N, Tanoue E. 2008. Assessing the dynamics of dissolved organic matter (DOM) in coastal environments by excitation and emission matrix fluorescence and parallel factor analysis (EEM-P ARAF AC). *Limnology and Oceanography* **53**:1900-1908 DOI 10.4319/lo.2008.53.5.1900.

Yokoyama H. 2013. Growth and food source of the sea cucumber *Apostichopus japonicus* cultured below fish cages. Potential for integrated multitrophic aquaculture. *Aquaculture* **372-375**:28–38 DOI 10.1016/j.aquaculture.2012.10.022.

Yokoyama H. 2015. Suspended culture of the sea cucumber *Apostichopus japonicus* below a Pacific oyster raft-potential for integrated multi-trophic aquaculture. *Aquaculture Research* **46**:825-832 DOI 10.1111/are.12234.

Zepp RG, Sheldon WM, Moran MA. 2004. Dissolved organic fluorophores in southeastern US coastal waters: correction method for eliminating Rayleigh and Raman scattering peaks in excitation- emission matrices. *Marine Chemistry* **89**:15–36 DOI 10.1016/j.marchem.2004.02.006.

Zhang Y, Yin Y, Feng L, Zhu G, Shi Z, Liu X. 2011. Characterizing chromophoric dissolved organic matter in Lake Tianmuhu and its catchment basin using excitation-emission matrix fluorescence and parallel factor analysis. *water research* **45**:5110-5122 DOI 10.1016/j.watres.2011.07.

General Discussion

The exponential growth of human population has increased the global demand of proteins and, consequently, of fish and for seafood. During last decades, human activities such as overfishing, addition of pollutants and climate change are substantially affecting species stocks and diversity in marine ecosystems (Halpern *et al.,* 2008; Purcell *et al.,* 2013). Since extractive fisheries are more and more limited, aquaculture currently accounts for more than 50% of human consumption of fish and seafood (FAO, 2016). Traditional aquaculture produces wastewater that usually contains high loads of organic and inorganic nutrients, antibiotic and uneaten food pellets (Black, 2001; Read & Fernandes, 2003; Klinger & Naylor, 2012) affecting the marine environment. Integrated multitrophic aquaculture and polycultures, unlike monospecific aquaculture, use extractive species that use the excretion, fecal and food wastes from the primary species are nutritional resources for the extractive species reducing environmental impact (Chopin *et al*., 2012).

In this dissertation, we explored the role of sea cucumbers species (*Holothuria tubulosa* and *H. Forskali*) as extractive species in cultures of sea anemones. In addition to their role as extractive species, sea cucumber culture is important for the food supply in many regions (Purcell *et al*., 2013), for conservation and restoration of some endangered species in natural environments in regions like Indo-Pacific or Mediterranean-North Atlantic waters (Froehlich *et al.,* 2017; González-Wangüemert *et al*., 2018) and for pharmaceutical bioprospecting (Bhatnagar & Kim, 2010; León-Palmero *et al*., 2018).

A. Influence of holothurians on mineral nutrients and particulate organic matter in aquaculture effluents

Previous works (Slater & Carton 2009; Nelson *et al.,* 2012; Yokoyama, 2013;2015) showed that sea cucumbers are species with a relevant extractive capacity for organic matter in accordance with our results. The comparison + holothurian and -holothurian showed undoubtedly the reduction of the concentration of POM, bacteria abundance, transparent exopolymer particles (TEP) and nitrate both the time-series and the short-term experiments.

The TEP reduction by holothurians is a very novel result. Both phytoplankton and bacteria release TEP precursors (Passow, 2002; Ortega-Retuerta *et al.,* 2010; Iuculano *et al.,* 2017). We observed a significant

correlation between TEP and chlorophyll-*a* concentration for inlet waters and the effluents of both tanks. Some studies have also demonstrated the digestion diatom cells by holothurians (Yingst, 1976; Hammond, 1983; Uthicke, 1999), however, we did not find a chlorophyll-a reduction in + holothurian tank. The remarkable reduction in the TEP concentration in the tanks containing holothurians indicates that TEP were used as a food source by the holothurians with potential implications for tank hygiene since TEP biofilms usually house bacteria (Bar-Zeev *et al*., 2012). Similarly, Wotton (2005, 2011) observed that the reduction of TEP in the water column is mostly due to marine invertebrate. Joyce and Utting (2015) have underlined the role of TEP in hatcheries sequestering micronutrients and toxins. TEP was not the only source of energy used by the holothurians. Bacteria are also considered as a food source for holothurians since have been reported previously (Moriarty, 1982; Moriarty *et al.,* 1985; Amon & Herndl, 1991) and we found in this study. We showed significant differences in the abundance of bacteria between the – holothurian effluent and the inlet waters. The bacterial abundance in the +holothurian tank was lower than in the –holothurian tank suggesting a net consumption of bacteria by holothurians. It seems, holothurian prefer to consume bacteria in compare with phytoplankton cells. Therefore, the holothurian controlling role on bacterial proliferation and TEP biofilm formation could minimize the risk of pathogenic outbreak (e.g., *Vibrio sp*.). That is, holothurians improve the hygiene conditions in aquaculture tank controlling both TEP biofilms and bacterial populations.

The direct consumption of particulate organic matter (POM) by holothurians diminishes the mineralization process and the release of mineral nutrients such as nitrate. In addition, due to direct absorption of holothurian on ammonium by their skin (Binyon, 1972) could slowdown nitrification process (converting ammonium to nitrite and nitrate form). The reduction of nitrate concentration by holothurian confirmed its environmental importance in IMTA installations. Therefore, our finding obviously confirmed that *H. tubolosa* individuals obtain their energy requirements via direct consumption of particulate compounds such as POM, bacteria and TEP improving tank hygiene and water quality. This information could help producers and aquatic scientist when *H. tubolosa* is

cultured (target species) in controlled condition for human consumption or /and sea-reaching (recovering of natural resources) especially in regions where this species have been overfished such as Turkey, Spain, Portugal (González-Wangüemert *et al*., 2018).

B. Influence of holothurians on chromophoric dissolved organic matter

Chromophoric dissolved organic matter (CDOM) includes humic like substances, lignin, phenol and amino acid-like substances that absorb UV and blue light affecting water transparency. Our results showed that CDOM parameters such as the absorption coefficient at 325 nm (a_{325}), the spectral slope from 275 nm to 295 nm ($S_{275-295}$) and the spectral ratio (S_R) were lower in the + *holothurian* tanks in comparison with - *holothurian* tanks both in the time-series and in the short term experiments. Therefore, the presence of holothurians in the tanks reduced absorption coefficients mostly of chromophoric compounds of low molecular weight as suggests the concomitant reduction in the spectral slope. This reduction improved water transparency. The underlying mechanisms of this water transparency improvement might be related to the POM consumption by holothurians as well as the direct assimilation of dissolved compounds of low molecular weight such as chromophoric amino acids.

POM consumption by holothurians can increase water transparency directly by reducing light absorption and scattering as Ibarra *et al*. (2012) and Del Bel Belluz *et al*. (2016) demonstrated; but also indirectly reducing the concentration of CDOM derived from POM disaggregation. POM disaggregation into dissolved components is a common process in coastal waters (He *et al*., 2016), particularly under sunny conditions (Shank *et al*., 2011; Pisani *et al*., 2011).

A direct uptake of free amino acids in several tissues as the respiratory trees, epidermis, and oral tentacles of a sea cucumber species (*Parastichopus californicus*) during the visceral regeneration has been demonstrated by Brothers et al. (2015) using a direct uptake of free ^{15}N-amino acids. Therefore, it might be possible that the reduction of the low molecular weight chromophoric compounds is due to assimilation of amino acids with chromophoric groups such as tyrosine, tryptophan and phenylalanine. This assimilation could be related to the presence of subcuticular bacteria in holothurians. These bacteria are common in holothurians

and are related to dietary supplementation (Roberts *et al*., 1991; Lawrence *et al*., 2010).

C. Influence of holothurians on fluorescence dissolved organic matter

Fluorescence spectroscopy (excitation and emission matrixes, EEMs) and parallel factor (PARAFAC) analysis are very powerful procedures to characterize DOM components in more refractory and humic-like compounds vs. more labile and amino acid-like compounds. We obtained six components: four (C1, C2, C3 and C4) humic-like components and two (C5 and C6) amino acid-like components. In the short-term experiments, we observed than holothurians reduced significantly both humic-like (C2 and C4) and amino acid-like components (C5 tryptophan-like and C6 tyrosine like). Like for the case of CDOM, this reduction in FDOM components can be attributed to two non-exclusive explanations. First, holothurians due to their great efficiency removing particulate organic matter and, consequently, FDOM derived from POM disaggregation (He *et al*., 2016). Second, we think that the layer of bacteria in the tentacle epidermis and below the cuticle in holothurians can directly assimilate tryptophan and tyrosine.

Yang *et al*. (2006) suggested that proteinaceous materials are essential for sea cucumbers growth. We think that the reduction of C5 and C6 was related with the assimilation of tryptophan and tyrosine, which have low molecular weight and have fluorescence. This assimilation could be due to symbiotic bacteria present in holothurians in diverse tissue such as epidermis, reparatory trees, oral tentacles, subcuticle layers and commensal gut microbiome (Brother *et al*., 2015; Lawrence *et al*., 2010; Robert *et al*., 1991; Deming & Colwell, 1982; Barlocher & Kendrick, 1978; Phillips, 1984). Hambly *et al*. (2015) concluded that the component (C5) as amino acid originated from feeding in recirculation aquaculture system. In another study, both tryptophan-like and tyrosine-like components were originated from feeding, food remains and fish faces (Nimptsch *et al*., 2015). However, we found this reduction in amino acid-like components even in the short-term experiments where we did not feed the sea anemones. Sibuet *et al*. (1982) estimated that almost 20% of holothurians energy requirements supply via proteinaceous compounds.

Biodegradability of amino acids and humic substances in tank effluents has environmental importance. Tryptophan-like has highly degradability in aquatic environment (Fellman *et al*., 2009; Mostofa *et al*., 2010). Nimptsch *et al*. (2015) concluded that amino acid-like tryptophan is a highly degradable substance in effluents of salmon mono-specific aquaculture. Therefore, the incorporation of extractive species as sea cucumbers can reduce the load of biodegradable organic matter with obvious improvements in water quality and oxygen demands.

References

Amon RMW, Herndl GJ. 1991. Interrelationship between *Holothuria tubulosa* (Holothurioida, Echinodermata) and the sediment microbial community. *Marine Ecology* **12**:163-174.

Bar-Zeev E, Berman-Frank I, Girshevitz O, Berman T. 2012. Revised paradigm of aquatic biofilm formation facilitated by microgel transparent exopolymer particles. *Proceedings of the National Academy of Sciences* **109**: 9119-9124.

Barlocher F, Kendrick B. 1978. Hyphomycetes as intermediaries of energy flow in streams. In, Recent advances in aquatic mycology, edited by E.B. Gareth Jones, Halsted Press, New York, 435-446

Bhatnagar I, Kim SK. 2010. Immense essence of excellence: Marine Microbial Bioactive Compounds. *Marine Drugs* **8**: 2673-2701 DOI 10.3390/md8102673.

Binyon G.1972. Physiology of Echinoderms. Chapter Three (Animalia), Echinodermata, Margulis L, Chapman MJ.Kingdoms and Domains. Academic Press (AP), UK, p 348-351

Black KD. 2001. Environmental Impacts of Aquaculture. *Aquaculture Research* **28**: 829- 839.

Brothers CJ, Lee RW, Nestler JR. 2015. The uptake of dissolved organic material by the sea cucumber *Parastichopus californicus* (Stimpson) and its potential role in visceral regeneration. *Journal of Experimental Marine Biology and Ecology* **469**:69–75 DOI 10.1016/j.jembe.2015.04.016.

Chopin T, Cooper JA, Reid G, Cross S, Moore C. 2012. Open-water integrated multitrophic aquaculture: environmental biomitigation and economic diversification of fed aquaculture by extractive aquaculture. *Reviews in Aquaculture* **4**:209–220 DOI 10.1111/j.1753-5131.2012.01074.x.

Del Bel Belluz J, Costa M, Reid G, Cross S. 2016. Bio-optical variability at a Vancouver Island aquaculture site. Limnology and Oceanography **61**:1686–1704 DOI 10.1002/lno.10332.

Demingt JW, Colwell RR.1982. Barophilic Bacteria Associated with Digestive Tracts of Abyssal Holothurians. Applied and Environmental Microbiology **44**: 12227–1230.

Food and Agriculture Organization of the United Nations (FAO). 2016. The State of World Fisheries and Aquaculture 2016. www.fao.org/documents/card/es/c/2c8bcf47-2214-4aeb-95b0-62ddef8a982a.

Fellman J, Hood E, D'Amore D, Edwards R, White D. 2009. Seasonal changes in the chemical quality and biodegradability degradation of dissolved organic matter exported from soils to streams in coastal temperate rainforest watersheds. *Biogeochemistry* **95**: 277–293 DOI 10.1007/s10533-009- 9336-6.

Froehlich, H. E., Gentry, R. R., & Halpern, B. S. 2017. Conservation aquaculture: Shifting the narrative and paradigm of aquaculture's role in resource management. *Biological Conservation* **215**: 162-168 DOI 10.1016/j.biocon.2017.09.012.

González-Wangüemert, M., Domínguez-Godino, J. A., & Cánovas, F. 2018. The fast development of sea cucumber fisheries in the Mediterranean and NE Atlantic waters: From a new marine resource to its over-exploitation. *Ocean & Coastal Management* **151**: 165-177 DOI 10.1016/j.ocecoaman.2017.10.002.

Halpern BS, Walbridge S, Selkoe KA, Kappel CV, Micheli F, D'Agrosa C, Bruno JF, Casey KS, Ebert C, Fox HE, Fujita R, Heinemann D, Lenihan HS, Madin EMP, Perry MT, Selig ER, Spalding M, Steneck R, Watson R. 2008. A global map of human impact on marine ecosystems. *Science Careers* **319**: 948–952 DOI 10.1126/science.1149345.

Hambly AC, Arvin E, Pedersen L-F, Pedersen PB, Seredynska-Sobecka B, Stedmon CA. 2015. Characterizing organic matter in recirculating aquaculture systems with fluorescence EEM spectroscopy. *Water Research* **83**:112-120 DOI 10.1016/j.watres.2015.06.037.

Hammond LS.1983. Nutrition of deposit-feeding holothuroids and echinoids (Echinodermata) from a shallow reef lagoon, Discovery Bay, Jamaica. *Marine Ecology Progress Series* **10**: 297–305.

He W, Chen M, Schlautman MA, Hur J. 2016. Dynamic exchanges between DOM and POM pools in coastal and inland aquatic ecosystems: a review. *Science of the Total Environment* **551–552**:415–428 DOI 10.1016/j.scitotenv.2016.02.031.

Ibarra DA, Cembella A, Grant, J. 2012. Attenuation of sunlight measured from moored radiometers to assess depletion of suspended particles caused by bivalve aquaculture. *Limnology and Oceanography Methods* **10**:1051–1069 DOI 10.4319/lom.2012.10.1051.

Iuculano F, Mazuecos IP, Reche I, Agustí S. 2017. Prochlorococcus as a possible source for Transparent Exopolymer Particles (TEP). *Frontiers in Microbiology* **8**:709 DOI 10.3389/fmicb.2017.00709.

Joyce A, Utting S. 2015. The role of exopolymers in hatcheries: an overlooked factor in hatchery hygiene and feed quality. *Aquaculture* **446**: 122-131 DOI 10.1016/j.aquaculture.2015.04.037.

Klinger D, Naylor R. 2012. Searching for solutions in aquaculture: charting a sustainable course. *The Annual Review of Environment and Resources* **37**: 247-276 DOI 10.1146/annurev-environ-021111-161531.

Lawrence SA, O'Toole R, Taylor MW, Davy SK. 2010. Subcuticular bacteria associated with two common New Zealand Echinoderms: characterization using 16S rRNA sequence analysis and fluorescence in situ hybridization. *The Biological Bulletin* **218**: 95-104.

León-Palmero E, Joglar V, Álvarez PA, Martín-Platero A, Llamas I, Reche I. 2018. Diversity and antimicrobial potential in sea anemone and holothurian microbiomes. *PloS one* **13**: 0196178 DOI 10.1371/journal.pone.0196178.

Moriarty DJW, Pollard PC, Hunt WG, Moriarty CM, Wassenberg TJ. 1985. Productivity of bacteria and microalgae and the effect of grazing by holothurians in sediments on a coral reef flat. *Marine Biology* **85**: 293-300. Available at: https://link.springer.com/article/10.1007(accessed 05 January 1985).

Moriarty DJW. 1982. Feeding of *Holoturia atra* and *Stichopus chloronotus* on bacteria, organic carbon and organic nitrogen in sediments of the Great Barrier Reef. *Australian Journal of Marine and Freshwater Research* **33**: 255-263 DOI 10.1071/MF9820255.

Mostofa KMG, Wu F, Liu C-Q, Fang WL, Yuan J, Ying WL, Wen L, Yi M. 2010. Characterization of Nanming River (southwestern China) sewerage-impacted pollution using an excitation- emission matrix and PARAFAC. *Limnology* **11**: 217–231 DOI 10.1007/s10201-009-0306-4.

Nelson EJ, MacDonald BA, Robinson SMC. 2012. The absorption efficiency of the suspension-feeding sea cucumber, *Cucumaria frondosa*, and its potential as an extractive integrated multi-trophic aquaculture (IMTA) species. *Aquaculture* **370-371**:19-25 DOI 10.1016/j.aquaculture.2012.09.029.

Nimptsch J, Woelfl S, Osorio S, Valenzuela J, Ebersbach P, Tuempling WV, Palma R, Encina F, Figueroa D, Kamjunke N, Graeber D. 2015. Tracing dissolved organic matter (DOM) from land-based aquaculture systems in North Patagonian streams. *Science of the Total Environment* **537**:129–138 DOI 10.1016/j.scitotenv.2015.07.160.

Ortega-Retuerta E, Duarte CM, Reche I. 2010. Significance of Bacterial Activity for the distribution and dynamics transparent exopolymer particles in the Mediterranean Sea. *Microbial Ecology* **59**:808–818 DOI 10.1007/s00248-010-9640-7.

Passow U. 2002. Transparent exopolymer particles (TEP) in aquatic environments. *Progress in Oceanography* **55**: 287 – 333.

Phillips N. 1984. Role of different microbes and substrates as potential suppliers of specific, essential nutrients to marine detritivores. *Bulletin of Marine Science* **35**: 283–298.

Pisani O, Yamashita Y, Jaffé R. 2011. Photo-dissolution of flocculent, detrital material in aquatic environments: contributions to the dissolved organic matter pool. *Water Research* **45**:3836–3844 DOI 10.1016/j.watres.2011.04.035.

Purcell SW, Mercier A, Conand C, Hamel JF, Toral-Granda MV, Lovatelli A, Uthicke S. 2013. Sea cucumber fisheries: global analysis of stocks, management measures and drivers of overfishing. *Fish and Fisheries* **14**:34–59 DOI 10.1111/j.1467-2979.2011.00443. x.

Read P, Fernandes T. 2003. Management of environmental impacts of marine aquaculture in Europe. *Aquaculture* **226**: 139–163 DOI 10.1016/S0044-8486(03)00474-5.

Roberts D, Gebruk A, Levin V, Manship BAD. 2000. Feeding and digestive strategies in deposit feeding holothurians. In: Barnes H, Gibson NH, Barnes M, eds. *Oceanography and Marine Biology*. London: Taylor and Francis, 257–310.

Shank GC, Evans A, Yamashita Y, Jaffé R. 2011. Solar radiation-enhanced dissolution of particulate organic matter from coastal marine sediments. *Limnology and Oceanography* **56**:577–588 DOI 10.4319/lo.2011.56.2.0577.

Sibuet M, Khripounoff A, Deming J, Colwell R, Dinet A. 1982. In: Lawrence JM, ed. Modification of the Gut Contents in the Digestive Tract of Abyssal Holothurians. Netherlands: International Echinoderms Conference, 421–428.

Slater MJ, Carton, AG. 2009. Effect of sea cucumber (*Australostichopus mollis*) grazing on coastal sediments impacted by mussel farm deposition. *Marine Pollution Bulletin* **58**:1123-1129 DOI 10.1016/j.marpolbul.2009.04.008.

Uthicke S, Karez R.1999. Sediment patch selectivity in tropical sea cucumbers analyzed with multiple choice experiments. Journal of Experimental Marine Biology and Ecology **236**:69-87 DOI 10.1016/S0022-0981(98)00190-7.

Wotton RS. 2005. The essential role of exopolymers (EPS) in aquatic systems. In: Barnes H, Gibson RN, Atkinson RJA, Gordon JDM, ed. Oceanography and Marine Biology. New York (NY): Taylor and Francis Group (CRC), 57–9.

Wotton RS. 2011. EPS (Extracellular Polymeric Substances), silk, and chitin: vitally important exudates in aquatic ecosystems. *Journal of the North American Benthological Society* **30**:762- 769 DOI 10.1899/10-120.1.

Yang H, Zhou Y, Zhang T, Yuan X, Li X, Liu Y, Zhang F. 2006. Metabolic characteristics of sea cucumber *Apostichopus japonicus* (Selenka) during aestivation. *Journal of Experimental Marine Biology and Ecology* **330**: 505–510 DOI 10.1016/j.jeme.2005.09.010.

Yingst JY. 1976. The utilization of organic matter in shallow marine sediments by an epibenthic deposit-feeding holothurian. *Journal of Experimental Marine Biology and Ecology* **23**: 55–69.

Yokoyama H. 2013. Growth and food source of the sea cucumber *Apostichopus japonicus* cultured below fish cages. Potential for integrated multitrophic aquaculture. *Aquaculture* **372-375**:28–38 DOI 10.1016/j.aquaculture.2012.10.022.

Yokoyama H. 2015. Suspended culture of the sea cucumber *Apostichopus japonicus* below a Pacific oyster raft-potential for integrated multi-trophic aquaculture. *Aquaculture Research* **46**:825-832 DOI *10.1111/are*.12234.

General Conclusions

1. The sea cucumber species *Holothuria tubulosa* and *Holothuria forskali* reduced total organic carbon, particulate organic matter, transparent exopolymer particles and bacteria in aquaculture tanks with *Anemonia sulcata* as primary species. Therefore, these species showed a great capacity as extractive species for integrated multritrophic aquaculture.

2.The consumption of transparent exopolymeric particles by holothurians was a result particularly remarkable and novel. This consumption is relevant in the maintenance of the tank hygiene and the control of potentially pathogenic bacterial outbreaks that can be associated to biofilm generation by exopolymeric particles.

3. The load of nitrogen (ammonium and nitrate) in the effluents was significantly higher in the tanks without holothurians than in the tanks with holothurians. Therefore, the presence of holothurians reduced the nitrogen loads in the tanks and effluents. This effect might be indirect through the consumption of bacteria, uneaten food from sea anemones, and detritus avoiding their complete mineralization up to nutrients such as ammonium and nitrate. This reduction in nitrate and ammonium in aquaculture effluents with holothurians can reduce environmental problems such as eutrophication and oxygen depletion in the coastal areas with aquaculture installations.

4. The presence of holothurians in the aquaculture tanks reduced significantly the concentration of chromophoric dissolved organic matter. A plausible mechanism to explain this effect is the consumption of particulate organic matter by holothurians that reduces the disaggregation of particulate organic matter into chromophoric dissolved compounds. This reduction in the concentration of particulate and chromophoric organic matter can affect positively to water transparency by reducing light scattering and absorption.

5. The reduction of chromophoric dissolved compounds under the presence of holothurians appeared to be particularly accentuated for low molecular weight components as it is suggested by the concomitant reduction of the spectral slopes $S_{275\text{-}295}$. We speculated that these chromophoric

compounds with relatively low molecular size might be chromophoric amino acids.

6. The use of fluorescence spectroscopy (excitation and emission matrixes, EEMs) and parallel factor (PARAFAC) analysis allowed us to characterize the components of the dissolved organic matter in two big groups: more refractory and humic-like compounds vs. more biodegradable and amino acid-like compounds. Holothurians were able to reduce significantly components of both groups likely due to their great efficiency uptaking particulate organic matter.

7. The presence of holothurians significantly reduced the fluorescence intensity of the two amino acid-like components C5 (tryptophan-like) and C6 (tyrosine-like). This remarkable reduction of the two chromophoric, amino acid-like components likely is related to the direct assimilation of amino acids by symbiotic bacteria in the tentacle epidermis and below the cuticle in holothurians.

8. Holothurians reduced the concentration of particulate organic matter, exopolymer particles, bacteria, chromophoric compounds as humic-like and amino acid-like substances and nitrogen. This extraordinary capacity to extract these organic compounds improves water quality and transparency in the effluents and the hygiene in the tanks. Therefore, holothurians have a relevant environmental role in multitrophic aquaculture beyond their marketable interest as food or biotechnological resource.

Conclusiones Generales

1. Los pepinos de mar *Holothuria tubulosa* y *Holothuria forskali* redujeron el carbono orgánico total, la materia orgánica particulada, las partículas exopoliméricas transparentes y las bacterias en tanques de acuicultura con *Anemonia sulcata* como especie principal. Por lo tanto, estas especies mostraron una enorme capacidad como especies extractivas para la acuicultura multitrófica integrada.

2. El consumo de partículas exopoliméricas transparentes por las holoturias fue un resultado particularmente reseñable y novedoso. Este consumo es importante en el mantenimiento de la higiene de los tanques y en control de brotes de bacterias potencialmente patógenas que pueden estar asociadas a la generación de biopelículas por los exopolímeros.

3. La carga de nitrógeno (amonio y nitrato) en los efluentes fue significativamente superior en los tanques sin holoturias que en aquellos que las contenían. Por lo tanto, la presencia de holoturias redujo las cargas de nitrógeno en los tanques y en sus efluentes. Este efecto puede ser indirecto a través del consumo de bacterias, de restos alimenticios sin consumir por la anémonas y de detritus impidiendo su mineralización completa hasta nutrientes inorgánicos como el amonio y el nitrato. Esta reducción en la carga de N en los efluentes de los tanques con holoturias puede reducir o evitar problemas ambientales como la eutrofización y la disminución de oxígeno en zonas costeras con instalaciones de acuicultura.

4. La presencia de holoturias en los tanques de acuicultura redujo significativamente la concentración de materia orgánica cromofórica. Un plausible mecanismo para explicar este efecto es el consumo de materia orgánica particulada por las holoturias que reduce la disgregación de la materia orgánica particulada en compuestos orgánicos cromofóricos. Esta reducción en la concentración de materia orgánica particulada y cromofórica puede afectar positivamente a la transparencia del agua reduciendo la dispersión y absorción de la luz.

5. La reducción de compuestos orgánicos cromofóricos bajo la presencia de holoturias pareció ser particularmente acentuada para los compuestos de bajo peso molecular como sugiere la reducción conjunta de

la pendientes espectrales $S_{275-295}$. Nosotros especulamos que estos compuestos cromofóricos de bajo peso molecular puedan ser aminoácidos cromofóricos.

6. El uso de la espectroscopía de fluorescencia (matrices de excitación y emisión, EEMs) y el análisis de factor paralelo (PARAFAC) nos permite caracterizar los componentes de la materia orgánica disuelta en dos grandes grupos: compuestos refractarios similares a compuestos húmicos vs compuestos más biodegradables similares a los aminoácidos. Las holoturias fueron capaces de reducir significativamente componentes de ambos grupos probablemente debido a su gran eficiencia incorporando materia orgánica particulada.

7. La presencia de holoturias redujo significativamente la intensidad de fluorescencia de los dos componentes como aminoácidos C5 (similar al triptófano) y C6 (similar a la tirosina). Esta reseñable reducción de los dos componentes aminoácidos cromofóricos probablemente está relacionada con la asimilación directa de aminoácidos por bacterias simbiontes en la epidermis de los tentáculos y por debajo de la cutícula de las holoturias.

8. Las holoturias redujeron la concentración de materia orgánica particulada, partículas exopoliméricas transparentes, bacterias, compuestos orgánicos cromofóricos tanto húmicos como aminoácidos y nitrógeno. Esta capacidad extraordinaria para extraer compuestos orgánicos mejora la calidad y transparencia de los efluentes y la higiene de los tanques. Por lo tanto, las holoturias tienen un papel ambiental muy relevante in la acuicultura multitrófica, más allá de su interés comercial como alimento o recurso biotecnológico.

Appendixes

Appendix I.

Table 1. Three replicate results of time- series experiments. R: replicate, M: mean, ammonium, nitrate and nitrite (µmol-N l^{-1}) and TP (µmol-P l^{-1})

Inlet water

Date (dd/mm/yy)	R	MTP	R	M Ammonium	R	M Nitrite	R	M Nitrate
17/07/13	0.5	0.3	0.01	0.01	0.02	0.02	6	7
	0.2		0.01		0.02		10	
	0.2				0.02		4	
30/07/13	0.1	0.2	0.01	0.01	0.07	0.07	9	9
	0.5		0.01		0.07		9	
	0.2		0.01		0.07			
29/08/13	0.2	0.2	0.01	0.01	0.07	0.07	9	9
	0.2		0.01		0.07		9	
	0.1		0.01		0.07			
13/09/13	0.2	0.2	0.01	0.01	0.03	0.03	9	9
	0.1		0.01		0.03		9	
	0.2				0.03			
27/09/13	0.5	0.2	0.01	0.01	0.03	0.03	6	6
	0.1		0.01		0.03		6	
	0.1							
15/10/13	0.2	0.2	0.01	0.01	0.03	0.03	6	6
	0.3		0.01		0.03		6	
	0.2				0.03			
30/10/13	0.3	0.9	0.01	0.01	0.07	0.07	6	6
	1.4		0.01		0.07		6	
	1.1				0.07		5	
13/11/13	0.9	1.0	0.03	0.03	0.04	0.04	6	5
	1.1		0.03		0.04		5	
	1.2						5	
2/12/13	1.2	1.2	0.01	0.01	0.02	0.02	4	4
	1.3		0.01		0.02		4	
	1.0				0.02		4	
17/12/13	0.8	0.7	0.01	0.01	0.04	0.04	4	3
	0.8		0.01		0.04		4	
	0.6		0.01		0.04		3	
30/12/13	0.3	0.2	0.01	0.01	0.04	0.04	6	6
	0.2		0.01		0.04		6	
	0.1				0.04		6	
15/01/14	0.1	0.2	0.01	0.01	0.06	0.06	6	6
	0.2		0.01		0.06		6	
	0.3		0.01				6	
30/01/14	0.1	0.1	0.01	0.01	0.05	0.05	4	4
	0.1		0.01		0.05		4	
	0.1		0.01				4	

+ holothurian tank

Date (dd/mm/yy)	R	M TP	R	M Ammonium	R	M Nitrite	R	M Nitrate
17/07/13	0.2	0.1	0.01	0.01	0.02	0.02	7	7
	0.1		0.01		0.02		7	
	0.1				0.01		7	
30/07/13	0.1	0.1	0.02	0.01	0.03	0.03	7	7
	0.1		0.01		0.05		7	
	0.2		0.01		0.03			
29/08/13	0.1	0.1	0.01	0.01	0.03	0.04	4	4
	0.0		0.01		0.04		4	
	0.1				0.03			
13/09/13	0.5	0.4	0.02	0.02	0.03	0.03	5	5
	0.1		0.02		0.04		5	
	0.6				0.03			
27/09/13	0.7	0.3	0.02	0.02	0.03	0.03	5	5
	0.1		0.02		0.03		5	
	0.1				0.03			
15/10/13	0.5	0.4	0.01	0.01	0.03	0.03	5	5
	0.3		0.01		0.03		5	
	0.3				0.03			
30/10/13	0.8	1.2	0.01	0.01	0.04	0.04	5	5
	0.4		0.01		0.04		5	
	2.5				0.04		5	
13/11/13	0.6	0.7	0.07	0.07	0.05	0.05	5	5
	0.7		0.07		0.04		5	
							5	
2/12/13	0.4	0.3	0.01	0.01	0.05	0.05	9	9
	0.2		0.01		0.05		9	
	0.4				0.05			
17/12/13	0.1	0.1	0.02	0.01	0.03	0.03	3	3
	0.1		0.01		0.03		3	
	0.1		0.01		0.03		3	
30/12/13	0.9	0.4	0.05	0.04	0.05	0.04	8	8
	0.1		0.01		0.04		8	
	0.3		0.05					
15/01/14	0.9	0.4	0.01	0.01	0.05	0.05	5	5
	0.1		0.01		0.05		5	
	0.4		0.01				5	
30/01/14	0.3	0.3	0.01	0.01	0.13	0.13	5	5
	0.2		0.02		0.13		5	
	0.4		0.01					

+ holothurian effluents

Date (dd/mm/yy)	R	M TP	R	M Ammonium	R	M Nitrite	R	M Nitrate
17/07/13	0.1	0.2	0.01	0.01	0.02	0.02	7	7
	0.4		0.01		0.02		7	
	0.1				0.01			
30/07/13	0.1	0.1	0.01	0.01	0.06	0.06	7	7
	0.1		0.01		0.06		7	
	0.2				0.07			
29/08/13	0.1	0.1	0.01	0.01	0.03	0.04	5	5
	0.1		0.01		0.04		5	
	0.1				0.03			
13/09/13	0.1	0.2	0.02	0.02	0.04	0.04	6	6
	0.1		0.02		0.04		6	
	0.5				0.04			
27/09/13	0.2	0.2	0.02	0.02	0.03	0.03	5	5
	0.3		0.02		0.03		5	
	0.1						5	
15/10/13	0.3	0.3	0.01	0.01	0.05	0.05	5	5
	0.1		0.01		0.05		5	
	0.5				0.05			
30/10/13	1.1	0.8	0.01	0.01	0.06	0.06	4	4
	1.1		0.01		0.05		4	
	0.2						5	
13/11/13	0.4	0.5	0.01	0.02	0.05	0.05	5	5
	0.7		0.03		0.05		5	
			0.02					
2/12/13	0.4	0.4	0.01	0.01	0.05	0.05	4	4
	0.4		0.01		0.05		4	
	0.4							
17/12/13	0.1	0.1	0.01	0.01	0.03	0.03	4	4
	0.1		0.01		0.03		4	
	0.1							
30/12/13	0.5	0.2	0.01	0.01	0.03	0.03	5	5
	0.1		0.01		0.03		5	
	0.1		0.01					
15/01/14	1.3	0.5	0.01	0.01	0.04	0.04	5	5
	0.0		0.01		0.04		5	
	0.0		0.01					
30/01/14	0.4	0.4	0.00	0.01	0.13	0.13	5	5
	0.4		0.01		0.13		5	

- holothurian tank

Date (dd/mm/yy)	R	M TP	R	M Ammonium	R	M Nitrite	R	M Nitrate
17/07/13	0.5	0.3	0.01	0.01	0.02	0.02	6	6
	0.2		0.01		0.02		6	
	0.1				0.02		6	
30/07/13	0.1	0.2	0.02	0.02	0.02	0.02	1	1
	0.2		0.02		0.02		1	
29/08/13	0.1	0.2	0.02	0.02	0.05	0.04	0	0
	0.1		0.02		0.03		0	
	0.3				0.03		0	
13/09/13	0.1	0.1	0.02	0.02	0.04	0.03	4	4
	0.1		0.02		0.03		4	
	0.1				0.03			
27/09/13	0.6	0.3	0.02	0.02	0.03	0.03	4	4
	0.0		0.02		0.03		4	
	0.2						4	
15/10/13	0.0	0.0	0.01	0.01	0.04	0.05	5	5
	0.0		0.01		0.05		5	
	0.0				0.05		5	
30/10/13	0.6	0.6	0.01	0.01	0.06	0.06	3	3
	0.6		0.01		0.06		3	
	0.5		0.01		0.06			
13/11/13	0.7	0.8	0.07	0.04	0.05	0.05	5	5
	0.8		0.02		0.05		5	
	0.7		0.02					
2/12/13	0.2	0.3	0.01	0.01	0.06	0.06	7	7
	0.4		0.01		0.06		7	
	0.2				0.06			
17/12/13	0.1	0.1	0.01	0.01	0.05	0.05	3	3
	0.1		0.01		0.05		3	
	0.1				0.05			
30/12/13	0.0	0.1	0.06	0.06	0.05	0.05	7	7
	0.1		0.06		0.05		7	
	0.1				0.05			
15/01/14	0.1	0.1	0.09	0.09	0.03	0.02	4	4
	0.1		0.09		0.02		4	
	0.1				0.03			
30/01/14	0.1	0.1	0.09	0.09	0.03	0.02	3	3
	0.1		0.09		0.02		3	
	0.1				0.02			

- holothurian effluents

Date (dd/mm/yy)	R	M TP	R	M Ammonium	R	M Nitrite	R	M Nitrate
17/07/13	0.3	0.5	0.01	0.01	0.03	0.03	6	6
	0.7		0.01		0.03		6	
					0.03		6	
30/07/13	0.1	0.1	0.01	0.02	0.03	0.03	1	1
	0.2		0.02		0.03		1	
	0.1				0.03			
29/08/13	0.1	0.1	0.02	0.02	0.03	0.03	1	1
	0.2		0.02		0.03		1	
	0.0				0.03		1	
13/09/13	0.2	0.2	0.02	0.02	0.06	0.05	4	4
	0.1		0.02		0.04		4	
	0.2							
27/09/13	0.3	0.2	0.02	0.02	0.03	0.02	4	4
	0.1		0.02		0.02		4	
	0.4							
15/10/13	0.2	0.3	0.01	0.01	0.03	0.03	5	5
	0.3		0.01		0.03		5	
	0.3				0.03			
30/10/13	0.1	0.1	0.01	0.01	0.04	0.04	4	4
	0.3		0.01		0.04		4	
	0.0		0.01		0.04		4	
13/11/13	0.5	0.6	0.03	0.09	0.04	0.04	5	5
	0.4		0.15		0.04		5	
	1.0		0.08					
2/12/13	0.2	0.2	0.04	0.04	0.04	0.04	7	7
	0.2		0.04		0.04		7	
	0.2				0.04			
17/12/13	0.1	0.1	0.04	0.04	0.04	0.04	3	3
	0.1		0.04		0.04		3	
	0.1							
30/12/13	0.7	0.7	0.09	0.09	0.03	0.03	7	7
	0.8		0.09		0.03		7	
	0.7				0.03			
15/01/14	0.1	0.1	0.07	0.07	0.02	0.02	4	4
	0.1		0.07		0.02		4	
	0.1				0.02			
30/01/14	0.1	0.1	0.08	0.08	0.03	0.03	4	4
	0.1		0.08		0.03		4	
	0.1				0.03		4	

Table 1(continued). Three replicate results of time- series experiments. R: replicate, M: mean, ammonium, nitrate and nitrite (µmol-N l^{-1}) and TP (µmol-P l^{-1})

Inlet waters

Date (dd/mm/yy)	R	MTP	R	M Ammonium	R	M Nitrite	R	M Nitrate
11/2/14	0.1	0.1	0.02	0.04	0.04	0.04	6	6
	0.2		0.08		0.04		6	
	0.1		0.01				6	
27/02/14	0.1	0.1	0.01	0.02	0.05	0.04	10	3
	0.1		0.04		0.04		10	
	0.0		0.01				10	
14/03/14	0.5	0.5	0.01	0.02	0.03	0.03	13	12
	0.4		0.01		0.03		12	
	0.6		0.03		0.03		13	
27/03/14	0.4	0.4	0.01	0.02	0.04	0.04	14	14
	0.4		0.02		0.04		14	
	0.4		0.02		0.04		14	
11/4/14	0.4	0.3	0.01	0.01	0.02	0.03	13	14
	0.4		0.01		0.03		15	
	0.3		0.01		0.03		14	
30/04/14	0.4	0.6	0.01	0.02	0.03	0.04	15	13
	0.7		0.03		0.04		8	
	0.6		0.03		0.04		14	
14/05/14	0.1	0.1	0.01	0.01	0.03	0.03	15	15
	0.0		0.01		0.03		15	
			0.01		0.03			
29/05/14	0.3	0.3	0.01	0.03	0.02	0.02	23	27
	0.3		0.01		0.02		30	
	0.3		0.06		0.02			
11/6/14	0.1	0.1	0.01	0.02	0.02	0.02	6	8
	0.2		0.02		0.02		10	
	0.1				0.02			
30/06/14	0.0	0.0	0.02	0.02	0.07	0.07	17	17
	0.0		0.01		0.07		17	
	0.0		0.02		0.08		17	
11/7/14	0.1	0.0	0.01	0.01	0.24	0.25	17	16
	0.0		0.01		0.25		17	
	0.0		0.01		0.25		15	
25/07/14	0.0	0.1	0.01	0.01	0.03	0.02	20	20
	0.3		0.01		0.02		20	
	0.0		0.01		0.03		20	
20/08/14	0.1	0.1	0.01	0.01	0.03	0.03	11	12
	0.0		0.01		0.03		13	
	0.1		0.01		0.03		13	

+ holothurian tank

Date (dd/mm/yy)	R	M TP	R	M Ammonium	R	M Nitrite	R	M Nitrate
11/2/14	0.1	0.2	0.08	0.09	0.06	0.06	5	5
	0.3		0.10		0.06		5	
	0.2		0.11				5	
27/02/14	0.1	0.1	0.08	0.05	0.06	0.06	11	11
	0.1		0.02		0.06		11	
	0.1		0.06				11	
14/03/14	0.3	0.3	0.03	0.02	0.02	0.02	13	13
	0.4		0.02		0.02		13	
	0.3		0.02		0.02		13	
27/03/14	0.4	0.3	0.01	0.01	0.05	0.06	16	16
	0.2		0.01		0.05		16	
	0.4		0.01		0.06			
11/4/14	0.1	0.0	0.01	0.01	0.02	0.03	15	15
	0.0		0.01		0.03		15	
	0.0		0.01		0.03			
30/04/14	0.2	0.2	0.01	0.02	0.01	0.01	14	14
	0.1		0.02		0.01		14	
	0.3		0.02		0.02			
14/05/14	0.1	0.3	0.01	0.01	0.02	0.02	14	14
	0.4		0.01		0.02		14	
			0.01		0.02			
29/05/14	0.2	0.2	0.01	0.01	0.02	0.02	25	25
	0.1		0.01		0.02		25	
	0.1		0.01		0.02			
11/6/14	0.1	0.3	0.01	0.01	0.14	0.17	8	8
	0.5		0.01		0.20		8	
			0.01		0.17			
30/06/14	0.0	0.1	0.01	0.01	0.05	0.05	12	13
	0.1		0.01		0.05		14	
	0.1		0.01		0.05		13	
11/7/14	0.1	0.1	0.01	0.01	0.35	0.35	17	17
	0.1		0.01		0.34		17	
	0.1		0.01		0.35		18	
25/07/14	0.1	0.1	0.01	0.01	0.02	0.02	23	23
	0.1		0.01		0.02		23	
	0.1		0.01		0.02		24	
20/08/14	0.2	0.2	0.02	0.01	0.03	0.03	15	15
	0.2		0.01		0.03		15	
	0.3		0.01		0.03			

+ holothurian effluents

Date (dd/mm/yy)	R	M TP	R	M Ammonium	R	M Nitrite	R	M Nitrate
11/2/14	0.1	0.1	0.06	0.10	0.03	0.03	6	6
	0.1		0.12		0.03		6	
	0.1		0.14				6	
27/02/14	0.1	0.1	0.03	0.03	0.06	0.06	3	3
	0.1		0.03		0.05		3	
	0.0		0.03				3	
14/03/14	0.1	0.1	0.01	0.03	0.02	0.03	3	3
	0.0		0.03		0.03		3	
	0.1		0.04		0.03			
27/03/14	0.2	0.2	0.01	0.01	0.05	0.05	2	2
	0.1		0.01		0.05		2	
	0.4		0.01		0.05			
11/4/14	0.2	0.1	0.01	0.01	0.02	0.03	1	1
	0.1		0.02		0.02		1	
	0.1		0.01		0.03		1	
30/04/14	0.2	0.2	0.01	0.01	0.01	0.01	7	7
	0.2		0.02		0.02		7	
			0.01					
14/05/14	0.1	0.1	0.01	0.01	0.02	0.02	5	5
	0.1		0.02		0.02		5	
	0.1		0.01		0.02			
29/05/14	0.2	0.2	0.01	0.01	0.03	0.03	18	18
	0.1		0.01		0.03		18	
	0.4		0.01		0.02		18	
11/6/14	0.3	0.2	0.01	0.01	0.16	0.16	26	26
	0.2		0.01		0.15		26	
	0.2				0.17		26	
30/06/14	0.1	0.1	0.01	0.01	0.05	0.05	4	4
	0.1		0.01		0.05		4	
	0.1		0.01		0.05		4	
11/7/14	0.3	0.1	0.01	0.01	0.03	0.03	4	4
	0.0		0.01		0.04		4	
	0.1		0.01		0.03		4	
25/07/14	0.2	0.1	0.01	0.01	0.02	0.02	10	9
	0.2		0.01		0.02		10	
	0.1		0.01		0.02		9	
20/08/14	0.2	0.2	0.02	0.01	0.03	0.03	9	9
	0.2		0.01		0.03		9	
	0.2							

- holothurian tank

Date (dd/mm/yy)	R	M TP	R	M Ammonium	R	M Nitrite	R	M Nitrate
11/2/14	0.1	0.1	0.06	0.08	0.03	0.04	6	6
	0.1		0.07		0.04		6	
	0.2		0.11					
27/02/14	0.0	0.1	0.02	0.01	0.05	0.05	9	9
	0.1		0.01		0.04		9	
	0.1		0.01					
14/03/14	0.2	0.2	x	x	x	x	12	12
	0.1		x		x		12	
	0.2		x		x			
27/03/14	0.2	0.2	x	x	x	x	13	13
	0.2		x		x		13	
			x		x			
11/4/14	0.1	0.1	0.01	0.00	0.04	0.04	14	14
	0.1		0.00		0.04		14	
	0.1				0.04			
30/04/14	0.1	0.1	0.01	0.01	0.04	0.04	12	12
	0.1		0.01		0.04		12	
	0.1		0.00		0.04			
14/05/14	0.2	0.2	0.01	0.01	0.03	0.03	12	12
	0.2		0.01		0.03		12	
	0.1		0.01		0.03		12	
29/05/14	0.1	0.1	0.02	0.03	0.03	0.02	23	23
	0.0		0.05		0.02		23	
	0.1		0.01		0.02		23	
11/6/14	0.3	0.2	0.01	0.01	0.02	0.02	7	7
	0.1		0.01		0.02		7	
	0.1				0.02		7	
30/06/14	0.0	0.0	0.01	0.01	0.04	0.04	14	14
	0.0		0.01		0.04		14	
			0.01		0.04		14	
11/7/14	0.1	0.4	0.03	0.02	0.32	0.30	16	16
	0.6		0.01		0.31		16	
	0.6		0.02		0.27		16	
25/07/14	0.0	0.2	0.04	0.04	0.03	0.03	18	18
	0.1		0.04		0.04		18	
	0.4		0.06		0.03		18	
20/08/14	x	x	x	x	x	x	11	11
	x		x		x		11	
	x		x		x		11	

- holothurian effluents

Date (dd/mm/yy)	R	M TP	R	M Ammonium	R	M Nitrite	R	M Nitrate
11/2/14	0.2	0.1	0.16	0.16	0.03	0.03	6	6
	0.1		0.16		0.03		6	
	0.1				0.03		6	
27/02/14	0.1	0.1	0.05	0.03	0.04	0.04	9	9
	0.0		0.02		0.03		9	
	0.1		0.01					
14/03/14	0.0	0.0	x		0.04	0.04	12	12
	0.0		x		0.04		12	
	0.0		x		0.04			
27/03/14	0.1	0.1	0.02	0.02	0.04	0.04	14	14
	0.1		0.02		0.04		14	
	0.1				0.04			
11/4/14	0.1	0.1	0.02	0.02	0.03	0.03	14	14
	0.1		0.02		0.03		14	
	0.1				0.03			
30/04/14	0.1	0.1	0.02	0.02	0.04	0.04	12	12
	0.1		0.02		0.04		12	
	0.1							
14/05/14	0.1	0.1	0.04	0.03	0.02	0.02	12	12
	0.1		0.02		0.02		12	
	0.2		0.01		0.02		12	
29/05/14	0.1	0.1	0.02	0.01	0.02	0.02	23	24
	0.3		0.01		0.02		24	
	0.0		0.01		0.02		24	
11/6/14	0.1	0.1	0.01	0.01	0.09	0.09	7	7
	0.1		0.01		0.09		7	
30/06/14	0.4	0.2	0.01	0.01	0.03	0.03	15	15
	0.1		0.01		0.03		15	
	0.1		0.01		0.03			
11/7/14	0.1	0.2	0.07	0.03	0.03	0.03	16	16
	0.3		0.02		0.03		16	
	0.1		0.02				16	
25/07/14	0.1	0.1	0.05	0.04	0.03	0.03	18	18
	0.1		0.06		0.03		18	
	0.1		0.02		0.03		18	
20/08/14	x	x	x	x	x	x	11	11
	x		x		x		11	
	x		x		x		11	

Table 2. Mean results of time- series experiments in inlet waters

Date (dd/mm/yy)	TP (μmol-PL^{-1})	Ammonium (μmol-NL^{-1})	Nitrate (μmol-NL^{-1})	Nitrite (μmol-NL^{-1})	TEP (μg XG eq. L^{-1})
17/07/13	0.3	0.01	7	0.02	
30/07/13	0.2	0.01	9	0.07	65
29/08/13	0.2	0.01	9	0.07	69
13/09/13	0.2	0.01	9	0.03	44
27/09/13	0.2	0.01	6	0.03	32
15/10/13	0.2	0.01	6	0.03	72
30/10/13	0.9	0.01	6	0.07	59
13/11/13	1.0	0.03	5	0.04	70
02/12/13	1.2	0.01	4	0.02	59
17/12/13	0.7	0.01	3	0.04	79
30/12/13	0.2	0.01	6	0.04	31
15/01/14	0.2	0.01	6	0.06	57
30/01/14	0.1	0.01	4	0.05	191
11/02/14	0.1	0.04	6	0.04	79
27/02/14	0.1	0.02	10	0.04	80
14/03/14	0.5	0.02	12	0.03	83
27/03/14	0.4	0.02	14	0.04	152
11/04/14	0.3	0.01	14	0.03	137
30/04/14	0.6	0.02	13	0.04	270
14/05/14	0.1	0.01	15	0.03	92
29/05/14	0.3	0.03	27	0.02	81
11/06/14	0.1	0.02	8	0.02	77
30/06/14	0.0	0.02	17	0.07	49
11/07/14	0.0	0.01	16	0.02	104
25/07/14	0.1	0.01	20	0.02	99
20/08/14	0.1	0.01	11	0.03	101

Table 3. Mean results of time- series experiments in the effluent of + holothurian effluents

Date (dd/mm/yy)	TP (μmol-PL^{-1})	Ammonium (μmol-NL^{-1})	Nitrate (μmol-NL^{-1})	Nitrite (μmol-NL^{-1})	TEP (μg XG eq. L^{-1})
17/07/13	0.2	0.01	7	0.02	
30/07/13	0.1	0.01	7	0.06	154
29/08/13	0.1	0.01	5	0.04	129
13/09/13	0.2	0.02	6	0.04	75
27/09/13	0.2	0.02	5	0.03	61
15/10/13	0.3	0.01	5	0.05	95
30/10/13	0.8	0.01	4	0.06	43
13/11/13	0.5	0.02	5	0.05	213
02/12/13	0.4	0.01	4	0.05	153
17/12/13	0.1	0.01	4	0.03	73
30/12/13	0.2	0.01	5	0.03	22
15/01/14	0.5	0.01	5	0.04	321
30/01/14	0.4	0.00	5	0.13	54
11/02/14	0.1	0.10	6	0.03	172
27/02/14	0.1	0.03	3	0.06	51
14/03/14	0.1	0.03	3	0.03	103
27/03/14	0.2	0.01	2	0.05	101
11/04/14	0.1	0.01	1	0.03	92
30/04/14	0.2	0.01	7	0.02	187
14/05/14	0.1	0.01	5	0.02	88
29/05/14	0.2	0.01	18	0.03	70
11/06/14	0.2	0.01	26	0.16	220
30/06/14	0.1	0.01	4	0.05	42
11/07/14	0.1	0.01	4	0.03	59
25/07/14	0.1	0.01	9	0.02	67
20/08/14	0.2	0.01	9	0.03	80

Table 4. Mean results of time- series experiments in the effluent of - holothurian effluents

Date (dd/mm/yy)	TP (μmol-PL^{-1})	Ammonium (μmol-NL^{-1})	Nitrate (μmol-NL^{-1})	Nitrite (μmol-NL^{-1})	TEP (µg XG eq. L^{-1})
17/07/13	0.5	0.01	6	0.03	
30/07/13	0.1	0.02	1	0.03	417
29/08/13	0.1	0.02	1	0.03	237
13/09/13	0.2	0.02	4	0.05	111
27/09/13	0.2	0.02	4	0.02	98
15/10/13	0.3	0.01	5	0.03	117
30/10/13	0.1	0.01	4	0.04	131
13/11/13	0.6	0.09	5	0.04	155
02/12/13	0.2	0.04	7	0.04	192
17/12/13	0.1	0.04	3	0.04	
30/12/13	0.7	0.09	7	0.03	35
15/01/14	0.1	0.07	4	0.02	522
30/01/14	0.1	0.08	4	0.03	180
11/02/14	0.1	0.16	6	0.03	171
27/02/14	0.1	0.03	9	0.04	
14/03/14	0.0		12	0.04	289
27/03/14	0.1	0.02	14	0.04	382
11/04/14	0.1	0.02	14	0.03	108
30/04/14	0.1	0.02	12	0.04	171
14/05/14	0.1	0.03	12	0.02	88
29/05/14	0.1	0.01	24	0.02	106
11/06/14	0.1	0.01	7	0.10	155
30/06/14	0.2	0.01	15	0.03	126
11/07/14	0.2	0.03	16	0.03	154
25/07/14	0.1	0.04	18	0.03	108
20/08/14			11		91

Table 5. Mean results of time- series experiments in the + holothurian tank

Date (dd/mm/yy)	TP (μmol-PL^{-1})	Ammonium (μmol-NL^{-1})	Nitrate (μmol-NL^{-1})	Nitrite (μmol-NL^{-1})	TEP (µg XG eq. L^{-1})
17/07/13	0.1	0.01	7	0.02	
30/07/13	0.1	0.01	7	0.03	80
29/08/13	0.1	0.01	4	0.04	42
13/09/13	0.4	0.02	5	0.03	38
27/09/13	0.3	0.02	5	0.03	58
15/10/13	0.4	0.01	5	0.03	75
30/10/13	1.2	0.01	5	0.04	64
13/11/13	0.7	0.07	5	0.05	43
02/12/13	0.3	0.01	9	0.05	277
17/12/13	0.1	0.01	3	0.03	111
30/12/13	0.4	0.04	8	0.04	268
15/01/14	0.4	0.01	5	0.05	67
30/01/14	0.3	0.01	5	0.13	50
11/02/14	0.2	0.09	5	0.06	120
27/02/14	0.1	0.05	11	0.06	101
14/03/14	0.3	0.02	13	0.02	101
27/03/14	0.3	0.01	16	0.06	169
11/04/14	0.0	0.01	15	0.03	159
30/04/14	0.2	0.02	14	0.01	48
14/05/14	0.3	0.01	14	0.02	82
29/05/14	0.2	0.01	25	0.02	185
11/06/14	0.3	0.01	8	0.17	93
30/06/14	0.1	0.01	13	0.05	113
11/07/14	0.1	0.01	17	0.03	139
25/07/14	0.1	0.01	23	0.02	183
20/08/14	0.2	0.01	15	0.03	229

Table 6. Mean results of time- series experiments in the - holothurian tank

Date (dd/mm/yy)	TP (μmol-PL^{-1})	Ammonium (μmol-NL^{-1})	Nitrate (μmol-NL^{-1})	Nitrite (μmol-NL^{-1})	TEP (µg XG eq. L^{-1})
17/07/13	0.3	0.01	6	0.02	
30/07/13	0.2	0.02	1	0.02	211
29/08/13	0.2	0.02	1	0.04	217
13/09/13	0.1	0.01	4	0.03	106
27/09/13	0.3	0.02	4	0.03	68
15/10/13	0.0	0.01	5	0.05	123
30/10/13	0.6	0.01	3	0.06	232
13/11/13	0.8	0.04	5	0.05	233
02/12/13	0.3	0.01	7	0.06	297
17/12/13	0.1	0.01	3	0.05	297
30/12/13	0.1	0.06	7	0.05	
15/01/14	0.1	0.09	4	0.03	197
30/01/14	0.1	0.09	3	0.03	152
11/02/14	0.1	0.08	6	0.04	170
27/02/14	0.1	0.01	9	0.05	
14/03/14	0.2		12		395
27/03/14	0.2		13		467
11/04/14	0.1	0.01	14	0.04	
30/04/14	0.1	0.00	12	0.04	188
14/05/14	0.2	0.01	12	0.03	105
29/05/14	0.1	0.03	23	0.02	123
11/06/14	0.2	0.01	7	0.02	324
30/06/14	0.0	0.01	14	0.04	216
11/07/14	0.4	0.02	16	0.02	334
25/07/14	0.2	0.04	18	0.03	373
20/08/14			11		292

Table 7. Three replicate results of short-term experiments. TP (µmol-P l^{-1}), nitrate (µmol-N l^{-1}), TEP (µg XG eq. l^{-1}) and bacteria abundance (cells ml^{-1})

	Date (dd/mm/yy)	TP	Nitrate	TEP	Bacteria		TP	Nitrate	TEP	Bacteria		TP	Nitrate	TEP	Bacteria		TP	Nitrate	TEP	Bacteria
Tank 1	6/10/17	1.4	127	523	853535	Tank 3	1.1	125	530	531566	Tank 5	1.2	124	788	608586	Tank 7	1.3	127	645	541667
		1.4	127	633	851010		1.2	125	532	599747		1.1	124	789	496212		1.4	127	646	532828
		1.3	127		553030		1.2	125		613636		1.2	122		506313		1.4	127		527778
	9/10/17	0.5	97	174	460859		0.4	103	122	503788		0.7	124	716	546717		0.8	120	649	531566
		0.4	97	128	556818		0.4	102	129	532828		0.5	124	710	549242		0.9	120	647	599747
		0.6	97		516414		0.4	102		463384		0.8	124		549242		0.8	120		613636
	27/10/17	0.9	128	491	1324495		0.8	129	498	1609848		0.8	128	500	1277778		0.9	128	507	1276515
		0.9	128	496	1337121		0.9	129	500	1328283		0.8	128	500	1460859		0.9	128	509	1291667
		0.9	128		1549242		0.9	129		1089646		1.0	128		873737		0.9	128		1327020
	30/10/17	0.8	132	141	743687		0.8	131	133	790404		1.0	149	485	1773990		0.8	146	490	1685606
		0.6	132	139	916667		0.8	131	140	790404		0.9	148	487	1814394		0.8	146	487	1760101
		0.7	131		1138889		0.8	132		1506313		0.9	149		1839646		0.8	145		1657828
	3/11/17	1.1	122	659	1723485		0.9	121	667	2017677		1.1	138	650	2434343		1.0	136	673	1546717
		1.0	121	665	1825758		1.0	121	667	2073232		1.0	137	650	2425505		1.0	136	678	1579545
		1.0	121		1821970		1.0	121				1.1	138		2309343		1.0	135		2099747
	6/11/17	1.1	124	148	1344697		0.9	127	140	1494949		1.1	156	779	1920455		1.0	146	812	1791667
		1.0	127	146	1391414		1.0	127	147	1500000		1.0	156	781	2037879		1.0	145	828	1791667
		1.0	127		1411616		1.0	128		1554293			156		1970960		1.0	145		1631313
Tank 2	6/10/17	1.2	125	528	518939	Tank 4	1.3	124	645	565657	Tank 6	1.2	130	637	608586					
		1.3	110	532	510101		1.2	124	646	611111		1.2	129	640	521465					
		1.2	110		540404		1.0	124		468434		1.2	129		559343					
	9/10/17	0.8	97	106	517677		0.7	126	649	853535		0.5	123	636	641414					
		0.9	97	118	516414		0.6	125	650	851010		0.6	123	635	546717					
		0.8	97		507576		0.6	125		553030		0.5	123		537879					
	27/10/17	0.8	128	496	1746212		0.8	128	508	1383838		0.8	128	496	1238636					
		0.8	128	498	1791667		0.8	128	509	1372475		0.8	128	500	1093434					
		0.8	128		1597222		0.7	128		1755051		0.8	128		1473485					
	30/10/17	1.4	131	112	1242424		0.8	142	494	1684343		0.9	145	480	1803030					
		1.5	131	124	1099747		0.6	143	504	1746212		0.9	145	496	1744949					
		1.4	131		753788		0.7	143		1791667		0.9	144		1643939					
	3/11/17	1.0	121	667	1866162		1.1	132	684	1613636		0.9	134	667	1223485					
		1.1	122	668	1750000		1.0	133	677	1520202		1.0	134	673	1204545					
		1.0	121		1741162		1.0	133		1508838		1.0	134		1250000					
	6/11/17	1.0	127	132	1412879		1.1	144	826	1772727		0.9	151	803	1828283					
		1.1	127	131	1448232		1.0	144	814	1790404		1.0	151	811	1859848					
		1.0	126		1582071		1.0	144		1859848		1.0	151		1949495					

Table 8. Mean results of short-term experiments. TP (µmol-P l^{-1}), nitrate (µmol-N l^{-1}), TEP (µg XG eq. l^{-1}) and bacteria abundance (cells ml^{-1})

	Date(dd/mm/yy)	TP	Nitrate	TEP	Bacteria x 10^6
Tank 1	6/10/17	1.4	127	578	0.8
	9/10/17	0.5	97	151	0.5
	27/10/17	0.9	128	494	1.4
	30/10/17	0.7	132	140	0.9
	3/11/17	1.0	121	662	1.8
	6/11/17	1.0	126	147	1.4
Tank 2	6/10/17	1.2	115	530	0.5
	9/10/17	0.8	97	112	0.5
	27/10/17	0.8	128	497	1.7
	30/10/17	1.4	131	118	1.0
	3/11/17	1.1	121	667	1.8
	6/11/17	1.1	127	131	1.5
Tank 3	6/10/17	1.1	125	531	0.6
	9/10/17	0.4	102	125	0.5
	27/10/17	0.8	129	499	1.3
	30/10/17	0.8	132	137	1.0
	3/11/17	1.0	121	667	2.0
	6/11/17	1.0	128	144	1.5
Tank 4	6/10/17	1.1	124	645	0.5
	9/10/17	0.6	125	704	0.8
	27/10/17	0.8	128	509	1.5
	30/10/17	0.7	143	535	1.7
	3/11/17	1.0	133	680	1.5
	6/11/17	1.0	144	729	1.8
Tank 5	6/10/17	1.1	123	788	0.5
	9/10/17	0.6	124	773	0.5
	27/10/17	0.8	128	500	1.2
	30/10/17	0.9	149	522	1.8
	3/11/17	1.1	138	650	2.4
	6/11/17	1.1	156	871	2.0
Tank 6	6/10/17	1.2	129	638	0.6
	9/10/17	0.5	123	690	0.6
	27/10/17	0.8	128	498	1.3
	30/10/17	0.9	145	524	1.7
	3/11/17	0.9	134	670	1.2
	6/11/17	0.9	151	898	1.9
Tank 7	6/10/17	1.4	127	645	0.5
	9/10/17	0.8	120	703	0.6
	27/10/17	0.9	128	508	1.3
	30/10/17	0.8	146	524	1.7
	3/11/17	1.0	136	676	1.7
	6/11/17	1.0	145	911	1.7

Appendix II.

Table 1. Physicochemical parameters, CDOM quantitative-qualitative parameters, TOC, POM, Chlorophyll *a* and bacteria abundance of time-series experiments in the inlet waters.

Date (dd/mm/yy)	pH	Temp (ºC)	Salinity (psu)	TDS	a_{325} (m^{-1})	$S_{275-295}$ (μm^{-1})	S_R	TOC (mg C l^{-1})	POM (mg l^{-1})	Chlorophyll *a* (µg l^{-1})	Bacteria abundance (x 10^6 cells ml^{-1})
17/07/13	8.16	21.02	35.7	26.55	0.38	20.0	1.85	0.11	5.07	x	9.76
30/07/13	8.18	21.58	35.91	27.14	0.70	19.0	1.31	0.35	3.85	1.25	18.94
29/08/13	8.09	25.58	37.48	28.21	0.43	20.0	2.05	0.23	x	0.42	8.19
13/09/13	8.11	23.74	38.8	29.05	0.83	16.3	1.52	x	3.21	0.72	5.98
27/09/13	8.06	23.2	35.8	20.27	0.20	26.7	1.89	0.12	2.98	0.29	5.55
15/10/13	8.15	19.86	37.68	18.26	0.50	18.7	1.35	0.14	2.40	0.46	6.52
30/10/13	8.15	16.78	37.45	28.14	0.46	18.2	1.40	0.13	2.51	0.27	4.24
13/11/13	8.15	17.28	37.93	28.48	0.25	23.5	1.93	0.19	2.31	0.57	0.50
02/12/13	8.27	16.29	39.92	29.75	0.24	20.5	2.63	0.21	2.33	0.29	4.13
17/12/13	8.31	16.28	38.11	28.56	0.18	28.0	2.15	0.36	2.31	0.97	4.79
30/12/13	8.22	14.29	41.54	30.84	0.21	24.1	1.94	0.37	1.20	0.74	20.74
15/01/14	8.23	14.8	41.39	30.79	0.20	24.3	1.76	0.28	1.20	0.87	1.64
30/01/14	8.05	13.87	38.25	28.7	0.45	15.2	1.21	0.09	0.60	2.35	2.33
11/02/14	7.87	13.58	41.65	30.94	0.18	22.8	2.04	0.09	1.28	1.07	1.58
27/02/14	7.71	14.31	41.3	30.71	0.06	19.3	2.05	0.31	1.39	0.41	3.82
14/03/14	7.89	15.2	40.47	30.14	0.08	24.0	1.63	0.43	0.39	0.28	14.29
27/03/14	7.9	14.37	38.24	28.64	0.06	24.3	1.76	0.13	1.08	0.50	3.17
11/04/14	8.04	17.18	36.44	27.43	0.07	38.1	0.64	0.17	1.75	0.34	2.78
30/04/14	7.97	18.03	38.25	28.65	0.07	24.7	1.79	0.17	6.27	2.62	4.22
14/05/14	7.79	19.2	37.47	28.13	0.14	10.3	0.63	0.26	2.25	0.37	2.93
29/05/14	7.94	16.41	37.2	27.96	0.40	19.0	1.44	0.18	1.80	0.48	5.68
11/06/14	8.05	18.94	36.96	27.78	0.36	21.6	1.58	0.09	2.47	0.57	4.83
30/06/14	7.56	17.17	36.22	27.28	0.36	19.7	1.59	x	1.60	0.33	7.39
11/07/14	8.1	17.96	38.22	20.62	0.58	18.5	1.25	x	2.45	0.66	10.24
25/07/14	7.98	19.82	36.95	27.78	0.18	27.9	1.22	0.17	2.50	0.70	8.96
20/08/14	8.06	25.04	38.88	29.15	0.31	23.1	1.22	0.13	2.45	0.84	x

Table 2. Physicochemical parameters, CDOM quantitative-qualitative parameters, TOC, POM, Chlorophyll *a* and bacteria abundance of time-series experiments in the effluents of + holothurian effluent water.

Date (dd/mm/yy)	pH	Temp (ºC)	Salinity (psu)	TDS	a_{325} (m^{-1})	$S_{275-295}$ (μm^{-1})	S_R	TOC (mgC l^{-1})	POM (mg l^{-1})	Chlorophyll *a* ($\mu g\ l^{-1}$)	Bacteria abundance ($x10^6$ cells ml^{-1})
17/07/13	8.18	21.04	35.71	26.96	0.38	15.2	1.57	0.12	4.59	x	1.09
30/07/13	8.19	21.48	35.86	27.7	0.59	13.6	1.32	0.20	3.87	1.04	20.24
29/08/13	8.13	25.29	37.39	28.15	0.31	18.0	2.07	0.20	4.49	0.62	8.13
13/09/13	8.14	23.84	38.72	28.55	0.45	15.7	1.99	0.09	3.30	0.12	4.77
27/09/13	8.09	22.8	35.89	21.03	0.41	16.7	1.82	0.15	4.17	0.32	8.41
15/10/13	8.2	19.73	37.57	28.17	0.53	13.1	1.32	0.15	2.13	0.52	7.93
30/10/13	8.15	17.28	37.46	28.12	0.34	16.2	2.03	0.16	3.86	0.20	4.33
13/11/13	8.14	16.69	38.16	28.6	0.32	14.5	2.34	0.18	2.59	1.01	5.94
02/12/13	7.93	12.36	40.36	30.15	0.32	12.2	3.13	0.26	2.01	0.48	13.96
17/12/13	8.2	14.51	37.67	28.29	x	-	x	x	2.24	0.37	10.34
30/12/13	7.87	14.07	41.67	30.96	0.13	17.2	2.07	0.29	1.70	0.32	7.23
15/01/14	8.23	14.76	41.59	30.89	0.21	22.5	2.59	0.32	1.60	2.48	3.92
30/01/14	8.04	12.77	38.23	28.61	0.37	12.6	1.66	0.29	0.07	1.39	6.73
11/02/14	7.78	13.21	41.62	30.65	0.20	18.3	2.13	0.29	1.40	0.63	2.63
27/02/14	7.69	13.96	41.35	30.55	0.07	17.4	1.81	0.31	1.52	0.34	5.76
14/03/14	7.86	14.93	40.68	30.28	0.14	15.1	2.19	0.55	2.32	0.53	18.10
27/03/14	7.88	13.88	37.91	28.47	0.10	19.4	1.48	0.21	1.28	0.87	3.51
11/04/14	7.99	17.21	36.52	27.45	0.06	19.6	x	0.20	1.11	0.52	6.13
30/04/14	8.03	18.63	38.03	27.1	x		x	x	2.09	1.87	7.97
14/05/14	8.05	19.25	37.68	28.25	0.17	17.3	1.43	0.33	2.70	0.60	1.23
29/05/14	8.02	16.12	37.26	28	0.45	12.9	1.48	0.27	3.80	0.79	8.78
11/06/14	7.7	20.84	27.75	21.43	0.79	5.5	0.49	0.11	2.20	1.70	15.79
30/06/14	8.09	16.49	36.39	27.13	0.51	11.8	1.26	0.09	1.55	0.50	6.76
11/07/14	8.18	18.47	38.05	28.52	0.35	19.3	1.65	0.09	2.20	0.87	9.90
25/07/14	8.06	19.61	36.8	27.15	0.20	28.7	1.63	0.15	3.05	1.22	10.42
20/08/14	7.83	24.55	38.32	28.12	0.56	15.1	1.68	0.19	3.19	x	x

Table 3. Physicochemical parameters, CDOM quantitative-qualitative parameters, TOC, POM, Chlorophyll *a* and bacteria abundance of time-series experiments in the effluents of - holothurian effluent water

Date (dd/mm/yy)	pH	Temp (ºC)	Salinity (psu)	TDS	a_{325} (m^{-1})	$S_{275-295}$ (μm^{-1})	S_R	TOC (mgC l^{-1})	POM (mg l^{-1})	Chlorophyll *a* (μg l^{-1})	Bacteria abundance (x10^6 cells ml^{-1})
17/07/13	8.18	21.04	35.69	22.96	0.72	34.9	2.34	0.15	x	x	9.36
30/07/13	8.24	19.63	36.08	28.03	x	x	x	x	x	0.79	x
29/08/13	8.19	25.26	37.43	28.29	0.58	39.8	2.60	0.15	x	0.53	8.68
13/09/13	8.12	23.93	38.74	28.99	1.27	30.8	2.80	0.10	x	0.47	4.21
27/09/13	8.09	22.8	35.9	25.83	0.85	33.4	2.55	0.19	3.36	0.29	5.91
15/10/13	8.2	19.74	37.57	28.19	0.88	33.1	2.35	0.22	3.80	0.47	6.53
30/10/13	8.15	17.16	37.47	28.13	0.72	35.1	2.85	0.20	2.21	0.40	4.21
13/11/13	8.14	16.67	38.15	28.59	0.54	38.0	3.30	0.27	2.73	0.57	5.71
02/12/13	8.1	12.85	40.37	35.65	0.54	36.0	3.64	0.42	1.67	x	15.23
17/12/13	8.25	14.63	37.78	30.16	0.68	34.3	3.90	0.42	1.67	x	9.23
30/12/13	7.86	14.09	41.8	32.25	x		x	x	2.64	x	9.19
15/01/14	8.21	14.83	41.62	31.32	x	x	x	x	3.54	2.31	7.9.8
30/01/14	8.03	12.8	38.25	29.41	0.54	13.7	1.66	0.21	2.94	0.92	8.8.8
11/02/14	7.8	13.13	41.61	30.95	0.54	36.5	2.97	0.23	1.92	0.52	2.2.9
27/02/14	7.7	13.9	41.35	30.75	0.37	40.4	3.16	0.24	2.35	x	14.21
14/03/14	7.88	14.91	40.68	30.29	0.49	34.3	2.74	0.51	0.72	1.91	17.60
27/03/14	7.87	13.89	37.93	28.97	x	x	x	x	2.28	2.19	x
11/04/14	8.04	17.08	36.28	27.89	x	x	x	x	1.98	x	10.58
30/04/14	8.03	18.63	38.04	28.5	0.44	37.2	0.40	0.23	1.12	0.37	12.16
14/05/14	8.08	19.23	37.7	28.28	0.45	39.3	2.43	0.37	2.76	0.34	4.89
29/05/14	8.05	16.08	37.27	28	0.67	33.6	2.45	0.20	0.66	0.98	7.99
11/06/14	7.7	20.84	27.75	21.51	1.13	31.3	2.22	0.15	2.80	1.26	9.13
30/06/14	8.1	16.55	36.36	27.39	0.90	30.5	2.48	0.09	2.52	0.83	7.13
11/07/14	8.2	18.14	38.2	28.61	0.74	36.1	2.44	0.12	2.82	1.69	14.51
25/07/14	8.04	19.88	36.74	27.64	0.57	39.1	1.75	0.15	2.88	1.16	13.48
20/08/14	7.83	24.55	38.32	28.64	x	x	x	x	x	0.81	x

Table 4. Physicochemical parameters, CDOM quantitative-qualitative parameters, TOC, POM, Chlorophyll *a* and bacteria abundance of time-series experiments in the + holothurian tank water

Date (dd/mm/yy)	pH	Temp (ºC)	Salinity (psu)	TDS	a_{325} (m^{-1})	$S_{275-295}$ (μm^{-1})	S_R	TOC (mg C l^{-1})	POM (mg l^{-1})	Chlorophyll *a* ($\mu g\ l^{-1}$)	Bacteria abundance (x 10^6 cell ml^{-1})
17/07/13	8.15	21.41	35.61	26.90	0.55	23.4	1.87	0.11	3.90	x	9.06
30/07/13	8.25	19.61	36.07	27.19	0.30	29.2	1.85	0.31	3.62	0.25	6.38
29/08/13	8.17	25.32	37.48	28.16	0.60	23.7	2.37	0.22	4.75	0.37	7.00
13/09/13	8.13	23.95	38.75	29.02	1.02	19.8	1.92	0.10	3.96	0.51	4.64
27/09/13	7.89	22.8	35.87	21.31	0.48	25.3	2.02	0.19	4.48	0.41	8.28
15/10/13	8.01	19.78	37.6	28.24	0.76	20.6	1.61	0.20	3.65	0.46	6.86
30/10/13	8.11	17.28	37.51	28.12	0.46	25.0	2.21	0.17	2.96	0.11	3.16
13/11/13	8.14	16.69	38.15	58.59	0.46	23.7	2.49	0.25	3.13	0.50	5.79
02/12/13	7.93	12.36	40.36	30.15	0.41	21.5	2.87	0.38	2.63	0.44	14.83
17/12/13	8.2	14.51	37.67	28.29	0.46	26.2	2.34	0.31	2.68	1.26	10.24
30/12/13	7.87	14.08	41.7	30.98	0.28	30.7	2.67	0.32	1.23	0.54	7.13
15/01/14	8.23	14.8	41.59	30.89	0.35	27.5	2.15	0.32	1.30	1.41	3.82
30/01/14	8.04	12.77	38.24	28.72	0.48	21.6	2.04	0.16	1.00	0.69	6.62
11/02/14	7.83	13.16	41.64	30.91	0.31	26.1	2.61	0.20	1.48	0.52	2.31
27/02/14	7.31	14.11	41.31	30.73	0.14	29.2	2.03	0.30	1.56	0.70	7.31
14/03/14	7.85	14.94	40.71	30.3	0.24	24.5	2.25	0.57	1.80	0.50	18.35
27/03/14	7.88	13.88	37.91	28.47	0.32	22.1	2.30	0.22	1.40	1.05	3.42
11/04/14	8.05	17.07	36.25	27.31	0.19	27.5	4.37	0.17	1.40	0.45	6.58
30/04/14	7.99	18.61	38.07	28.53	0.48	22.1	1.66	0.29	2.93	1.84	8.05
14/05/14	8.04	19.22	37.63	28.23	0.30	25.0	1.88	0.30	2.60	0.39	3.92
29/05/14	8.01	16.22	37.3	28.02	0.49	23.0	1.74	0.11	2.25	0.66	6.77
11/06/14	7.7	20.84	27.75	21.51	0.93	14.5	1.08	0.27	2.27	0.99	7.01
30/06/14	8.09	16.47	36.4	27.42	0.62	21.0	1.57	0.06	2.30	0.45	6.90
11/07/14	8.17	17.99	57.25	28.63	0.56	23.6	1.77	0.08	2.55	0.92	9.76
25/07/14	8.04	19.6	36.8	27.68	0.34	36.6	2.10	0.12	3.20	1.17	12.20
20/08/14	7.83	24.55	38.32	28.64	0.67	24.1	2.01	0.16	3.05	2.07	x

Table 5. Physicochemical parameters, CDOM quantitative-qualitative parameters, TOC, POM, Chlorophyll *a* and bacteria abundance of time-series experiments in the - holothurian tank water

Date (dd/mm/yy)	pH	Temp (ºC)	Salinity (psu)	TDS	a_{325} (m^{-1})	$S_{275-295}$ (μm^{-1})	S_R	TOC (mgC l^{-1})	POM (mg l^{-1})	Chlorophyll *a* (μg l^{-1})	Bacteria abundance (x 10^6 cells ml^{-1})
17/07/13	8.18	21.09	35.68	26.93	0.79	26.4	2.18	0.16	3.72	x	10.22
30/07/13	8.23	19.63	36.08	27.93	x	x	x	x	x	x	x
29/08/13	8.18	25.33	37.43	28.16	0.90	26.0	2.17	0.21	x	2.99	7.60
13/09/13	8.14	23.92	38.80	29.05	0.82	27.5	2.20	0.08	4.44	0.47	4.20
27/09/13	7.96	22.8	35.92	25.16	0.76	27.1	2.19	0.17	3.40	0.35	9.30
15/10/13	8.16	19.78	37.62	28.23	0.93	24.7	1.93	0.19	3.46	0.31	7.35
30/10/13	8.14	17.15	37.47	28.13	0.77	26.3	2.68	0.17	2.32	0.31	2.80
13/11/13	8.14	16.67	38.17	28.6	0.62	29.2	3.04	0.15	2.77	0.57	5.84
02/12/13	8.11	12.83	40.36	35.65	0.62	26.3	3.81	0.25	3.42	1.18	16.93
17/12/13	8.26	14.62	37.77	28.36	0.75	29.2	2.56	0.34	3.12	1.18	10.06
30/12/13	7.86	14.09	41.8	32.15	x	x	x	x	4.14	x	7.13
15/01/14	8.22	14.82	41.61	31.19	x	x	x	x	3.60	2.56	3.88
30/01/14	8.03	12.79	38.24	29.01	0.56	29.2	3.32	0.31	2.82	0.92	6.66
11/02/14	7.85	13.06	41.63	30.97	0.56	30.0	2.65	0.22	2.28	0.90	2.31
27/02/14	7.66	13.93	41.34	30.75	0.44	32.0	2.60	0.30	1.97	x	14.11
14/03/14	7.88	14.91	40.6	30.28	0.54	26.2	2.50	0.74	1.54	2.45	18.34
27/03/14	7.87	13.89	37.92	28.91	x	x	x	x	2.34	3.09	x
11/04/14	8.04	17.08	36.26	27.82	x	x	x	x	2.04	x	10.59
30/04/14	8.03	18.63	38.05	28.51	0.52	29.2	2.68	0.20	3.12	0.31	12.07
14/05/14	8.08	19.22	37.69	28.27	0.52	30.3	1.99	0.30	2.34	0.44	4.73
29/05/14	8.04	16.06	37.28	28.01	0.82	25.1	2.07	0.20	2.28	0.76	8.08
11/06/14	7.7	20.84	27.75	21.51	1.16	22.2	1.79	0.12	3.30	1.33	10.02
30/06/14	8.1	16.5	36.37	27.4	0.93	22.4	2.11	0.07	2.58	0.71	7.19
11/07/14	8.2	18.06	38.21	28.02	0.81	26.3	2.17	0.07	3.12	1.14	12.15
25/07/14	8.04	19.84	36.75	27.64	0.62	32.1	1.51	0.13	3.28	1.27	x
20/08/14	x	x	x	x	x	x	x	x	x	x	x

Table 6. Mean results of the CDOM quantitative-qualitative parameters of short-term experiments.

	Date(dd/mm/yy)	a_{325}	a_{443}	$S_{275\text{-}295}$
Tank 1	6/10/17	7.7	3.3	3
	9/10/17	5.0	0.5	2
	27/10/17	2.9	0.3	4
	30/10/17	1.5	0.7	3
	3/11/17	4.0	0.4	5
	6/11/17	2.5	0.3	4
Tank 2	6/10/17	7.8	3.3	3
	9/10/17	5.2	0.5	2
	27/10/17	2.9	0.5	4
	30/10/17	1.9	1.1	3
	3/11/17	3.9	0.3	5
	6/11/17	2.4	0.4	4
Tank 3	6/10/17	7.4	3.3	3
	9/10/17	5.1	0.5	1
	27/10/17	2.3	0.2	4
	30/10/17	2.6	0.2	3
	3/11/17	4.0	0.4	5
	6/11/17	1.4	0.2	3
Tank 4	6/10/17	7.4	3.2	3
	9/10/17	7.4	3.3	3
	27/10/17	3.3	0.4	3
	30/10/17	3.9	0.4	4
	3/11/17	4.0	0.4	5
	6/11/17	4.0	0.4	5
Tank 5	6/10/17	7.4	3.3	3
	9/10/17	7.5	3.4	3
	27/10/17	3.4	0.4	4
	30/10/17	4.1	0.4	4
	3/11/17	3.9	0.4	5
	6/11/17	4.1	0.4	5
Tank 6	6/10/17	7.4	3.3	3
	9/10/17	7.3	3.2	3
	27/10/17	2.9	0.3	4
	30/10/17	4.1	0.4	4
	3/11/17	4.0	0.4	5
	6/11/17	4.3	0.5	5
Tank 7	6/10/17	7.4	3.3	3
	9/10/17	7.5	3.4	3
	27/10/17	1.9	0.4	5
	30/10/17	4.1	0.5	4
	3/11/17	3.9	0.4	5
	6/11/17	1.9	0.3	5

Appendix III.

Table 1. Component concentration (x 10^{-3} RU) of time- series experiments in the inlet

Date (dd/mm/yy)	C1	C2	C3	C4	C5	C6
17/07/13	6.5	8.0	17.1	8.5	5.6	12.1
30/07/13	7.1	8.0	17.1	8.5	13.0	10.0
29/08/13	8.0	5.2	11.8	5.8	26.8	23.3
13/09/13	8.5	8.3	10.3	6.8	9.3	27.7
27/09/13	10.7	9.2	11.7	6.3	7.4	25.5
15/10/13	29.1	37.9	24.4	10.8	11.2	24.8
30/10/13	26.2	29.3	23.4	9.9	3.3	17.0
13/11/13	16.0	10.1	21.4	8.4	8.7	30.2
02/12/13	13.2	6.2	14.8	9.0	8.1	22.2
17/12/13	8.8	3.6	10.6	5.4	4.0	9.1
30/12/13	10.9	3.5	12.5	6.2	7.3	17.7
15/01/14	10.8	3.9	12.3	6.4	9.0	19.9
30/01/14	2.6	3.0	21.2	5.7	6.9	13.1
11/02/14	11.1	3.3	12.6	6.3	6.3	16.2
27/02/14	10.9	3.8	13.4	6.3	18.4	5.4
14/03/14	9.9	5.4	13.4	6.5	7.1	16.5
27/03/14	9.6	3.0	11.8	6.0	6.2	17.0
11/04/14	13.0	2.2	13.4	7.0	6.2	17.0
30/04/14	10.0	2.9	12.7	6.7	4.7	16.3
14/05/14	3.8	12.4	37.8	17.7	5.6	8.1
29/05/14	21.6	14.9	21.6	9.9	14.9	6.1
11/06/14	14.6	5.5	14.9	7.9	7.0	7.2
30/06/14	14.4	10.2	15.2	7.5	8.8	9.9
11/07/14	23.4	5.4	35.3	8.0	19.9	5.8
25/07/14	4.0	9.8	65.2	9.4	10.9	5.1
20/08/14	X	X	X	X	X	X

Table 2. Component concentration (x 10^{-3} RU) of time- series experiments in the effluents of +holothurian effluent

Date (dd/mm/yy)	C1	C2	C3	C4	C5	C6
17/07/13	8.0	7.6	17.4	10.4	7.0	14.7
30/07/13	9.0	3.0	12.4	5.4	6.2	14.7
29/08/13	15.7	2.5	13.7	7.3	8.2	16.5
13/09/13	11.8	1.4	16.4	6.3	9.3	10.4
27/09/13	19.8	10.5	18.4	8.7	8.9	16.4
15/10/13	X	X	X	X	X	X
30/10/13	14.1	8.0	15.9	7.2	6.8	13.4
13/11/13	13.4	7.0	16.7	7.6	0.6	12.9
02/12/13	13.3	3.5	22.3	10.6	11.4	10.2
17/12/13	12.6	4.2	19.7	9.2	5.2	3.7
30/12/13	10.9	2.8	13.0	5.8	7.0	10.8
15/01/14	11.3	2.1	13.5	6.9	4.1	11.6
30/01/14	16.5	2.8	22.0	7.2	8.6	10.5
11/02/14	10.6	2.1	12.5	6.3	4.9	11.1
27/02/14	9.2	1.5	12.5	5.8	5.4	18.4
14/03/14	9.5	0.9	14.8	6.9	5.9	13.2
27/03/14	10.3	3.6	14.5	6.9	3.9	18.6
11/04/14	9.2	4.5	13.9	6.7	5.3	18.6
30/04/14	13.9	4.1	20.4	2.4	3.6	8.2
14/05/14	18.1	9.1	20.3	6.4	4.7	7.9
29/05/14	20.2	11.4	20.0	9.6	4.8	8.3
11/06/14	4.8	8.0	60.2	2.9	7.4	19.7
30/06/14	22.5	24.0	23.0	9.9	7.1	17.4
11/07/14	X	X	X	X	X	X
25/07/14	9.6	6.0	15.0	7.7	1.1	14.2
20/08/14	X	X	X	X	X	X

Table 3. Component concentration (x 10^{-3} RU) of time- series experiments in the effluents of -holothurian effluent

Date (dd/mm/yy)	C1	C2	C3	C4	C5	C6
17/07/13	25.0	14.5	18.4	10.9	13.6	19.3
30/07/13	X	X	X	X	X	X
29/08/13	11.6	3.8	13.0	7.8	9.5	19.9
13/09/13	9.8	9.6	15.1	6.6	149.0	79.1
27/09/13	17.5	24.3	15.4	10.5	102.8	19.4
15/10/13	28.4	39.5	25.1	10.9	7.4	49.6
30/10/13	13.6	13.0	14.5	7.8	9.4	18.1
13/11/13	13.2	11.5	15.9	9.3	4.8	33.7
02/12/13	14.7	6.7	18.6	12.9	14.6	24.7
17/12/13	11.3	6.2	16.7	11.9	7.8	27.9
30/12/13	11.4	3.2	12.2	6.3	11.6	27.2
15/01/14	11.4	3.2	12.2	8.3	11.9	29.7
30/01/14	X	X	X	X	X	X
11/02/14	X	X	X	X	X	X
27/02/14	9.4	3.1	12.8	7.7	6.6	29.8
14/03/14	9.8	5.8	13.9	7.3	14.3	18.4
27/03/14	X	X	X	X	X	X
11/04/14	X	X	X	X	X	X
30/04/14	10.3	4.1	14.5	8.7	10.5	17.6
14/05/14	11.9	9.3	12.9	7.2	5.9	15.2
29/05/14	20.9	11.6	20.9	14.3	7.1	18.9
11/06/14	15.3	8.5	21.8	14.3	8.8	28.2
30/06/14	19.6	24.7	20.2	11.4	8.3	28.2
11/07/14	22.0	7.7	37.2	9.9	5.2	23.2
25/07/14	10.3	4.1	14.5	8.7	10.5	17.6
20/08/14	X	X	X	X	X	X

Table 4. Component concentration (x 10^{-3} RU) of time- series experiments in the +holothurian tank

Date (dd/mm/yy)	C1	C2	C3	C4	C5	C6
17/07/13	X	X	X	X	X	X
30/07/13	18.2	10.9	34.3	17.4	8.2	44.3
29/08/13	10.9	5.5	14.3	6.2	21.0	8.7
13/09/13	9.7	9.6	12.9	6.3	188.8	27.3
27/09/13	18.6	24.7	18.2	9.0	82.1	35.7
15/10/13	28.8	41.4	24.2	10.7	82.0	18.4
30/10/13	14.0	11.6	14.6	6.9	19.3	17.2
13/11/13	13.8	10.5	17.2	8.7	6.6	21.5
02/12/13	13.3	8.5	22.3	10.6	8.7	13.7
17/12/13	12.6	7.0	19.7	9.2	8.1	30.2
30/12/13	9.1	2.3	10.3	4.9	18.7	22.2
15/01/14	11.1	5.6	12.9	6.6	7.2	13.7
30/01/14	16.5	9.2	22.0	10.1	9.0	17.5
11/02/14	11.1	3.9	12.9	6.7	7.0	19.9
27/02/14	9.1	3.2	12.6	5.8	5.7	12.4
14/03/14	10.4	6.5	12.1	7.5	6.4	15.5
27/03/14	11.2	3.8	14.7	7.3	6.9	16.3
11/04/14	8.5	4.8	13.6	6.5	7.3	15.0
30/04/14	8.5	4.8	13.6	6.5	6.9	16.4
14/05/14	14.7	10.9	16.9	7.9	3.2	37.6
29/05/14	19.3	12.6	20.1	10.0	23.2	26.4
11/06/14	45.4	23.5	59.6	28.8	16.3	20.5
30/06/14	21.7	26.0	20.6	9.2	16.0	24.3
11/07/14	17.2	8.1	24.0	9.5	16.1	23.0
25/07/14	14.1	6.9	21.7	8.4	19.2	X
20/08/14	31.3	10.6	55.5	15.2	10.9	57.2

Table 5. Component concentration (x 10^{-3} RU) of time- series experiments in the -holothurian tank

Date (dd/mm/yy)	C1	C2	C3	C4	C5	C6
17/07/13	25.0	14.5	18.4	10.9	13.6	19.3
30/07/13	X	X	X	X	X	X
29/08/13	11.6	3.8	13.0	7.8	9.5	19.9
13/09/13	9.8	9.6	15.1	6.6	14.9	79.1
27/09/13	17.5	24.3	15.4	10.5	10.3	19.4
15/10/13	28.4	39.5	25.1	10.9	7.4	49.6
30/10/13	13.6	13.0	14.5	7.8	9.4	18.1
13/11/13	13.2	11.5	15.9	9.3	4.8	33.7
02/12/13	14.7	6.7	18.6	12.9	14.6	24.7
17/12/13	11.3	6.2	16.7	11.9	7.8	27.9
30/12/13	11.4	3.2	12.2	6.3	11.6	27.2
15/01/14	11.4	3.2	12.2	8.3	11.9	29.7
30/01/14	X	X	X	X	X	X
11/02/14	X	X	X	X	X	X
27/02/14	9.4	3.1	12.8	7.7	6.6	29.8
14/03/14	9.8	5.8	13.9	7.3	14.3	18.4
27/03/14	X	X	X	X	X	X
11/04/14	X	X	X	X	X	X
30/04/14	10.3	4.1	14.5	8.7	10.5	17.6
14/05/14	11.9	9.3	12.9	7.2	5.9	15.2
29/05/14	20.9	11.6	20.9	14.3	7.1	18.9
11/06/14	15.3	8.5	21.8	14.3	8.8	28.2
30/06/14	19.6	24.7	20.2	11.4	8.3	28.2
11/07/14	22.0	7.7	37.2	9.9	5.2	23.2
25/07/14	10.3	4.1	14.5	8.7	10.5	17.6
20/08/14	X	X	X	X	X	X

Table 6. Component concentration (x 10^{-3} RU) of short- term experiments in tank 1

Date (dd/mm/yy)	C1	C2	C3	C4	C5	C6
06/10/17	345.6	242.3	278.6	136.2	222.0	37.2
09/10/17	359.3	252.6	290.5	144.6	205.3	36.7
27/10/17	298.4	204.6	231.2	111.9	121.2	39.9
30/10/17	304.2	213.2	231.2	113.6	117.4	42.1
03/11/17	297.9	204.7	233.8	108.5	100.2	32.2
06/11/17	306.5	210.1	234.6	116.0	106.4	42.6

Table 7. Component concentration (x 10^{-3} RU) of short- term experiments in tank 2

Date (dd/mm/yy)	C1	C2	C3	C4	C5	C6
06/10/17	341.3	241.0	273.4	136.7	218.9	32.6
09/10/17	373.5	262.1	298.8	148.8	213.4	43.3
27/10/17	290.6	202.5	225.8	107.5	119.4	44.2
30/10/17	311.3	217.9	236.3	115.1	120.7	39.8
03/11/17	297.8	205.7	228.3	110.4	102.6	23.5
06/11/17	X	X	X	X	X	X

Table 8. Component concentration (x 10^{-3} RU) of short- term experiments in tank 3

Date (dd/mm/yy)	C1	C2	C3	C4	C5	C6
06/10/17	X	X	X	X	X	X
09/10/17	370.1	258.7	295.9	147.4	208.6	35.7
27/10/17	294.9	207.7	229.5	109.8	121.4	41.9
30/10/17	309.6	215.2	237.4	114.9	121.0	39.3
03/11/17	295.6	205.3	226.3	110.5	103.0	22.6
06/11/17	309.9	212.9	238.9	117.4	108.0	40.4

Table 9. Component concentration (x 10^{-3} RU) of short- term experiments in tank 4

Date (dd/mm/yy)	C1	C2	C3	C4	C5	C6
06/10/17	336.4	238.0	269.7	135.5	216.9	33.4
09/10/17	369.5	259.4	292.5	146.5	208.3	33.5
27/10/17	293.9	207.4	227.1	110.1	122.2	43.6
30/10/17	302.6	210.7	231.7	112.3	119.0	38.0
03/11/17	295.1	204.5	226.4	110.7	102.1	22.9
06/11/17	308.9	213.9	237.3	116.7	106.9	40.5

Table 10. Component concentration (x 10^{-3} RU) of short- term experiments in tank 5

Date (dd/mm/yy)	C1	C2	C3	C4	C5	C6
06/10/17	336.4	239.5	268.3	135.6	217.4	37.2
09/10/17	373.3	268.2	299.3	149.9	214.1	36.0
27/10/17	294.2	206.9	227.1	110.9	120.8	39.1
30/10/17	310.2	217.8	239.7	113.0	118.9	41.9
03/11/17	294.2	203.4	226.1	109.9	102.8	23.1
06/11/17	311.2	212.5	239.5	116.8	106.9	40.7

Table 11. Component concentration (x 10^{-3} RU) of short- term experiments in tank 6

Date (dd/mm/yy)	C1	C2	C3	C4	C5	C6
06/10/17	335.8	234.6	271.5	136.0	216.1	32.0
09/10/17	376.3	265.2	300.8	149.2	212.8	38.6
27/10/17	292.8	206.3	225.2	110.3	120.1	39.3
30/10/17	308.9	214.9	236.0	113.6	119.5	41.7
03/11/17	293.8	205.6	224.0	109.8	109.0	41.1
06/11/17	307.9	212.6	237.1	116.3	106.4	40.1

Table 12. Component concentration (x 10^{-3} RU) of short- term experiments in tank 7

Date (dd/mm/yy)	C1	C2	C3	C4	C5	C6
06/10/17	337.9	241.5	272.1	136.3	217.7	31.6
09/10/17	X	X	X	X	X	X
27/10/17	292.4	206.1	225.9	110.4	120.3	38.7
30/10/17	308.8	217.7	233.9	113.2	119.3	42.7
03/11/17	X	X	X	X	X	X
06/11/17	306.1	214.9	270.2	107.2	107.2	39.6

Appendix IV.

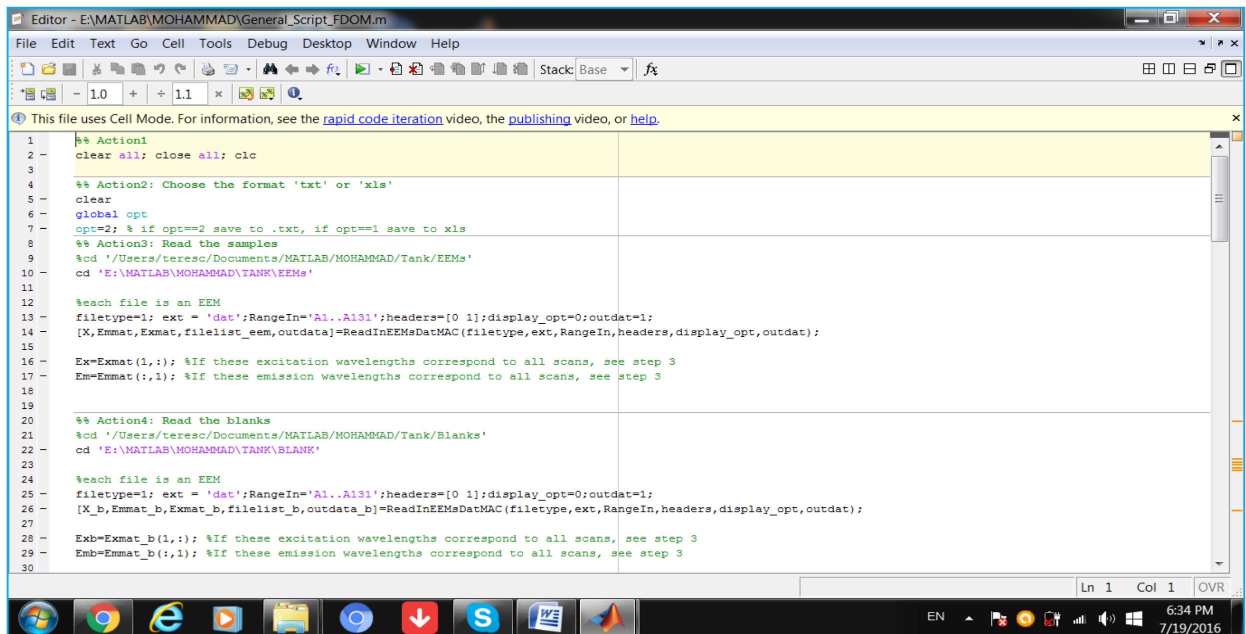


Fig.1. Part of using general script by Matlab program (Ver. R,2016b) to obtain Coble Peaks and final components (Time series experiments)

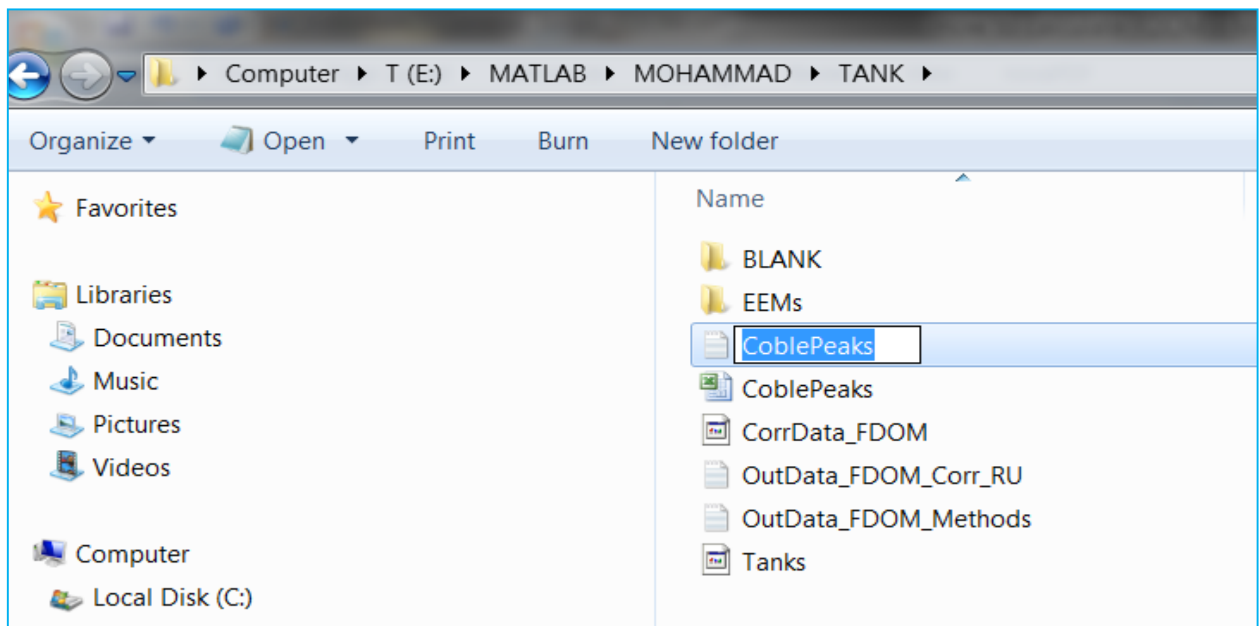


Fig.2. Coble Peaks output file format obtained by Matlab (Time series

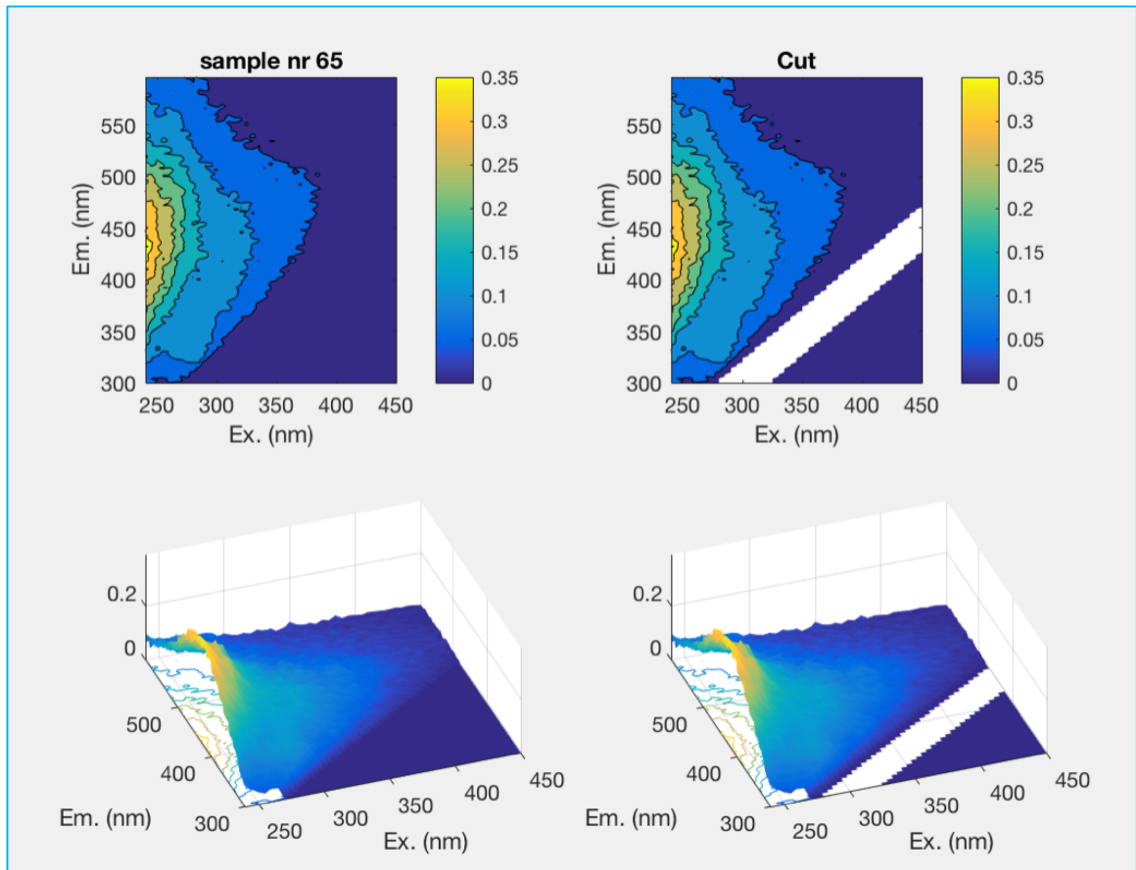


Fig.3. Action cutting the region of the spectra influenced by scatter peaks([CutData]= EEMCut (OriginalData,20, 20, NaN, NaN,'No')). The graphs will plot automatically from the first sample to the last. The function deletes the data in the region of no fluorescence (where emission wavelength is less than excitation wavelength) and the regions greatly influenced by first order scatter (where Rayleigh and Raman peaks dominate the signal).

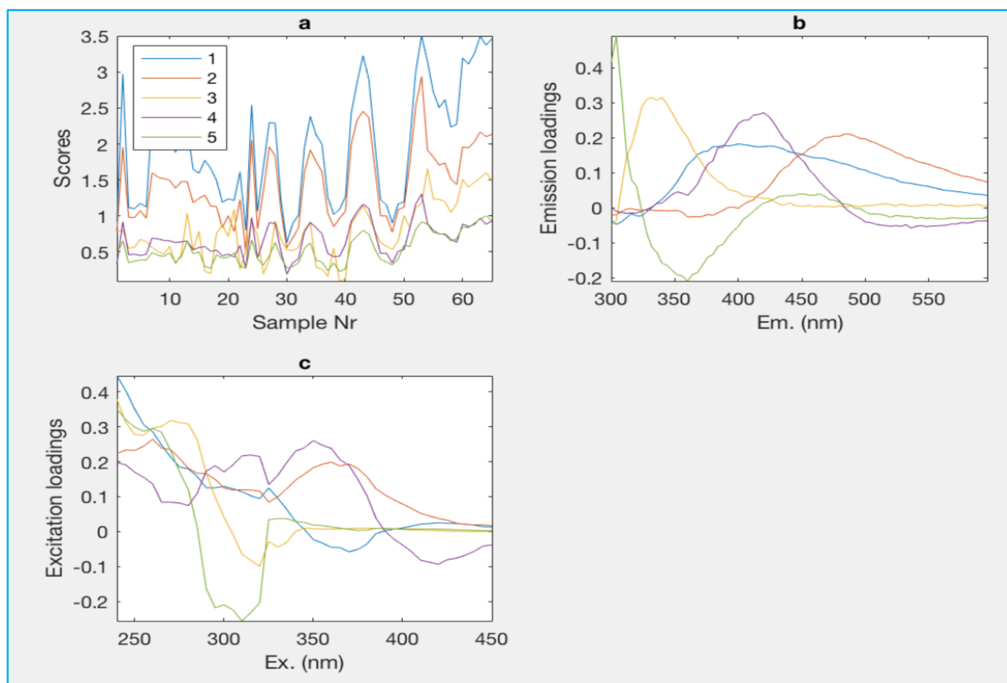


Fig.4. Plots *b* and *c* show the emission and excitation loadings of the five components and Plot *a* shows how the concentration of the two components varies between samples (Time series experiments).

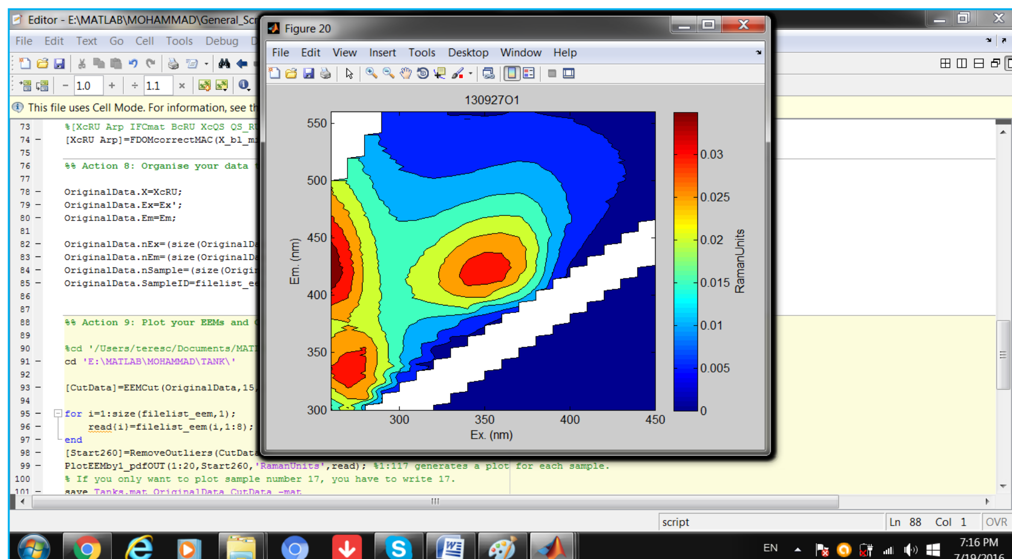


Fig.5. Plots generation action

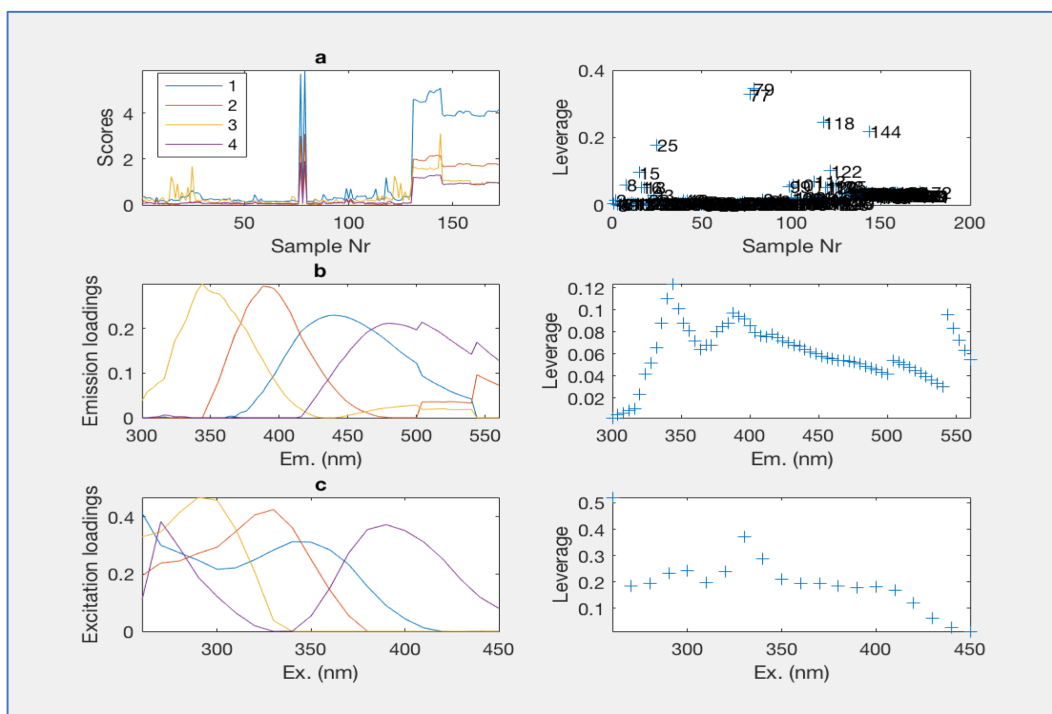


Fig.6. Action plot Leverage (Plot LL, Test 1.5). The Leverage Plot seems to suggest that Sample number 40 showed extreme Leverage and maybe is problematic.

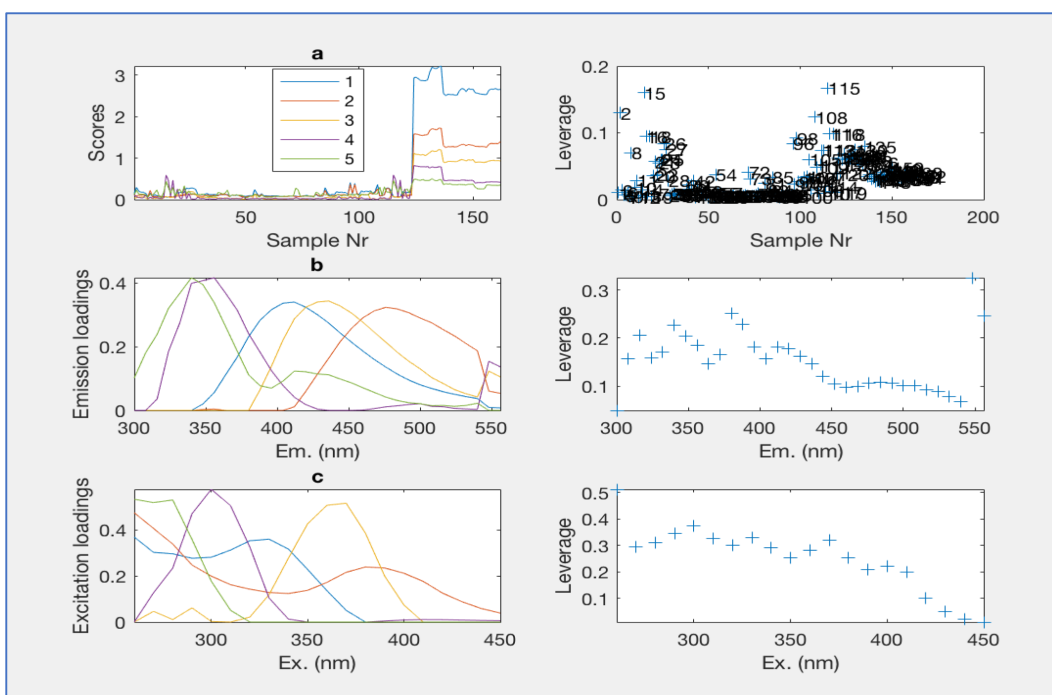


Fig.7. Action remove outlier ([Test3] =Remove Outliers (CutData,[5 30],[],[])).

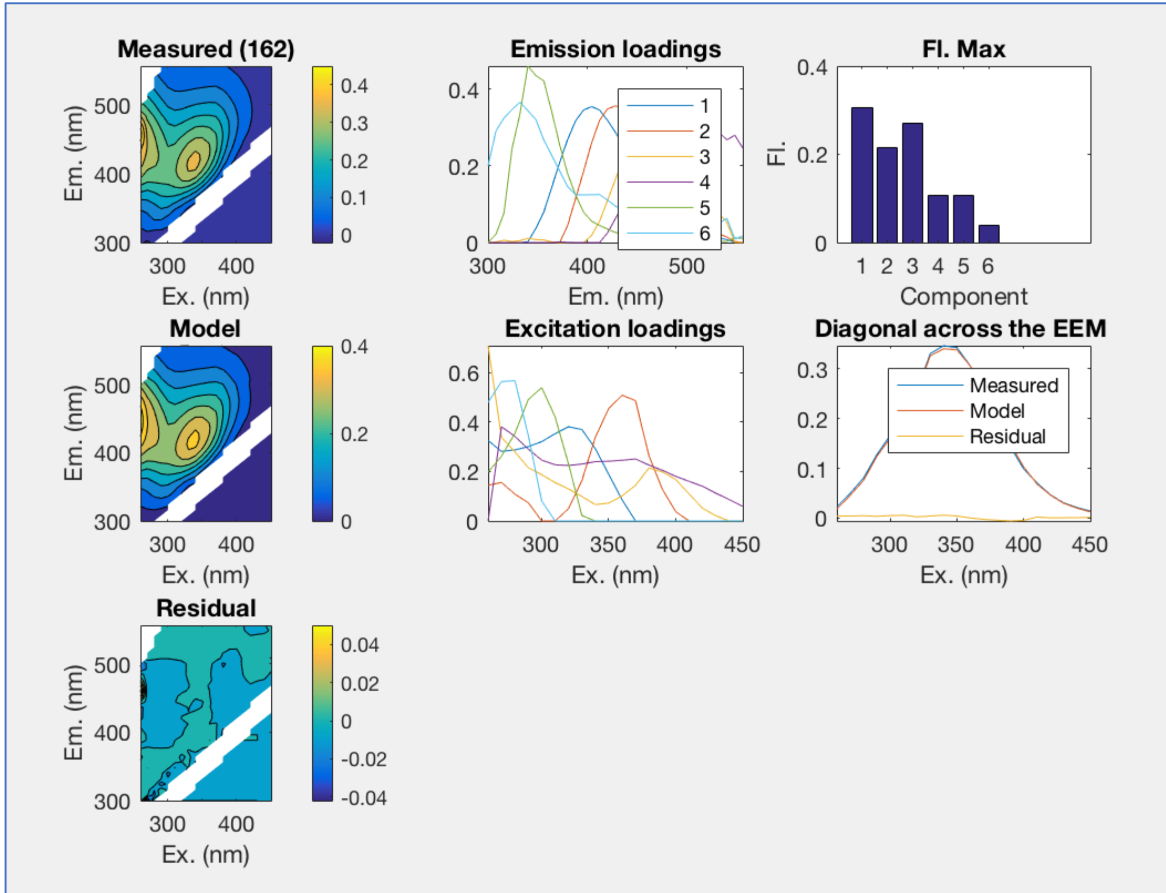


Fig.8. Action `EvalModel` which creates a series of graphs which we can use to evaluate the model fit by looking at the residuals (measured minus modeled data)

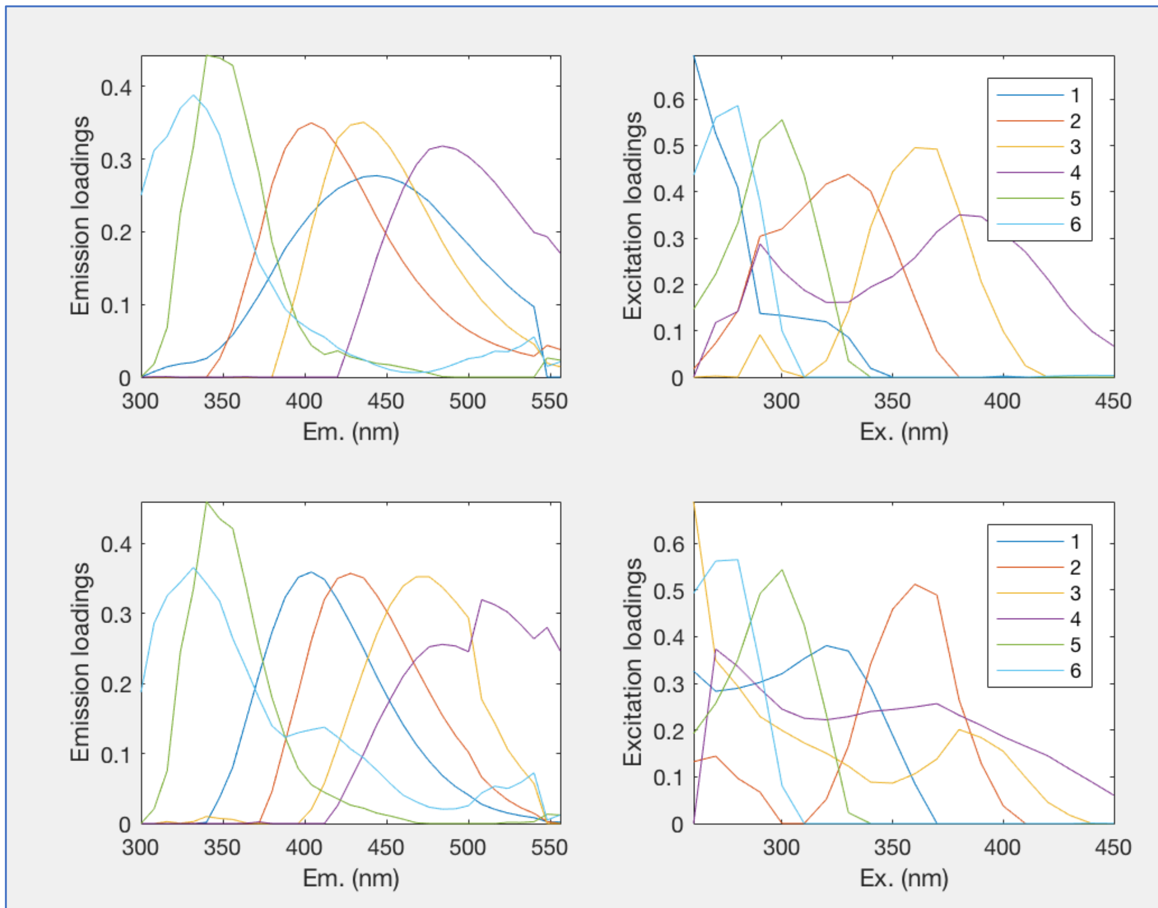


Fig.9. Action Split-Half model (SplitHalfValidation(AnalysisData,'1-2',3)). This function mathematically compares the excitation and emission loadings of the models run on separate splits of the data using Tucker Congruence Coefficients as described by (Lorenzo-Seva and Berge, 2006) and states in the Command Window whether the model is validated or not.

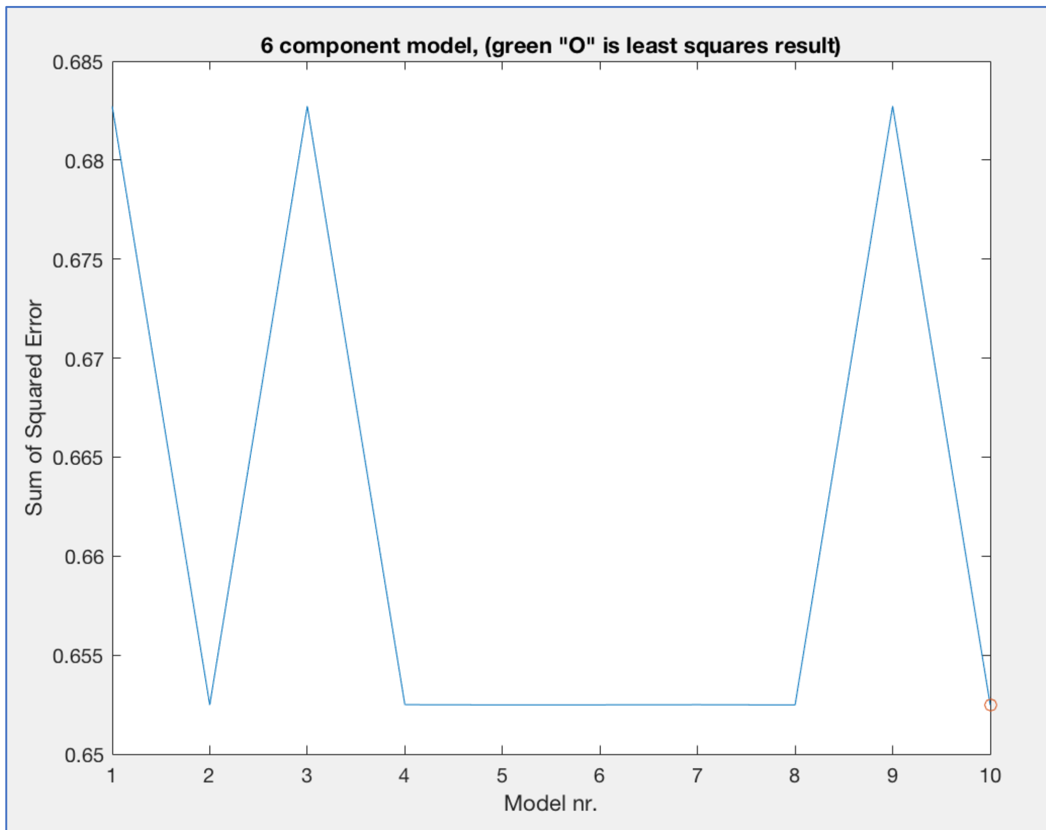


Fig.10. Action Random initialization (RandInitAnal (Analysis Data,4,10). The model with the least squares result is highlighted with a green circle.

References

APHA. 1992. Standard Methods for the Examination of Water and Wastewater. 18th edition. Washington,D.C.: American Public Health Association.

Bortone G. 2009. Integrated anaerobic/aerobic biological treatment for intensives swine production. *Bioresource Technology* **100**: 5424–5430 DOI 10.1016/j.biortech.2008.12.005.

Buhmann AH, Papenbrock J. 2013. Biofiltering of aquaculture effluents by halophytic plants: Basic principles, current uses and future perspectives. *Environmental and Experimental Botany* **92**:122-133 DOI 10.1016/j.envexpbot.2012.07.005.

Chopin T, Buschmann AH, Halling C, Troell M, Kautsky N, Neori A, Kraemer GP, Zertuche-Gonzalez JA, Yarish C, Neefus C. 2001. Integrating seaweeds into marine aquaculture systems: a key toward sustainability. *Journal of Phycology* **37**: 975–986 Available at: https://www.researchgate.net.

Fontenot Q, Bonvillain C, Kilgen M, Boopathy R. 2007. Effects of temperature, salinity, and carbon: nitrogen ratio on sequencing batch reactor treating shrimp aquaculture wastewater. Bioresource Technology **98**:1700–1703 DOI 10.1016/j.biortech.2006.07.031.

Gasol JM, del Giorgio PA. 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Scientia Marina* **64**:197-224 DOI:10.3989/scimar.2000.64n2197.

Lorenzo-Seva U, Berge JMFT. 2006. Tucker's congruence coefficient as a meaningful index of factor similarity. *Methodology* **2**: 57–64 DOI 10.1027/1614-1881.2.2.57.

Oladoja NA, Adelagun ROA, Ahmad AL, Ololade IA. 2015. Phosphorus recovery from aquaculture wastewater using thermally treated gastropod shell. *Process Safety and Environmental Protection* **98**:296-308 DOI 10.1016/J.PSEP.2015.09.006.

Passow U, Alldredge AL. 1995. A dye-binding assay for the spectrophotometric measurement of transparent exopolymer particles (TEP). *Limnology and Oceanography* **40**:1326-1335. Available at: http://onlinelibrary.wiley.com.

Zhang Q, Achal V, Xu Y, Xiang WN. 2014. Aquaculture Wastewater quality improvement by water Spinach (*Ipomoea aquatica Forskall*) floating bed and ecological benefit assessment in ecological agriculture district. *Aquacultural Engineering* **60**:48-55 DOI 10.1016/j.aquaeng.2014.04.002.