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Toxigenic effects of benthic diatoms upon grazing activity of the sea urchin *Paracentrotus lividus*

Tutors:

Dr. Maria Costantini, Stazione Zoologica Anton Dohrn, Naples

Dr. Angelo Fontana, Istituto di Chimica Biomolecolare, ICB-CNR, Pozzuoli

Dr. Valerio Zupo, Stazione Zoologica Anton Dohrn, Ischia

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Nadia

Abstract

Diatoms are unicellular algae playing a key role as photosynthetic organisms in the world's ocean food web. The chemical ecology of planktonic diatoms is well documented, but few studies have reported on the effects of benthic diatoms on their consumers, also due to difficulties in the collection, quantification and massive culturing of benthic species. This study investigates, for the first time, the effects of diets based on four benthic diatoms, *Cylindrotheca closterium*, *Nanofrustulum shiloi*, *Cocconeis scutellum* and *Diploneis* sp. isolated from the leaves of the seagrass *Posidonia oceanica*, on the sea urchin *Paracentrotus lividus*. The results demonstrate a toxigenic effect on embryos generated by females fed for one month on these benthic diatoms by multidisciplinary approaches. (i) Morphological observations by microscope revealed a noxious effects of *C. closterium*, *N. shiloi* and *Diploneis* sp. on embryos deriving from adult sea urchin *P. lividus* fed for one month on these diets, with *N. shiloi* showing the strongest effects; on the contrary, *C. scutellum* showed no effects, producing embryos as those deriving from the control diet. Malformations of these embryos were very similar to those observed after treatment with planktonic diatom-derived oxylipins. (ii) Metabolomic analysis by *Nuclear Magnetic Resonance* (^{1}H-NMR) demonstrated that feeding on these diatoms induced variations in the levels of lipids and/or amino acids in the gonads of *P. lividus*. (iii) Molecular analysis by *de novo* transcriptome and *Real Time qPCR* showed that benthic diatoms were able to affect the expression levels of several genes, involved in different cellular processes. (iv) Chemical analyses by Gas Chromatography–Mass Spectrometry (GC-MS) and Liquid Chromatography–Mass Spectrometry have been focused on two classes of secondary metabolites isolated in

planktonic diatoms: the oxylipins and sterol sulfates. All benthic diatoms analysed in this work produced oxylipins with the only exception of *Diploneis* sp., which in turn produced some unknown compounds deriving from polyunsaturated fatty acids metabolism. Moreover, all four benthic diatoms showed the presence of StS.

This study is the first demonstration of the toxic effects of benthic epiphytic diatoms on embryos and larvae of the sea urchin *P. lividus* due to the feeding of adults during gonadal maturation. Furthermore, the present work assumes a considerable ecological relevance, opening new perspectives on the study of diatom-derived secondary metabolites influencing their grazers.

List of abbreviations

°C degree Celsius

µL microlitre

11,10-EHETE 11,12-epoxy-10-hydroxy-eicosa-5*Z*,8*Z*,14*Z*,17*Z*-tetraenoic acid

11-HEPE 11-hydroxy-eicosa-5*Z*,8*Z*,12*E*,14*Z*,17*Z*-pentaenoic acid

14*S*,13*R*-EHETE 14*S*,15*S*-epoxy-13*R*-hydroxy-eicosa-5*Z*,8*Z*,11*Z*,17*Z*-tetraenoic acid

15*S*-HEPE 15*S*-hydroxy-eicosa-5*Z*,8*Z*,11*Z*,13*E*,17*Z*-pentaenoic acid

16,15-EHDoPE 16,17-epoxy-15-hydroxy-docosa-4*Z*,7*Z*,10*Z*,13*Z*,19*Z*-pentaenoic acid

17-HDoHE 17-hydroxy-docosa-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-hexaenoic acid

19,18-EHDoPE 19,20-epoxy-18-hydroxy-docosa-4*Z*,7*Z*,10*Z*,13*Z*,16*Z*-pentaenoic acid

20-HDoHE 20-hydroxy-docosa-4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,18*Z*-hexaenoic acid

5-HEPE hydroxy-eicosa-6*E*,8*Z*,11*Z*,14*Z*,17*Z*-pentaenoic acid

6-HHTrE 6-hydroxy-hexadeca-7E,9Z,12Z-trienoic acid

9-HHME 9-hydroxyhexadec-7*E*-enoic acid

9-KHME 9-ketohexadec-7*E*-enoic acid

9*S*,11*R*-EHHDE 9*S*,10*S*-epoxy-11*R*-hydroxy-hexadecadienoic acid

9*S*-HHTrE 9*S*-hydroxy-hexadeca-6*Z*,10*E*,12*Z*-trienoic acid

9*S*-HHTtE 9*S*-hydroxy-hexadeca-6*Z*,10*E*,12*Z*,15-tetraenoic acid

AA arachidonic acid

ANOVA analysis of variance

BHT butylated hydroxytoluene

BLAST basic local alignment search tool

BMRB biological magnetic resonance database

bp base pairs

BP biological process

C/N carbon/nitrogen ratio

CC cellular component

cDNA complementary deoxyribonucleic acid

CHNS carbon, hydrogen, nitrogen, sulfur

CHOS cholesterol sulfate

cm centimetre

CO_2 carbon dioxide

CTAB cetyltrimethylammonium bromide

DE differentially expressed

DEPC diethylpyrocarbonate

DHA docosa-4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-esenoic acid

DHBS dihydrobrassicasterol sulfate

DNA deoxyribonucleic Acid

DNase deoxyribonuclease

dNTP deoxynucleotide triphosphate

DSP death specific proteins

dw dry weight

EAS epoxyalcohol synthase

EPA eicosa-5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-pentaenoic acid

ESI electrospray ionization

ESTs expressed sequence tags

FAHs fatty acid hydroperoxides

FDR false discovery rate

fmol femtomole

FOX2 ferrous oxidation-xylenol orange 2

FSW filtered sea water

g gram

GC-MS gas chromatography-mass spectrometry

GI gonadal index

GO gene ontology

GTPC guanidinium thiocyanate-phenol-chloroform

h hour

H_20 dihydrogen monoxide

HCO_3^- bicarbonate

HMDB human metabolome database

hpf hours post fertilization

HPL hydroperoxide lyase

HR hydroperoxide reductase

HR-MS high resolution - mass spectrometry

Hz hertz

IPA ingenuity pathway analysis

KCl postassium chloride

kDa kilodalton

Kg kilogram

LAH lipolytic acyl hydrolase

LC-MS liquid chromatography-mass spectrometry

log logarithm

LOX lipoxygenase

m metre

M molar

MeOH methanol

MF molecular function

mg milligram

min minute

mL millilitre

mM millimolar

ms millisecond

MS/MS tandem mass spectrometry

MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Na^{+} sodium

NaCl sodium chloride

NCBI national center for biotechnology information

NGS next generation sequencing

nm nanometre

NMR nuclear magnetic resonance

NO nitric oxide

NVOs non-volatile oxylipins

OPLS-DA orthogonal partial least squares-discriminant analysis

PAPS 3'-phosphoadenosine-5'-phosphosulfate

PCA principal component analysis

PCD programmed cell death

PCR polymerase chain reaction

pg picogram

ppm parts per million

PUAs polyunsaturated aldehydes

PUFAs polyunsaturated fatty acids

qPCR quantitative polymerase chain reaction

qTOF quadrupole time of flight

REST relative expression software tool

RIN RNA integrity number

RNA ribonucleic acid

RNase ribonuclease

RNAseq RNA sequencing

ROS reactive oxigen species

rRNA ribosomal RNA

RT retention time

s second

SCUBA self-contained underwater breathing apparatus

SD standard deviation

SEM scanning electron microscopy

SiO_2 silicon dioxide

SSH suppression subtractive hybridization

STD standard

StS sterol sulfates

SULT sulfotransferase

TCEP tris(2-carboxyethyl)phosphine

TUNEL terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling

UHPLC ultra-high-performance liquid chromatography

UV_{max} UV maxima

VIP variable importance in projection

VOCs volatile organic compounds

ww wet weight

XO xylenol orange

β-ME 2-mercaptoethanol

B-SITS β-sitosterol sulfate

µg microgram

µm micrometre

µM micromolar

1. State of the art and thesis objectives

1.1 Marine diatoms: an overview of planktonic and benthic taxa

Diatoms are unicellular algae formally classified in the phylum *Bacillariophyta*, class *Bacillariophyceae*. They are highly abundant in nearly every habitat where water is found (oceans, lakes, streams, mosses, soils, even the bark of trees), colonizing the planktonic and benthic environments of marine and freshwater habitats. The term *plankton* is referred to organisms that live in the water column and, because they are non-motile or too weak to swim against the current, exist in a drifting state. Phytoplankton is the portion of plankton that grows autotrophically, using CO_2 as its carbon source and light as its energy source. Diatoms are considered a fundamental component within phytoplankton, because they play a key role in the global carbon cycle (**Figure 1.1**), constituting a rich diet for many herbivores, due to their lipid content (Pohnert, 2005). They can be placed at the bottom of food webs in marine and freshwater habitats, contributing for up to 40-50% of marine primary productivity (Nelson et al., 1995; Falkowski and Raven, 2007). In the ocean, marine phytoplankton comprises photoautotrophic organisms from 12 taxonomic divisions and 3 Kingdoms (Falkowski et al., 1998), including several algae, bacteria, protozoans, crustaceans, molluscs and coelenterates, as well as representative other organisms from almost every other phylum.

Differently from plankton, *benthos* consists of sessile or creeping organisms, such as prochlorophytes and cyanobacteria (Giovannoni and Rappè, 2000) and eukaryotic

microalgae, such as chromophytes (brown algae), rhodophytes (red algae), and chlorophytes (green algae) (Van Den Hoek et al., 1997).

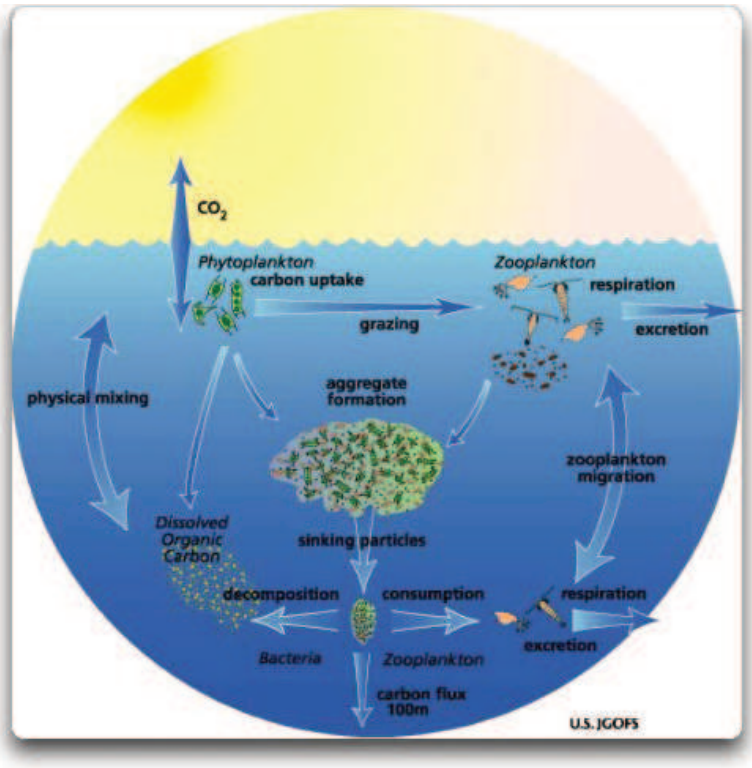


Figure 1.1. Schematic representation of the carbon cycle in the oceans. Marine ecosystems are a major sink for atmospheric CO_2 and take up similar amount of CO_2 as terrestrial ecosystems, currently accounting for the removal of nearly one third of anthropogenic CO_2 emissions from the atmosphere. The net transfer of CO_2 from the atmosphere to the oceans and then sediments is mainly a direct consequence of the combined effect of the solubility and the biological pump. While the solubility pump serves to concentrate dissolved inorganic carbon in the deep oceans, the biological carbon pump transfers both organic and inorganic carbon fixed by primary producers (phytoplankton) in the euphotic zone to the ocean and subsequently to the underlying sediments, maintaining atmospheric CO_2 at significantly lower levels.

The benthic environment of marine or freshwater habitats includes the bottom, such as the ocean floor or the bottom of a lake, the sediment surface and some sub-surface layers. Benthos can be divided in phytobenthos, the primary producers (algae, aquatic plants) living on the bottom, and zoobenthos, all consumers (from protozoa to all other benthic animals) living on/or in close relationship with the bottom. Benthic diatoms

constitute a part of phytobenthos where they represent a food source for several marine consumers (Miller et al., 1996). As already mentioned for planktonic species, benthic diatoms are implicated in the biogeochemical cycles of carbon, nitrogen, phosphorus and silicon, contributing for about the 20% of benthic primary production (Falkowski and Raven, 1997). Because of the diversity of substrates, benthic diatoms have numerous different microhabitats, as opposed to phytoplankton, whose environment is more homogenous. Benthic diatoms can range from large motile forms that are common in the intertidal sands, to sessile species that are attached to various substrates (periphyton). The latter may be attached to rocks (epilithon) and sand (episammon), as well as loosely associated with mud (epipelon), macrophytes, seagrasses and artificial substrates (epiphytic) or even growing on living animals (epizoic). Moreover, semi-aquatic habitats of temporal pools and tide pools are observed to contain some taxa of benthic diatoms. Despite the ability of benthic diatoms to adapt to different microenvironments, they tend to prefer natural substrates, especially vegetation, compared to artificial substrates, because of the selective nature of the physical and chemical properties of artificial substrates (Bere, 2010).

Considering both planktonic and benthic species, diatoms are commonly between 20-200 μm in diameter or length, although sometimes they reach 2 mm in length. They display a rigid cell wall made of silicon dioxide (SiO_2), called frustule. The diatom frustule is composed of two overlapping thecae (the larger is called epitheca and the smaller hypotheca), consisting of a valve and an accompanying series of girdle bands. Since silica is impervious, diatoms have evolved elaborate patterns of perforations in their valves to allow nutrient and waste exchange with the environment (Kröger and

Poulsen, 2008). The valve face of the diatom frustule is decorated with pores (areolae), processes, spines, hyaline areas and other distinguishing features normally used for diatom classification. Diatoms are commonly divided into two Orders: i) the Centrales, which have valve striae arranged basically in relation to a point, an annulus or a central areola and tend to appear radially symmetrical; ii) the Pennales, which have valve striae arranged in relation to a line and tend to appear bilaterally symmetrical (Simonsen, 1979; Von Stosch, 1982; Hasle and Syvertsen, 1997; **Figure 1.2**).

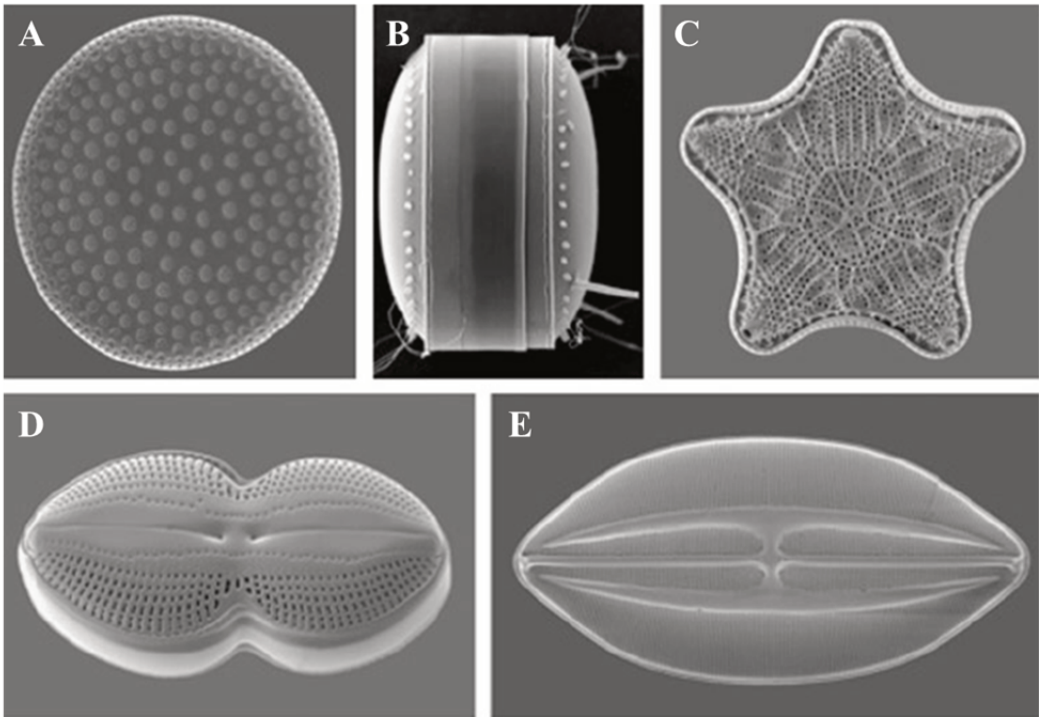


Figure 1.2. Scanning Electron Microscopy (SEM) images of centric diatoms (A-C) and pennate diatoms (D-E). (From Brayner et al., 2011)

Centric diatoms include three sub-orders based primarily on the shape of the cells, the polarity and the arrangement of the processes: i) the *Coscinodiscineae*, with a marginal ring of processes and no polarity to the symmetry; ii) the *Rhizosoleniineae*, with no marginal ring of processes and unipolar symmetry; iii) the *Biddulphiineae*, with no

marginal ring of processes and bipolar symmetry (**Figure 1.3**; Simonsen, 1979). Pennate diatoms are generally elongated, and many of them exhibit bilateral symmetry, and even helical symmetry is possible (Round et al., 1990). They are divided into two sub-orders based on the presence or absence of a paired slit system (the raphe) in the valves: *Bacillariineae* (raphid diatoms) and *Fragilariineae* (araphid diatoms; Hasle and Syvertsen, 1997).

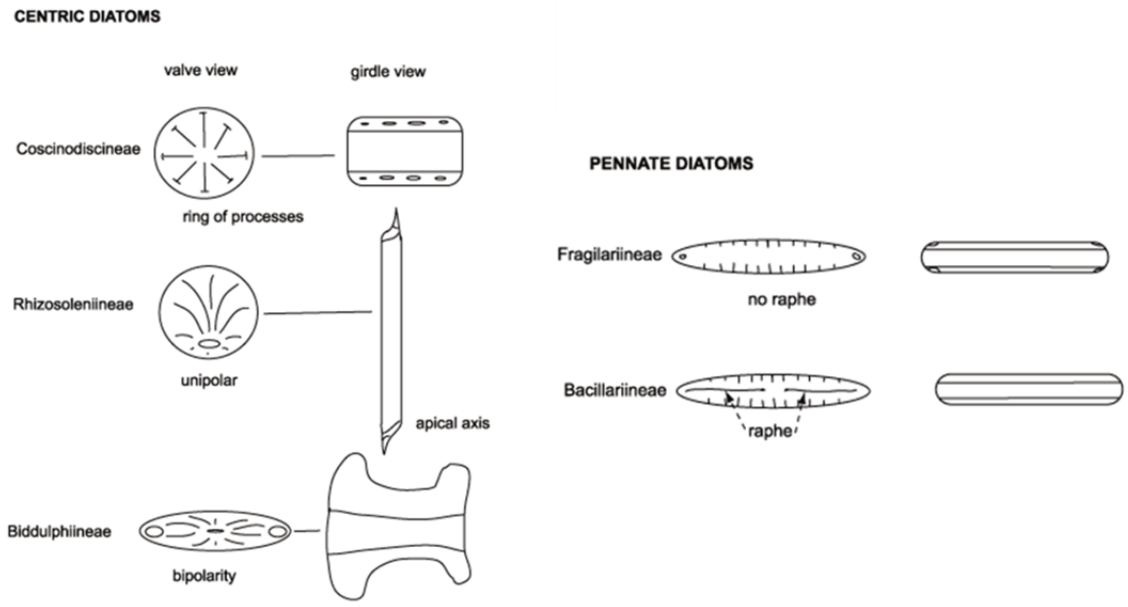


Figure 1.3. Schematic representation of centric, raphid and araphid pennate diatoms. (From Hasle and Syvertsen, 1997).

The siliceous cell wall has been used for classification of diatoms. The diatom taxonomy has been almost exclusively based upon frustule characteristics: shape, size, symmetry, structure and density of striae, nature of raphe and its position, copula and processes on the valves. However, recently ultrastructure and features of living cells, such as

chloroplastids, life cycle pattern and reproductive biology have been extensively used in systematic studies.

In fact, the scientific community acquired a greater awareness regarding the need to describe the structure of the living cells of diatoms, the protoplast, especially the plastids as part of the diagnosis of species. Plastids are the most conspicuous organelles featuring in living diatoms and their number and shape are consistent features of taxonomic importance (Cox, 1996; Round et al., 1990). Chloroplastids may be rounded or lobed discs or large plate-like with or without lobed margins, and may range from one to two, four or more. A typical centric diatom has many disc-shaped plastids, closely arranged to the periphery surrounding a large central vacuole or scattered throughout the cell. The raphid diatoms tend to have large chloroplasts (one to four), lying along the girdle with central nucleus flanked by two large vacuoles. The species concept in biology is sometimes based upon reproductive isolation but it is difficult to apply to diatoms (Round et al., 1990).

Diatom taxonomy is in a continuous state of revolutionary change, involving the splitting of genera, creation of new ones and revision of criteria for classification. This is mainly due to advancement in cultivation technologies and in molecular techniques, which have made taxonomy of diatoms a very dynamic field of research, in addition to the significance of diatoms as recognized worldwide biomonitoring tools for aquatic systems (John, 1998, 2000).

From 20.000 to 2 million species of diatoms (Round et al., 1990; Hasle and Syvertsen, 1997; Mann, 2005) are presently estimated. This range is large because scientists are still working to understand basic aspects about "what is a diatom species" and because

new and diverse forms are still being discovered and described in scientific publications (Simonsen, 1979; Von Stosch, 1982; Hasle and Syvertsen, 1997).

The diatom reproduction process can occur by either sexual or asexual mechanisms (**Figure 1.4**). All diatoms pass through a seed-like or a spore phase called the resting spore. The asexual reproduction occurs by binary fission. In this process, the DNA undergoes replication that causes the chromosomes to divide into two identical halves. This leads to the formation of two frustules or theca. Each daughter cell receives one of the parent cell frustules, the hypotheca, and forms a new frustule, the epitheca. The parent cell grows larger until dividing into two daughter cells by pushing out of the valve. Each daughter cell produces a new cell wall, each receiving one valve. Availability of dissolved silica limits the rate of vegetative reproduction, but also because this method gradually reduces the average size of the diatom frustule.

For this reason, the size of diatoms tends to decrease along asexual generations and there is a certain threshold at which restoration of frustule size is necessary in populations (Hasle and Syvertsen, 1997; Zhang et al., 2005; Peng et al., 2014). The algae need to restore their original cell size, and to this end they use sexual reproduction. The vegetative cells of diatoms are diploid (2n), and hence, they undergo meiosis with males and females producing gametes, respectively. The female cells tend to bend and create an opening in the cell wall helping the male gamete to enter the female cell and fertilize it. The zygote gets encased in an envelope-like structure, which produces its own shell and nucleus. Then, the diatom cell starts to grow until it reaches its full size. The parent cell and the new diatom can form auxospores, which are cells that possess a different wall structure, lacking the siliceous frustule, and enlarge to the maximum

frustule size. This helps the cells to survive for long periods under unfavourable conditions. Once the cells get proper conditions to grow, they continue with their asexual reproduction (Round et al., 1990).

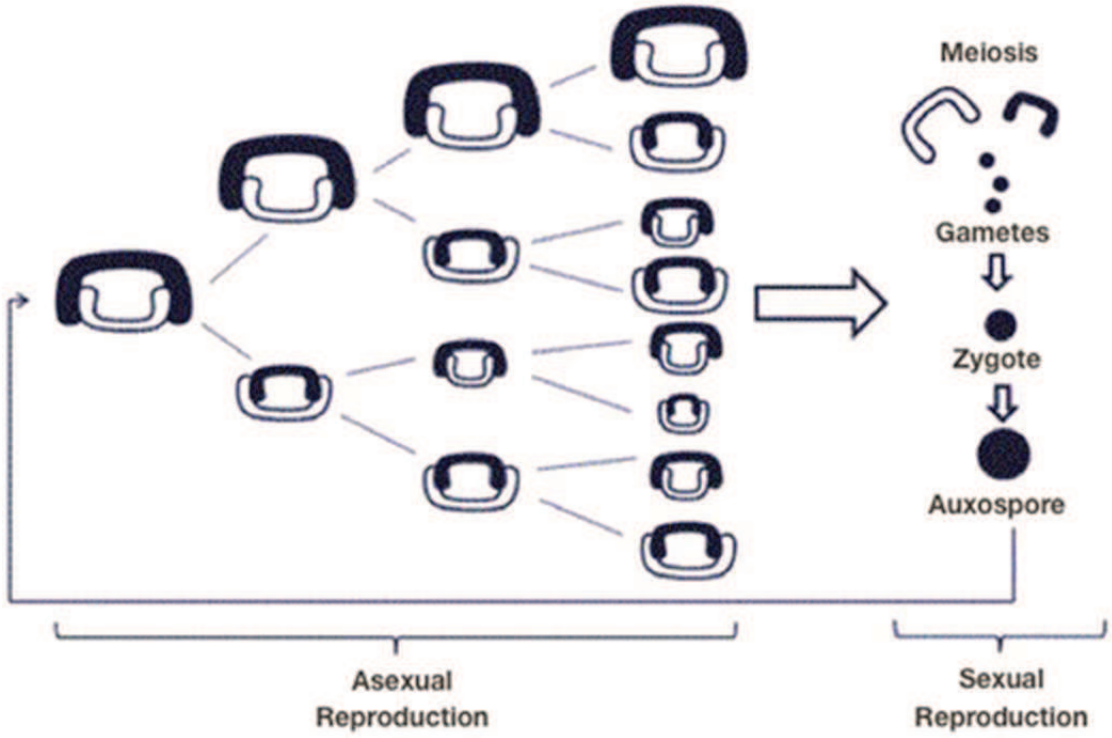


Figure 1.4. Diatoms reproduce through asexual reproduction or cell division. Each half of the new cell gets half of the frustule, which then has to fit inside the other half when it is reformed. As they keep getting smaller each time, the diatoms must then produce sexually to make an entirely new frustule.

Diatoms use the pigments chlorophyll a and c to collect energy from the sun through photosynthesis. They also contain the accessory pigments, fucoxanthin and β-carotene, which give them a characteristic golden colour (Bertrand, 2010). Diatom cells store energy from photosynthesis in the form of chrysolaminarin (a carbohydrate) and lipids (fats in the form of oils). The high production of lipids in many diatom species give great interest in diatoms as a source of biofuels and prospective materials for nanotechnology (Drum and Gordon, 2003; Gordon et al., 2009; Stonik and Stonik,

2015; Prabandono and Amin, 2015). Indeed, as one of the important global sources of carbon fixation, diatoms already are an important biofuel for aquatic food webs (Falkowski et al., 2008).

Diatom abundance is generally highest at the beginning of spring and in the autumn, when light intensity and day length are optimal for their photosynthesis. In some regions of the oceans, the annual production of fixed carbon can be up to 2 kg m^{-2} equivalent to a cereal or corn crop (Field et al., 1998). When the population of these organisms increases up to a concentration of hundreds to thousands of cells per mL, the water is coloured green-blue, yellow-brown or red: this phenomenon is known as "algal bloom" (**Figure 1.5**). These events mainly involve toxic phytoplankton, such as dinoflagellates of the genera *Alexandrium* and *Karenia*, or diatoms of the genus *Pseudo-nitzschia*. Of the over 5000 known species of marine phytoplankton, about 300 species can, under certain circumstances, proliferate in exponential numbers and only about 2% of these species have the capacity to produce potent toxins, which can negatively affect the local ecosystem, as well as fishing and aquaculture activities (Landsberg, 2002). Although these events are particularly known in planktonic species, recent evidences of benthic diatom blooms in marine and freshwater habitats were also recorded (Galland and Pennebaker, 2012; Ahn et al., 2016; Bothwell and Taylor, 2017). For example, a massive blooming of several benthic species, including *Cocconeis* spp., *Paralia sulcata*, *Achnanthes* spp., *Amphora* sp., *Eucampia antarctica*, *Navicula* spp., *Odontella litigiosa*, P*leuorosigma* sp. have been detected in the shallow seafloor of an Antarctic Fjord (King George Island) (Ahn et al., 2016).

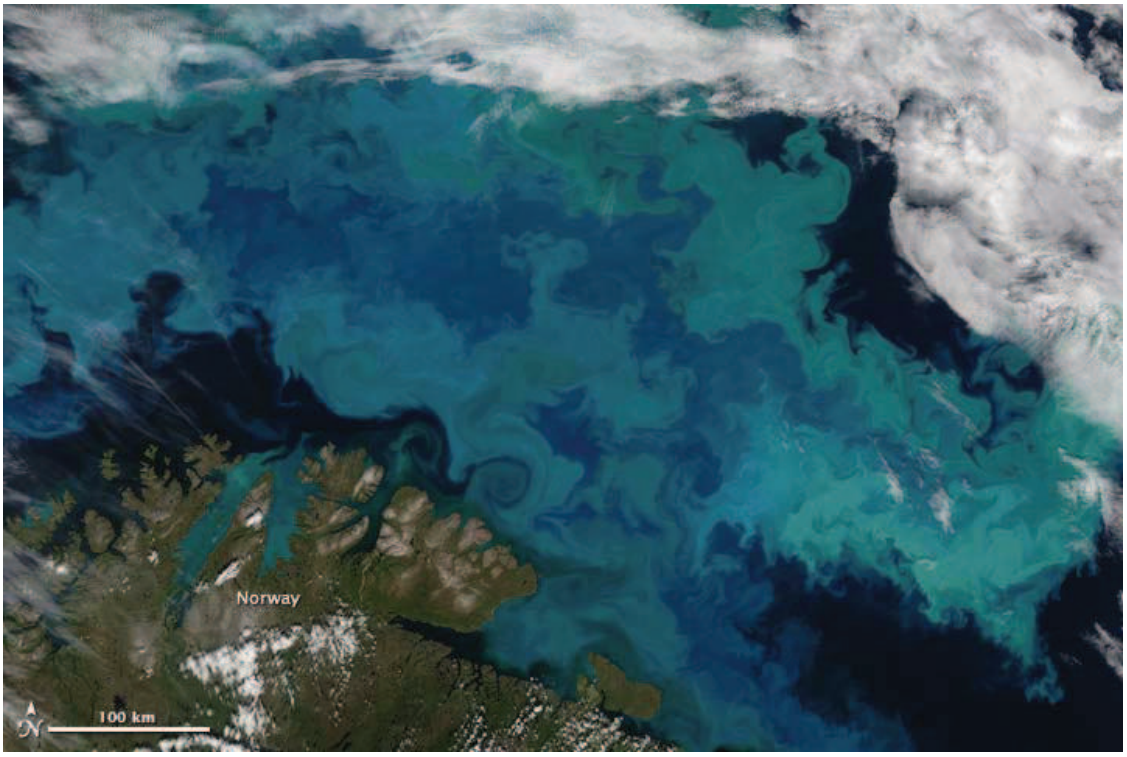


Figure 1.5. Image taken on August 14, 2011, by the Moderate Resolution Imaging Spectroradiometer (MODIS) on the Aqua satellite show brilliant shades of blue and green across the Barents Sea. The color was created by a massive bloom of phytoplankton that are common in the area each August.

Fundamentally, diatoms are unicellular, but several taxa form chains or filaments due to daughter cells, remaining connected even after cell division, often by polysaccharide pads or linking spines. The colonies may be straight, zigzagged, fan-shaped, star-shaped, or radiate. The production of exopolysaccharides (EPSs) normally reaches high levels in planktonic diatoms (for example *Skelertonema costatum*), to cause the formation of the co-called "marine snow" (Riebesell, 1991a,b). One of the first chemical studies in benthic diatoms of mucilage and their influence on growth rate were performed by Buzzelli et al. (1996), who collected samples from the coastal area of Cesenatico (Adriatic Coast) containing cells of the diatom *Amphora coffeaeformis* var. *perpusilla*.

EPSs are secreted into the environment as a reserve of carbohydrates, as well as cell wall constituents within the cells, which form adhering biofilms (**Figure 1.6**), contributing to biostabilization of sediments (Paterson and Black, 1999). The sediment stabilization efficiency of benthic diatoms is due to the high water retention capacity of EPSs, which may protect the organisms against desiccation (Decho, 1990).

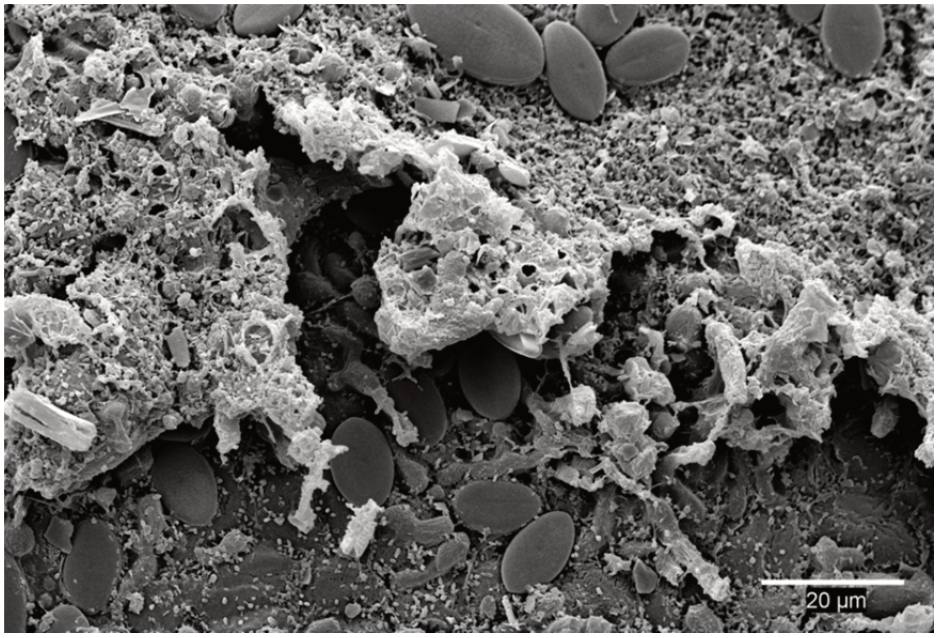


Figure 1.6. Scanning electron micrograph of *Ascophyllum nodosum* surface. The brown alga, collected on the rocky shores of the North Atlantic Ocean, is covered by a thin biofilm of diatoms, bacteria, and fungi. (From Canadian Science Publishing Blog).

Paterson (1994) studying the sediment-stabilizing effect of pennate diatoms on quartz-sandy tidal flats, differentiated between epipelic (immotile diatoms, living semi-permanently attached to sand grains) and epipsammic diatoms (motile diatoms, moving freely through the sediment).

In relation to the life-form of the benthic diatoms, two effects of sediment stabilization are observed:

1) Cohesive effect: the mucus, secreted by epipsammic diatoms, epipelic diatoms and bacteria, on sand grains improves the cohesion of the particles. The effect of the organic coating on the sand grains was studied in the laboratory in a small circular flume, using samples from the field. An organic coating on the grains was found to cause a 5-17% increase in critical erosion velocity, relative to sterile sediment cleaned with H_2O_2;

2) Network effect: when a large number of epipelic diatoms migrate to the sediment surface, they can form an extensive network of mutual attachment and mucus threads. In laboratory conditions the formation of an epipelic diatom mat leads, in 24 hours, to an increase in the sediment motion by 25% to 100%. In the field the network formation by epipelic diatoms and the physical processes are interdependent. The network protects the sediment surface against erosion by tidal currents and wave action, and conversely, the diatom population depends on these factors.

Benthic microalgae biofilms can be found in different arrangements. The structure of a biofilm is characteristic: it is primarily regulated by inundation, sediment dynamics, light, and water availability (Gerdes and Wehrmann, 2008) and depends on the degree of microalgal development. Even though the range of diatoms in biofilms is diverse, their ecology is poorly understood because of the difficulty in sampling and enumeration. Scraping or brushing are the traditional methods used for removal of diatoms from biofilms developed on solid substrata.

The biological and ecological characteristics of diatoms make them good indicators of water quality, and their use is widespread and well developed for evaluating the quality of flowing water (Prygiel et al., 1999) and standing waters, particularly lakes (Kitner and Poulícková, 2003; Blanco et al., 2004; DeNicola et al.,

2004; Denys, 2004; King et al., 2006). Analysis of diatom communities constitutes a valuable tool for general water quality monitoring and for the evaluation of more specific phenomena, such as eutrophication and acidification (Prygel and Coste, 1993; Danilov and Ekelund, 2000; Kauppila et al., 2002; Kitner and Poulícková, 2003).

1.1.2 Interactions of benthic diatoms with seagrasses and their grazers

Epiphytic community on seagrasses is constituted of various diatom species along with red, brown, green algae, cyanophyta and detritus (Frankovich and Zieman, 1994), which is called periphyton. It is obvious that benthic diatoms are the most important elements of the periphyton on seagrasses, because they take the greatest share in the epiphytic biomass (Jacobs and Noten, 1980). Most diatom species found on marine angiosperms are classified as obligate epiphytes (McIntyre and Moore, 1977) and, under nutrient limited conditions, can be strongly influenced by host metabolism. Nutrient uptake by the roots of seagrasses and, subsequent release via the leaves (McRoy and Barsdate, 1970; McRoy et al., 1972), bathe the periphyton in a nutrient-rich medium and individual diatom species exhibit a highly varied response to different nutrients (Saks et al., 1976). Biochemical, mechanical and fisical interactions may be partially responsible for the observation that the pennate diatom *Cocconeis scutellum* is the sole pioneer species on *Zostera marina* leaves, forming a uni-algal mat over newly formed blades (Sieburth and Thomas, 1973).

Benthic diatoms appear also to be specific in their selection of a suitable-sized substrate. For example, the species *C. scutellum*, which has a larger surface area of attachment, was shown to avoid finely branched algal thalli in preference for more thickly branched

species (Ramm, 1977). In fact, the genus *Cocconeis* is dominant on *Z. marina* (Sieburth and Thomas, 1973; Jacobs and Noten, 1980) and *Thalassia testudinum* (DeFelice and Lynts, 1978; Reyes-Vasquez, 1970), whereas *Navicula pavillarsi*, a narrow diatom, is highly abundant on *Ruppia maritima* leaves (Sullivan, 1977).

Both beneficial and adverse effects of epiphytes on macrophytes have been mentioned in scientific literature. Algal mats have shown to have a limiting effect on the growth of *R. maritima* and have been observed to cause temperature stratification in the water column due to shading (Richardson, 1980). Such stratification can postpone flowering, fruiting and seed production in *R. maritima*. If shading by algal mats is severe, active photosynthesis is restricted to the upper layer of the water column. As a result of thermal stratification, the photosynthetically oxygenated water does not reach the lower portions of the water column.

The effects of epiphytes on photosynthesis of *Z. marina* have been investigated by Sand-Jensen (1977). The authors have showed that epiphytes, including benthic diatoms (for example *C. scutellum*), form a crust over the leaves of *Z. marina* and reduce photosynthesis by limiting the availability of light and acting as a barrier to carbon uptake. Photosynthesis was reduced (31%) by epiphytes under optimal light conditions and environmental HCO_3^- concentrations. Light attenuation by epiphytes is therefore responsible for premature senescence and a strong decrease in both vegetative and sexual reproductive capabilities (Richardson, 1980; Rice et al., 1983).

Trocine et al. (1981) demonstrated a protective role of epiphytes against ultraviolet-β radiation. Fouling by algal epiphytes enabled *Halophila engelmannii* to colonise shallow water dominated by high levels of ultraviolet-β radiation.

Colonization of seagrass blades by epiphytes commonly follows a pattern that may have further beneficial consequences for host plant. In fact, epiphytes normally grow at the tips of seagrass leaves which are the preferred food for grazers. The selective removal of highly epiphytized and senescent leaf tips cause minimal damage to basal leaf tissues and increase light penetration (Ott and Maurer, 1976; Lobel and Ogden, 1981). Thus, epiphytes may divert direct grazing from the primary photosynthetic tissue (more basal) of seagrasses to senescent leaf tips.

Grazers from diverse taxonomic groups including gastropods, amphipods, isopods, decapods, echinoderms and fish basically find most of their nutrition from seagrass epiphytes. Zupi and Fresi (1984) observed the gut content of echinoderms (23 species) living on *Posidonia oceanica* meadows collected in 35 sites around Ischia (Gulf of Naples, Italy). This analysis revealed the presence of *P. oceanica* fragments, several species of diatoms, molluscs, macrophytes, foraminiferans, poriferans, bryozoans and polychaetes, with *P. oceanica* dominating the gut content of the sea urchins *Parancentrotus lividus* and the echinoderm *Ophiura texturata* individuals.

Through their feeding activities, some grazers may increase epiphytic dominance of adhesive diatoms, such as *C. scutellum* (van Montfrans et al., 1982), which is tightly attached and generally not removed by grazers (Lamberti and Resh, 1983). On the contrary, grazing can also reduce epiphyte abundance on seagrass leaves. For example, Caine (1980) demonstrated that periphyton biomass was dramatically reduced on ungrazed *Z. marina* with respect to plants grazed by the caprellid amphipod *Caprella laeviscula*.

Concluding, benthic diatoms are important food sources for benthic grazers such as sea urchins, fishes, gastropods and crustaceans. Without diatoms and other epiphytes, there will be no food for herbivores in seagrass beds, being epiphytes their main food source. Seagrass itself has poor nutritional value and is made up of cellulose, which several grazers are unable to metabolize. Consequently, some herbivorous prefer to eat only the epiphytes, eating the seagrass solely as a means for consuming the epiphytes and therefore selecting for seagrass leaves that only have an abundant amount of epiphytes and diatoms (Del Río, 2016). Contemporarily, diatoms are not able to adapt to local environmental changes becoming tolerant to drought and low light conditions and able to respond strongly to large-scale climatic variables and fluctuations by finding suitable habitats and having efficient nutrient uptake from seagrass leaves (Pajunen et al., 2016).

1.2 Secondary metabolites in diatoms

Oceans, due to the area they represent and the ecosystem services they provide, are fundamental to the planet, harbouring a huge biodiversity of life. Some estimates suggest that the probability of discovering new bioactive molecules from marine sources is approximately three times higher than that from terrestrial ones, mainly due to the great biodiversity of marine organisms and their chemical products, many of which have no terrestrial counterpart. In the last decades, metabolites produced by marine organisms, especially micro-organisms, have received increasing attention for their role in shaping interactions and communities (Leflaive and Ten-Hage, 2009). These metabolites, called secondary metabolites, are not required for the growth and maintenance of the cellular functions (primary metabolites) and are the end products of

the primary metabolism. Marine organisms, including diatoms, have been shown to produce a variety of bioactive secondary metabolites acting as chemical signals within communities (Leflaive and Ten-Hage, 2009; Stonik and Stonik, 2015).

In the plankton, marine diatoms, dinoflagellates and other green and yellow-brown flagellates are considered a potentially valuable source of new biologically active molecules that can be useful for applications in several biotechnology sectors (food, energy, health, and environment). Several studies have shown that microalgal peptides can exert a large variety of activities, such as, antioxidant, anticancer, antihypertensive, antiatherosclerotic, anti-UV radiation, and antiosteoporosis (Fan et al., 2014). However, a few of these have so far been addressed for evaluation in clinical phases and even fewer have managed to reach the market. A successful example is Dermochlorella®, an oligopeptide purified from *Chlorella vulgaris* which is used in cosmaceutical field. Dermochlorella® increases the expression of collagen, elastin, laminin, and the inhibitors (elafin and tissue inhibitors of metalloproteinase) of enzymes that degrade the extracellular matrix and restore skin elasticity (Martins et al., 2014). Furthermore, Lauritano et al. (2016) have recently reported that the diatom *Skeletonema marinoi* arrested the growth of melanoma A2058 cells, whereas the diatoms *Cylindrotheca closterium*, *Odontella mobiliensis* and *Pseudonitzschia pseudodelicatissima* had anti-inflammatory effects, and *Leptocylindrus aporus* and *danicus* and *S. marinoi* had antibacterial properties.

For these reasons, despite diatoms have been regarded as beneficial to the growth and survival of primary consumers (such as copepods and benthic filter feeders), several authors have placed in doubt the positive role of diatoms, demonstrating that fecundity

and/or hatching success were reduced when herbivorous copepods were fed on diatom-based diets (Ianora and Poulet 1993; Poulet et al., 1994; Chaudron et al., 1996; Buttino et al., 1999; Ianora and Miralto, 2010). Such evidences collected over the years, is known as "paradox of the diatoms-copepods interaction" in the pelagic food web (Ban et al., 1997), because the known negative plant-animal interactions are generally related to repellent or poisoning processes, but never to reproductive failure.

The present Ph. D. project has focused on two classes of diatom secondary metabolites with interesting biological activities, oxylipins and the sterol sulfates (StS), which will be described in detail in the following paragraphs.

1.2.1 Oxylipins

The first studies demonstrating the inhibitory effect of diatoms on the reproduction of copepods were reported by Ianora and Poulet (1993). They showed that when the copepod *Temora stylifera* was fed with mono-algal diet of diatom *Thalassiosira rotula*, eggs productions remained high for more than 15 days but hatching success was seriously compromised, compared to animals fed with the dinoflagellate *Prorocentrum minimum*. The authors proposed that these differences were not due to the biochemical composition of the two algae, because both *T. rotula* and *P. minimum* contained high levels of proteins, vitamins and fatty acids essential for growth and development of copepods. It has been hypothesized that the reduction of hatching rates was caused by the presence of unknown anti-mitotic compounds, which arrested embryogenesis in the copepod *Calanus helgolandicus* (Poulet et al., 1994). The embryonic development of copepods was blocked when eggs were exposed to diatom extracts in a dose-dependent

manner (Poulet et al., 1994; Ianora et al., 1995; Miralto et al., 1995; Uye, 1996). Depending on the incubation time, fusion of the female and male pronuclei was arrested and eggs remained either at the one-cell stage, or zygotes underwent abnormal development characterized by the presence of dispersed chromatin and accompanied by anomalous cellular division (Poulet et al., 1995). Cellular anomalies rejected erroneous synchronization between nuclear and cellular division. In a few cases, embryos developed up to hatching, but nauplii were deformed and displayed marked anatomical anomalies, such as asymmetrical bodies or small number of appendages for swimming and feeding (Poulet et al., 1995; Uye 1996; Starr et al., 1999; Ianora et al., 2004). Those nauplii did not survive long, dying a few hours after hatching because they were not able to swim or feed. The inhibition of hatching was also shown to be diatom density-dependent (Chaudron et al., 1996; Ban et al., 1997; Starr et al., 1999). The unknown compounds, responsible for these deleterious effects on copepods, were demonstrated to be produced by the diatom cells and not by bacteria associated with diatom cultures (Ianora et al., 1996). Ban et al. (1997) showed that hatching success and/or fecundity were reduced when seventeen copepod species were fed with sixteen different diatoms, representing a variety of marine, estuarine and freshwater habitats, demonstrating that this phenomenon occurs on a world-wide scale. Among the seventeen species, only one did not result in reduced egg production rates in comparison to dinoflagellate diets. Testing different diatom-copepod combinations, diatoms reduced average fecundity by 90% and hatching success by 80%. It is important to remember, however, that the bulk of evidence for diatom toxicity on copepods is based on laboratory studies.

In other studies, the attention has been focused on the effects of maternal diet on the production of eggs, the hatching success and growth and differentiation of copepods. Carotenuto et al. (2002) have shown that *T. stylifera* was not able to complete development from hatching to adult, after the feeding of larvae with three different species of diatoms (*T. rotula*, *Skeletonema costatum* and *Phaeodactylum tricornutum*), showing a high mortality rate. Ianora et al. (2004) have also analysed the effects of maternal diatom-diet on the fitness of copepod offspring. They have shown that the development was stopped in all larvae of *C. helgolandicus* females fed with the diatom *S. marinoi*. In addition, the mortality remained high even if the larvae, generated by females fed with *S. marinoi*, were nursed with only the dinoflagellate *P. minimum*. On the other hand, when the females were fed with *P. minimum* and the nauplii were grown with *S. marinoi*, the survival rate increased considerably. On the contrary, when females were fed with *S. marinoi* and then with *P. minimum*, the offspring mortality increased. As a result, maternal-feeding quality was demonstrated to be more important than nauplia sustenance for progeny survival.

These surprising findings have led researchers to perform chemical studies in order to identify the molecules responsible for these deleterious effects. Thus, it has been demonstrated that these uncharacterized compounds were the end-products of a lipoxygenase (LOX) metabolic pathway (**Figure 1.7**) initiated by cell damage occurring during senescence or grazing. Cell damage activates specific lipases, such as phospholipase or lipolytic acyl hydrolases (LAHs), which release PUFAs from cell and chloroplast membranes. Then LOXs, iron non-heme enzymes, catalyse the insertion of oxygen into the 1,3-pentadienyl moiety of PUFAs to produce fatty acid hydroperoxides

(FAHs). FAHs are finally modified through several enzymatic activities catalysed by hydroperoxide lyase (HPL), epoxyalcohol synthase (EAS) and hydroperoxide reductase (HR) to form a plethora of metabolites, such as polyunsatured aldehydes (PUAs), hydroxy fatty acids and epoxyalcohols, collectively termed oxylipins (Fontana e al., 2007a,b; Romano et al., 2010).

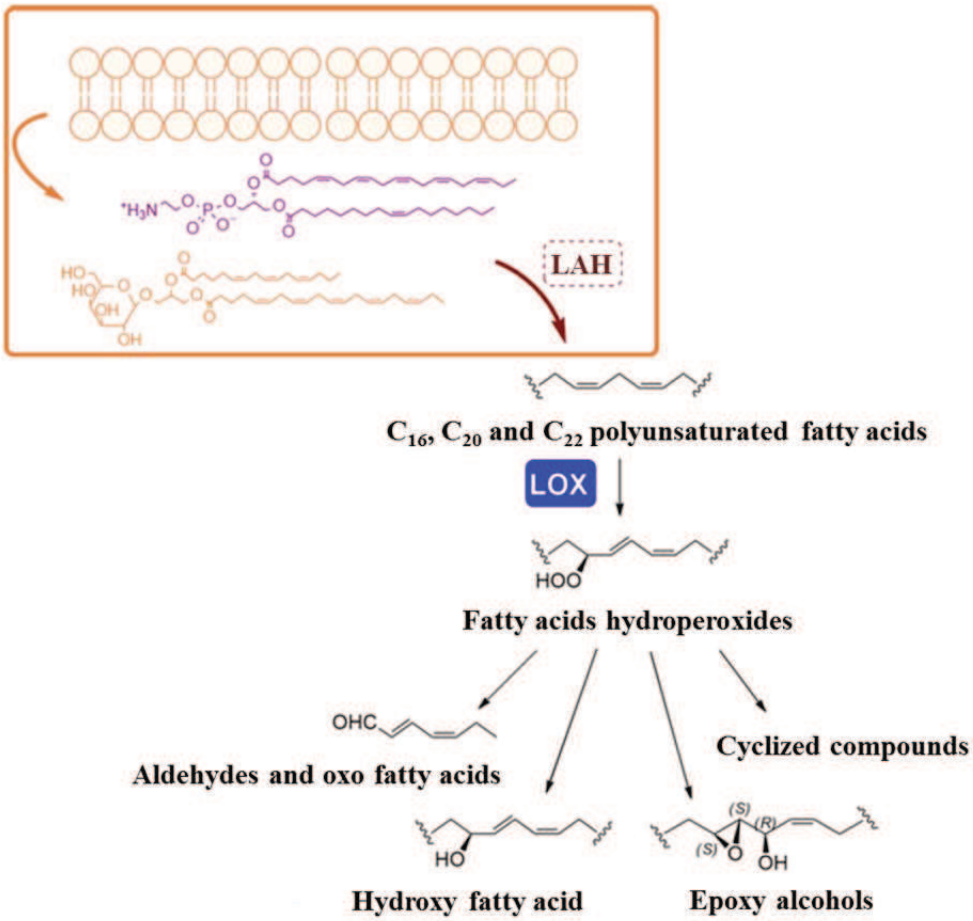


Figure 1.7. Schematic representation of the oxylipins' synthesis of from C_{16}, C_{20} and C_{22} polyunsatured fatty acids (PUFAs) through lipoxygenase enzymes (LOXs) pathway in marine diatoms. (From d'Ippolito et al., 2018).

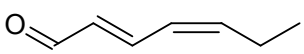
Among oxylipins, PUAs have been described only in a small number of diatom species. Firstly, Miralto et al. (1999) isolated and identified PUAs from the bloom-forming

diatoms *T. rotula*, *S. costatum* (identified as *S. marinoi* in Sarno et al., 2005) and *Pseudo-nitzschia delicatissima* as two C_{10} short chain PUAs: 2*E*,4*E*/*Z*-decadienal and 2*E*,4*E*/*Z*,7*Z*-decatrienal. These findings have been further confirmed in several studies, using different methodologies and identifying a more complete range of PUAs from *S. marinoi* and *T. rotula* (Pohnert, 2000, 2002; d'Ippolito et al., 2002a,b, 2003; Wichard et al., 2005a,b). Afterwards, an expanded range of PUAs were identified including 2*E*,4*E*-heptadienal, 2*E*,4*Z*-octadienal, 2*E*,4*E*-octadienal, 2*E*,4*E*/*Z*,7*Z*-octatrienal and 2*E*,4*Z*-decadienal (Pohnert, 2000, 2002; Pohnert et al., 2002; d'Ippolito et al., 2002a,b, 2003) (**Figure 1.8**).

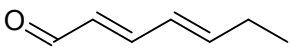
Despite PUAs have been the first oxylipins identified, almost all diatoms possess LOX pathways for the synthesis of other oxylipins, including fatty acid derivatives with hydroxy-, keto-, oxo-, and hydroxy-epoxy functionalities. In order to distinguish them from PUAs, these compounds are generically named as non-volatile oxylipins (NVOs).

In addition to PUAs and NVOs, FAHs (the precursors) are also suspected to play different ecophysiological functions, including the detrimental effects on grazers and the chemical communication between diatom cells, which may contribute to regulate the oceanic phytoplankton blooms. FAHs, the primary LOX products, are reactive oxygen species (ROS) that could operate in addition to other reactive radicals to induce DNA and protein damage contributing to cell aging. These compounds also induce apoptosis and morphological abnormalities in copepods at concentrations significantly lower than PUAs and, in consideration of their almost universal presence in diatoms, it is possible that FAHs more than PUAs are responsible for the negative response and reproductive failure of copepods (Barreiro et al., 2011; Fontana et al., 2007a,b).

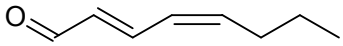
2*E*,4*Z*-heptadienal



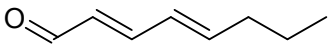
2*E*,4*E*-heptadienal



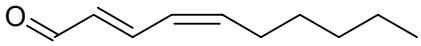
2*E*,4*Z*-octadienal



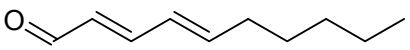
2*E*,4*E*-octadienal



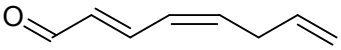
2*E*,4*Z*-decadienal



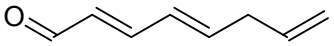
2*E*,4*E*-decadienal



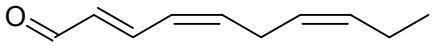
2*E*,4*Z*,7*Z*-octatrienal



2*E*,4*E*,7*Z*-octatrienal



2E,4*Z*,7*Z*-decatrienal



2*E*,4*E*,7*Z*-decatrienal

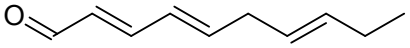


Figure 1.8. Structure of diatom-derived polyunsaturated aldehydes (From Ribalet et al., 2007).

Type and quantity of oxylipins are species-specific (Pohnert et al., 2002, Taylor et al., 2009), due to a variety of precursor PUFAs and enzymes (d'Ippolito et al., 2003; d'Ippolito et al., 2004; Fontana et al., 2007a,b), with variable effects on zooplankton grazers (Ianora and Miralto, 2010).

Oxylipins and LOX enzymes are classified in different groups, according to the substrate specificity, as well as the position and the stereochemistry of the oxygen addition along the PUFA carbon chains.

Oxylipin pathways of diatoms are mostly based on the oxygenation of eicosapentaenoic acid (EPA, C20:5 ω-3) (Barreiro et al., 2011; d'Ippolito et al., 2005, 2009; Fontana et al., 2007a,b), with highly diverse oxidation regiochemistry associated to species specificity (Lamari et al., 2013). The most common pathway shared by different genera of diatoms rely on a 15*S*-LOX activity, leading to the biosynthesis of the epoxyalcohol *trans*,*threo*,14*S*,15*S*-epoxy-13*R*-hydroxy-epoxyeicosa-5*Z*,8*Z*,11*Z*-tetraenoic acid (14*S*,13*R*-EHETE) from 15*S*-hydroperoxy-eicosa-5*Z*,8*Z*,11*Z*,13*E*,17*Z*-pentaenoic acid (15*S*-HpEPE). Other EPA-derived epoxyalcohols reported in diatoms are the products of 5-LOX in *Pseudo-nitzschia delicatissima*, *Tenuicylindrus belgicus* and *S. costatum* (d'Ippolito et al., 2009; Fontana et al., 2007a,b; Lamari et al., 2013; Nanjappa et al., 2014); 8-LOX in *Pseudo-nitzschia pseudodelicatissima* (Lamari et al., 2013); 9*S*-LOX in *Chaetoceros socialis* (Fontana et al., 2007a,b); 12-LOX in *Pseudo-nitzschia arenysensis* (Lamari et al., 2013); and 14-LOX in *Pseudo-nitzschia fraudulenta*, *Pseudo-nitzschia multistriata*, *Leptocylindrus aporus*, and *Chaetoceros affinis* (Fontana et al., 2007a,b; Lamari et al., 2013; Nanjappa et al., 2014).

Considering PUAs, EPA oxygenation at C_{14} (14-LOX) and C_{11} (11-LOX) forms the corresponding hydroperoxides (HpEPEs), which are then modified by HPL to heptadienal and decatrienal, respectively (**Figure 1.9**).

In a few species, such as, *T. rotula* and *S. costatum*, oxylipins derive from hexadeca-6*Z*,9*Z*,12*Z*-trienoic acid (HTrA, C16:3 ω4) and hexadeca-6*Z*,9*Z*,12*Z*,15-tetraenoic acid (HTtA, C16:4 ω1). In analogy to the synthesis of heptadienal and decatrienal from EPA, LOX and HPL metabolism of C_{16} PUFAs produce octadienal and octatrienal, whose biosynthesis has been extensively investigated in *S. marinoi* and *T. rotula* (d'Ippolito et

al., 2003, 2005; Romano, et al., 2003). Feeding experiments and stereochemical analysis of the main intermediates of C_{16} LOX activity proved the occurrence of a 9*S*-LOX, responsible for the synthesis of 9*S*-hydroperoxy-6*Z*,10*E*,13*Z*-hexadecatrienoic acid (9*S*-HpHTrE) and 9S-hydroperoxy-6*Z*,10*E*,13*Z*,15-hexadecatetraenoic acid (9*S*-HpHTtE), from which the two C_8 PUAs derives.

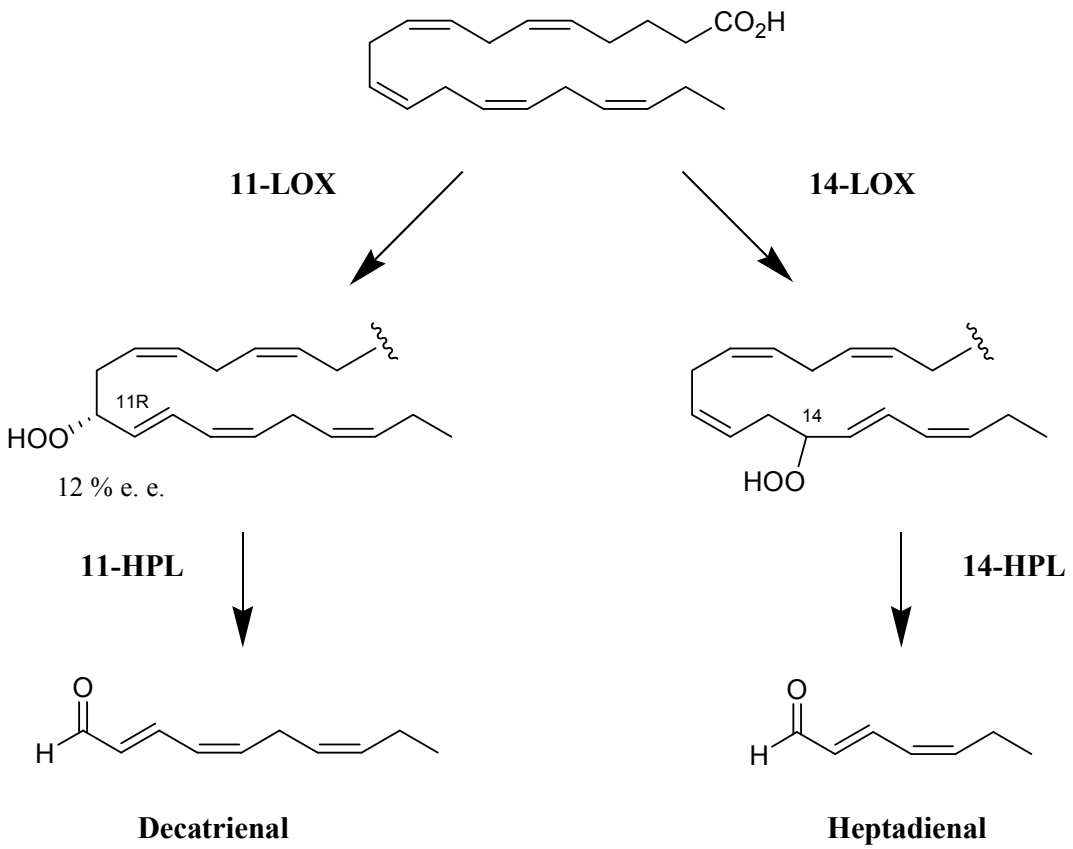


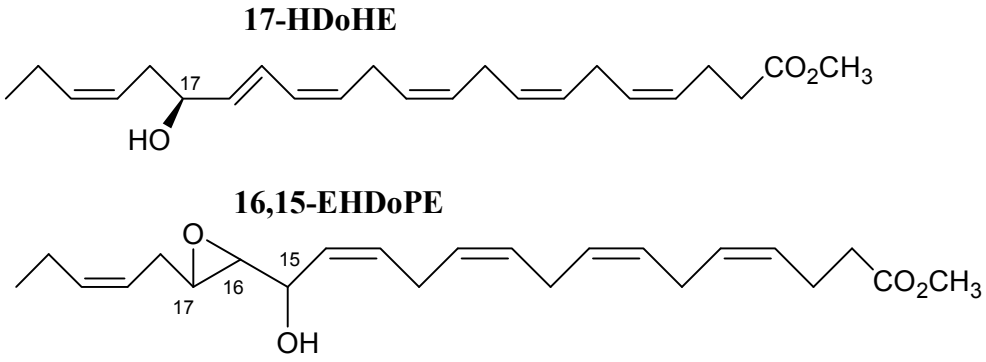
Figure 1.9. Precursors and stereochemistry of the synthesis of decatrienal and heptadienal. (From d'Ippolito et al., 2018).

Other typical oxylipins of this LOX pathway are 9*S*-hydroxy-hexadeca-6*Z*,10*E*,12*Z*-trienoic acid (9*S*-HHTrE), 9*S*-hydroxy-hexadeca-6*Z*,10*E*,12*Z*,15-tetraenoic acid (9*S*-HHTtE), and 9*S*,10*S*-epoxy-11*R*-hydroxy-hexadecadienoic acid (9*S*,11*R*-EHHDE).

A new pathway has been described by Nanjappa et al. (2014), showing several oxylipins (epoxyalcohols and hydroxy fatty acids) that derive from docosahexaenoic acid (DHA, C22:6 ω3) from three species of Leptocylindraceae diatoms collected in the Gulf of Naples. *Leptocylindrus danicus* and *Leptocylindrus hargravesii* showed 15*S*-LOX products, corresponding to 17-hydroxy-docosa-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-hexaenoic acid (17-HDoHE) and 16,17-epoxy-15-hydroxy-docosa-4*Z*,7*Z*,10*Z*,13*Z*,19*Z*-pentaenoic acid (16,15-EHDoPE), while *Leptocylindrus convexus* displayed 18-LOX derived compounds named 20-hydroxy-docosa-4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,18*Z*-hexaenoic acid (20-HDoHE) and 19,20-epoxy-18-hydroxy-docosa-4*Z*,7*Z*,10*Z*,13*Z*,16*Z*-pentaenoic acid (19,18-EHDoPE) (**Figure 1.10**).

Since Miralto et al. (1999) reported PUA production and the clear correlation between diatom diets and reproduction failure in copepods, several studies aimed to understand how diatoms regulate the rapid production of reactive PUAs. As mentioned above, PUA production is initiated upon wounding in sea water, indicating that the transcription and *de novo* biosynthesis of the enzymes involved in lipoxygenase pathway is quite unfeasible (Pohnert, 2000). Pohnert (2002) observed that the addition of free PUFA precursors to wounded diatom boosted the PUA production, hypothesizing that the formation of these secondary metabolites was apparently substrate limited. Nevertheless, this increase of PUA production can also be due to the fact that the equilibrium between free PUFAs and PUAs is disturbed after addition of PUFA molecules, activating again lipoxygenase pathway and the subsequent aldehyde formation (Fontana et al., 2007b).

Leptocylindrus danicus and *Leptocylindrus hargravesii*



Leptocylindrus conversus

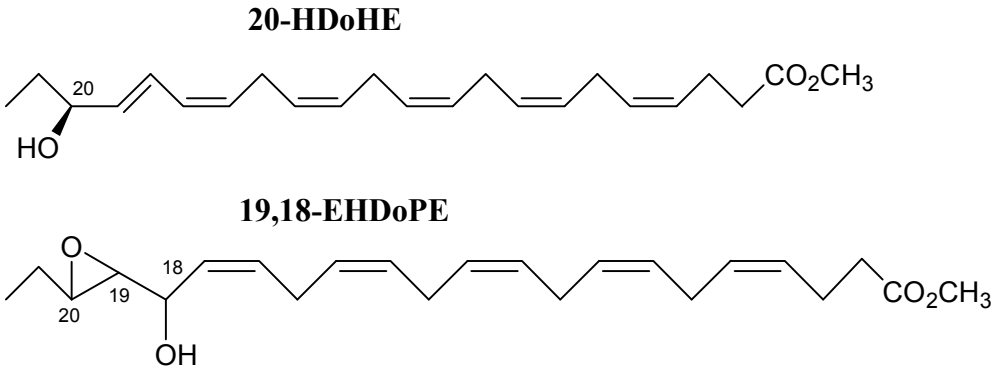


Figure 1.10. C_{22} oxylipins characterized in Leptocylindraceae species. (Modified from Nanjappa et al., 2014).

These findings emphasized the importance of the availability of free PUFAs for leading to the formation of PUAs. Thus, any mechanism that regulates PUFAs discharge from cells can be involved in PUAs formation. Due to their cytotoxicity, small amounts of PUFAs are present within cells in free form but they are bound to phospholipids and galactolipids (Pohnert, 2002; d'Ippolito et al., 2004).

Mechanisms regulating the activity of phospholipase A_2 and galactolipase enzymes can influence the production of free fatty acids and subsequent oxylipins. The phospholipase

A_2 is modulated by many cellular factors in higher terrestrial plants such as: intracellular Ca^{2+}, the degree of the enzyme phosphorylation and also the presence of ROS (Chandra et al., 1996; Chapman, 1998; Narvaez-Vasquez et al., 1999; Wang, 2001). It is presumable that these cellular signals act in the same way in phytoplankton. The production of PUAs was also observed in freshwater chrysophytes under light stress, iron, nitrogen and phosphorus limitation (Watson and Satchwill, 2003). However, the influence of these environmental stresses on the mechanisms of PUAs production, including the PUFAs availability and/or the enzyme activities is still unclear and debated. Enzymes involved in the PUAs production seem to be activated only after grazer wounding and thus PUAs have been considered as "anti-predator" molecules. However, in terrestrial plants, it has been demonstrated that activation of phospholipase and galactolipase are essential to cell function and very important for regulating cell growth and differentiation (by reorganizing the cellular membrane) as well as apoptosis and senescence (by controlling the membrane entirety) (Chapman, 1998).

Several evidences indicated that oxylipins may be released not only as chemical defences against grazers, but also as signalling molecules within diatom populations. Spiteller (2003) proposed that lipid hydroperoxides (oxylipins precursors) and PUAs, induce two types of physiological responses: moderate amounts of oxylipins (nanomolar levels or lower) would allow cell division and cell proliferation, while large amounts of oxylipins (micromolar or higher) would induce necrosis and apoptosis, respectively. For example, the marine diatom *Thalassosira weissflogii* incubated with large amounts of decadienal (up to 6.4 μM) showed a clear growth inhibition, activating programmed cell death (PCD; Casotti et al., 2005). Recently it was hypothesized that *S. marinoi* (PUAs

producer) recognizes PUAs as intra-population infochemicals, while *P. tricornutum* (non-PUAs producer) perceives them as allelochemicals. The ability of *S. marinoi* to produce and use PUAs as infochemicals may modulate ecological success in natural communities (Gallina et al., 2014).

This assumption should be supported by understanding other causes, which trigger oxylipins production and their subsequent release in the marine environment without a cell disruption occurring during grazing, as observed in the marine diatom *S. marinoi* (Ribalet et al., 2014).

PUAs were produced by 38% of the cultivated isolates in a range of concentrations from 0.01 to 9.8 fmol $cell^{-1}$ (Wichard et al., 2005b). Among them, 2*E*,4*E*-decadienal has been widely used as a model aldehyde to show deleterious effects on the reproduction of several marine invertebrates, such as echinoderms, polychaetes, ascidians, crustaceans and molluscs (Caldwell, 2009).

Several treatments with the pure molecule demonstrated that the PUA decadienal induced apoptosis in sea urchin embryos (Romano et al., 2003). As a biochemical marker for apoptotic processes in sea urchin embryos treated with decadienal, the ability of this PUA to activate caspase-like enzymes had been tested, using several commercially available kits developed for vertebrate caspases. A caspase-3-like activity was detected only in sea urchin embryos after 60 minutes of exposure to 5 µg mL^{-1} of decadienal, with maximum activity after incubation for 120 minutes (Romano et al., 2003). Romano et al. (2010) confirmed the deleterious effect of PUAs, such as decadienal, octadienal, octatrienal and heptadienal on early and late developmental stages of *P. lividus*. PUAs blocked sea urchin cell cleavage in a dose dependent manner,

but at different concentrations depending on the chain length of the molecules. The percentage blockage of cell cleavage increased with increasing chain length from C_7 to C_{10} PUAs, with arrest occurring at 27.27 μM with heptadienal, 16.13 μM with octadienal, 11.47 μM with octatrienal (which was slightly more active compared to octadienal), and 5.26 μM of decadienal. The saturated aldehyde tridecanal, also found in diatoms, did not interfere with first cleavage up to 25 μM. Higher concentrations were not tested due to scarce solubility of this compound in sea water. The effect of PUAs on sea urchin hatching success were also tested, showing that all three PUAs exerted a very strong dose-dependent effect, with decadienal showing strongest effects than the other two aldehydes.

Incubation of eggs soon after elevation of fertilization membrane, with lower doses of decadienal (1.32 to 5.26 μM) than those inducing the arrest of cell division (6.58 μM), increased the number of abnormal sea urchin plutei and delayed the development of larvae or embryos which showed various degrees of malformations with increasing concentrations tested (**Figure 1.11**). At lower concentrations (1.32-2.63 μM), malformations were less severe with a shortening of spicules and arms. At higher concentrations (3.95-5.26 μM), larvae were similar to blastula and gastrula stages, showing severe abnormalities or blebbing associated with apoptosis.

Moreover, these studies revealed that decadienal not only slowed down development but also induced apoptosis in a dose-dependent manner. At 1.32 μM decadienal, the number of embryos, presenting a high degree of apoptosis, was more or less the same as in the control. It is interesting to note that, at this concentration, larvae completely negative to TUNEL (Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling)

staining were also recorded, suggesting the possible alteration of the cell cycle and apoptotic machinery. Already at decadienal 2.63 µM, the proportion of embryos presenting apoptotic nuclei in the whole body considerably increased, reaching almost 80% of the total number of embryos examined. At 3.95 µM decadienal, there was a stronger effect and almost all of the larvae were positively stained.

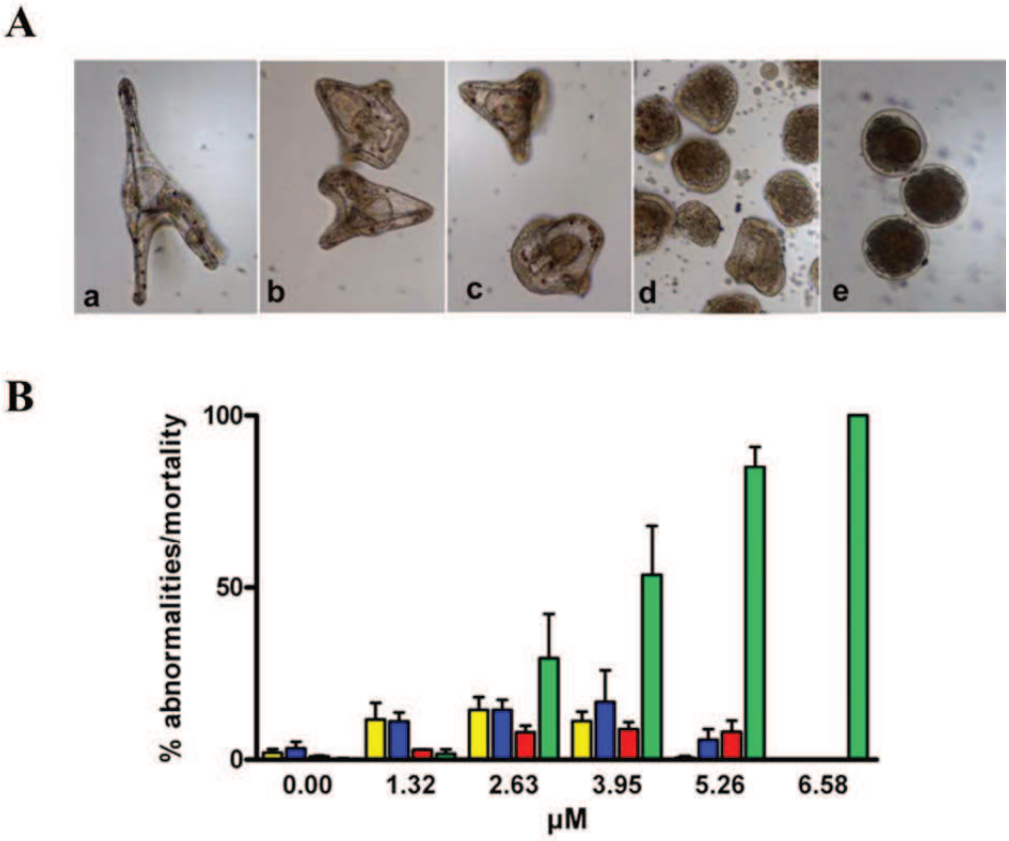


Figure 1.11. A) *P. lividus* embryos at the pluteus stage (48 hours post fertilization) incubated with decadienal at different concentrations: (a) control embryos in sea water without decadienal; (b) 1.32, (c) 2.63, (d) 3.95 and (e) 5.26 µM. B) Percentages of abnormal plutei (blue bars), abnormal blastulae and gastrulae (red bars), retarded larvae (yellow bars) and dead pre-hatched embryos (green bars) at different decadienal concentrations from 1.32 µM to 6.58 µM. (From Romano et al., 2010).

Recent studies confirmed the apoptotic effects of PUAs on cancer cell lines (Sansone et al., 2014). The effects of the PUAs decadienal, octadienal and heptadienal have been

tested on the adenocarcinoma cell lines lung A549 and colon COLO 205, and the normal lung/brunch epithelial BEAS-2B cell lines. Using the viability MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)/Trypan blue assays, the results revealed that PUAs have a toxic effect on both A549 and COLO 205 tumor cells but not BEAS-2B normal cells. Decadienal was the strongest among the three PUAs tested, at all time-intervals considered, but heptadienal was as strong as decadienal after 48 hours. Octadienal was the least active of the three PUAs.

Surprisingly, although this sea urchin is extensively used as a model system for ecotoxicological studies, the complete genome is not yet available. This represents a significant limitation to the use of this sea urchin for molecular studies and explains the large number of morphological studies conducted to date. Sea urchin *P. lividus* has been used as a model system for the first studies on molecular response to PUAs, reported in Romano et al. (2011) and in Marrone et al. (2012). The authors treated sea urchin embryos with a low concentration of decadienal (0.25 μg mL^{-1} producing about 35% of abnormal embryos) and followed the expression levels of sixteen genes using *Real Time qPCR*, in order to identify genes that were activated in response to this teratogen. The results showed that at low decadienal concentrations (in the range 0.15-0.35 μg mL^{-1}) the sea urchin places in motion different classes of genes to defend itself against this toxic aldehyde, activating heat shock proteins and several genes involved in skeletogenesis (formation of the skeleton). These molecular data were in accordance with morphological ones, demonstrating that developmental abnormalities mainly affected skeleton morphogenesis (Marrone et al., 2012). Moreover, sea urchin embryos

treated with increasing decadienal concentrations (in the range 0.15-0.35 μg mL^{-1}) revealed a dose-dependent response of activated target genes.

These studied have been extended to the other two commercialized PUAs, heptadienal and octadienal, the most common of the PUAs produced by diatoms, in comparison with decadienal, the most studied (Wichard et al., 2005a,b). The effect was dose-dependent for the three PUAs tested, even if the range of concentrations inducing malformations on *P. lividus* embryos differed (from 0.5 to 2.5 μM for decadienal, from 1.0 to 6.0 μM for heptadienal and from 2.0 to 9.0 μM for octadienal) (**Figure 1.12**; Varrella et al., 2014). Decadienal was the strongest of the three, because of the very narrow range (2.0 μM) that affected embryonic development; while heptadienal and octadienal required higher ranges of concentrations to reach the same effects as decadienal.

Treatments with the three PUAs induced similar malformations on sea urchin *P. lividus* plutei at 48 hours post-fertilization (hpf) (**Figure 1.13**), with a poorly-formed apex and arms appearing longer and asymmetrical or completely degenerated, compared to controls (sea urchins grown in sea water without PUAs) (Varrella et al., 2014).

Post-recovery experiments showed that embryos can recover after treatment with all three PUAs, indicating that negative effects depended both on PUA concentrations and the exposure time of the embryos to these metabolites. The time range during which PUAs exert the greatest effect on sea urchin embryogenesis has also been defined, corresponding to 10 min before fertilization and/or 10 min after fertilization. The addition of PUAs in later developmental stages does not seem to affect the embryonic development of *P. lividus*.

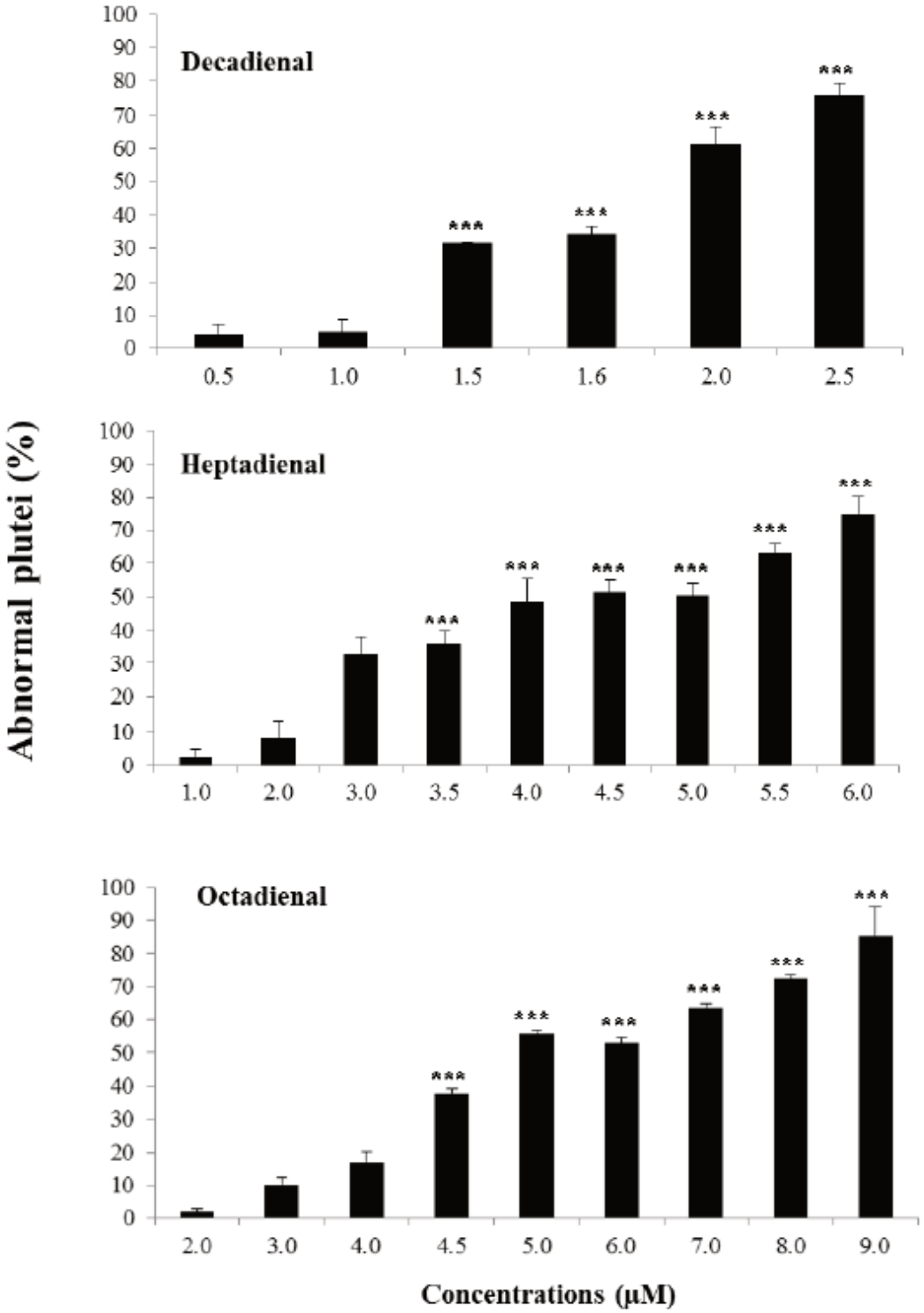


Figure 1.12. Percentage of abnormal *P. lividus* plutei observed after exposure to different concentrations of heptadienal, octadienal and decadienal. (From Varrella et al., 2014).

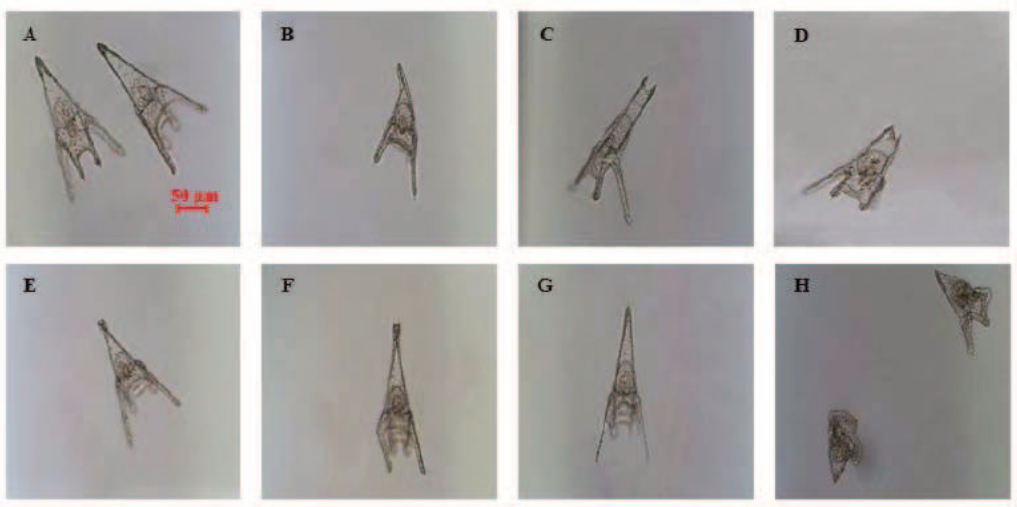


Figure 1.13. Examples of malformations of *P. lividus* plutei affecting: the apex (B) with spicules either parallel or disjoined (C, D) or crossed at the tip (E); the arms longer and broader or crooked and asymmetrical (F) or completely degenerated (G); the whole body plan of the plutei strongly compromised and malformed (H). All observations were compared to the control (A). (From Varrella et al., 2014).

Very little information is available on the morphological and molecular effects of NVOs on sea urchin embryos. Very recently, it has been demonstrated that increasing concentrations of two hydroxy fatty acids (abbreviated as HEPEs), 5-hydroxy-eicosa-6*E*,8*Z*,11*Z*,14*Z*,17*Z*-pentaenoic acid (5-HEPE) and 15*S*-hydroxy-eicosa-5*Z*,8*Z*,11*Z*,13*E*,17*Z*-pentaenoic acid (15*S*-HEPE) induce dose-dependent developmental malformations on sea urchin embryos but at higher concentrations when compared with PUAs (range of 6-15 μM; **Figure 1.14**; Varrella et al., 2016b). The observed malformations were similar to those induced by PUAs (see above; Varrella et al., 2014). Interestingly, HEPEs also induced a marked developmental delay in sea urchin embryos, which has not hitherto been reported for PUAs. In particular, the percentage of delayed embryos increased with increasing HEPE concentrations to become the only class of embryos present at the highest concentrations of 30 μM (**Figure 1.15**; modified from Varrella et al., 2016b). Moreover, the degree of delay was different with increasing

concentrations: from 6.0 to 10 μM the delay in the development of embryos was manifested by a shortening of the body and, at the pluteus stage, the morphology of the embryo closely resembled that of the control, with only a slight reduction in body length; at 15 and 30 μM, the development of embryos was much more delayed, with embryos still at the stage of early pluteus.

PUAs and HEPEs molecular targets have also been identified by *Real Time qPCR*, analysing the variation in expression levels of 39 genes, implicated in a broad range of functional responses, such as stress, development, differentiation, skeletogenesis and detoxification processes (**Figure 1.16**; Varrella et al., 2014, 2016b).

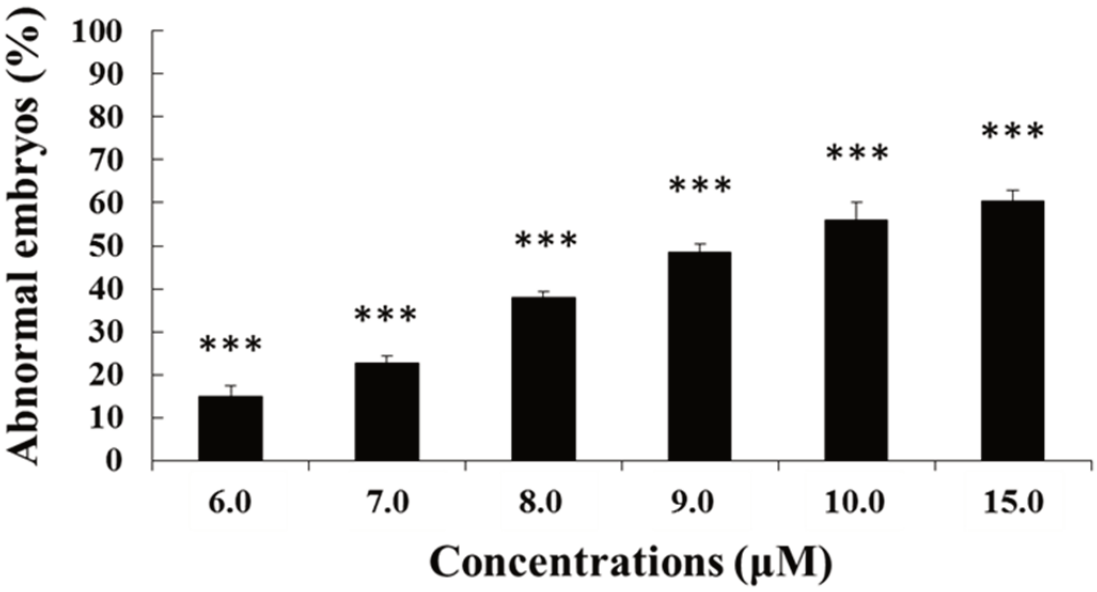


Figure 1.14. Percentage of abnormal plutei when *P. lividus* newly-fertilized eggs (10 minutes after the elevation of the fertilization membrane) were exposed to different concentrations of the four HEPEs (6, 7, 8, 9, 10 and 15 μM). (From Varrella et al., 2016b).

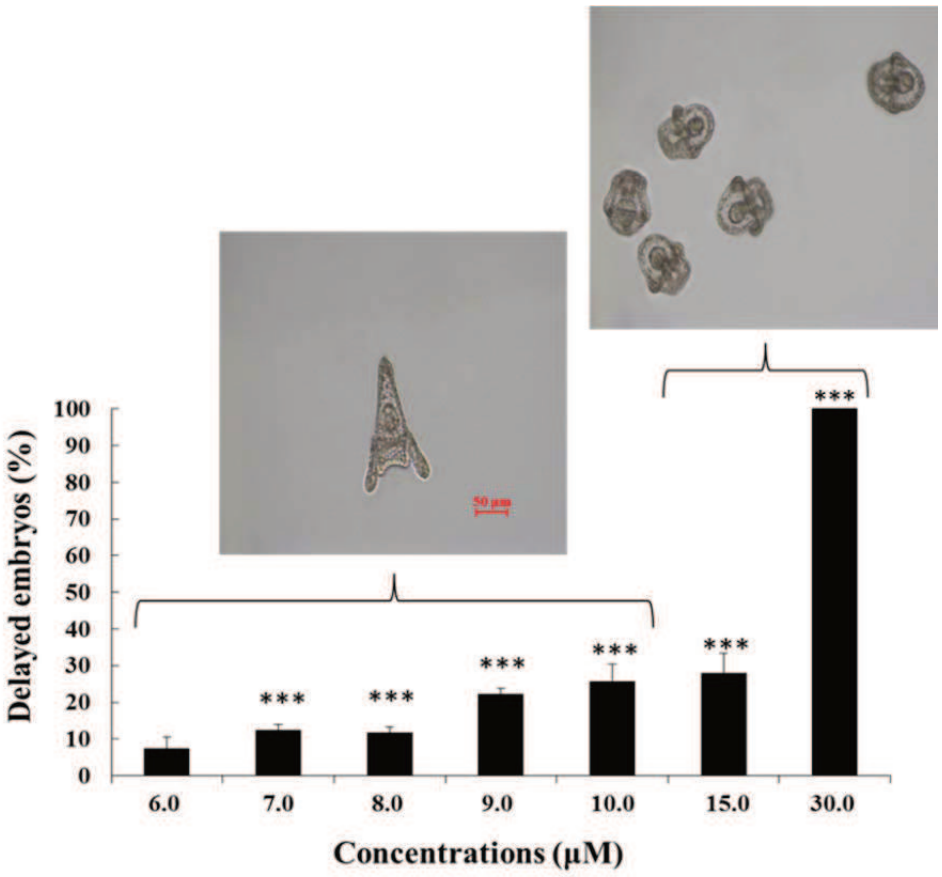


Figure 1.15. Percentage of delayed *P. lividus* embryos when newly-fertilized eggs were exposed to different HEPEs concentrations (6, 7, 8, 9, 10, 15 and 30 µM) at 48 hpf respect to the control (not shown). (Modified from Varrella et al., 2016b).

PUAs had several common molecular targets in all the functional classes tested. Moreover, among PUAs heptadienal seemed to be the strongest one, affecting the largest number of genes, mainly those involved in development and differentiation processes. The results also showed that 15*S*-HEPE switched on fewer genes than 5-HEPE, being the weakest of the oxylipins tested. These findings highlight the differences between HEPEs and PUAs and also have important ecological implications because many diatom species do not produce PUAs, but rather these other chemicals are derived from the oxidation of fatty acids.

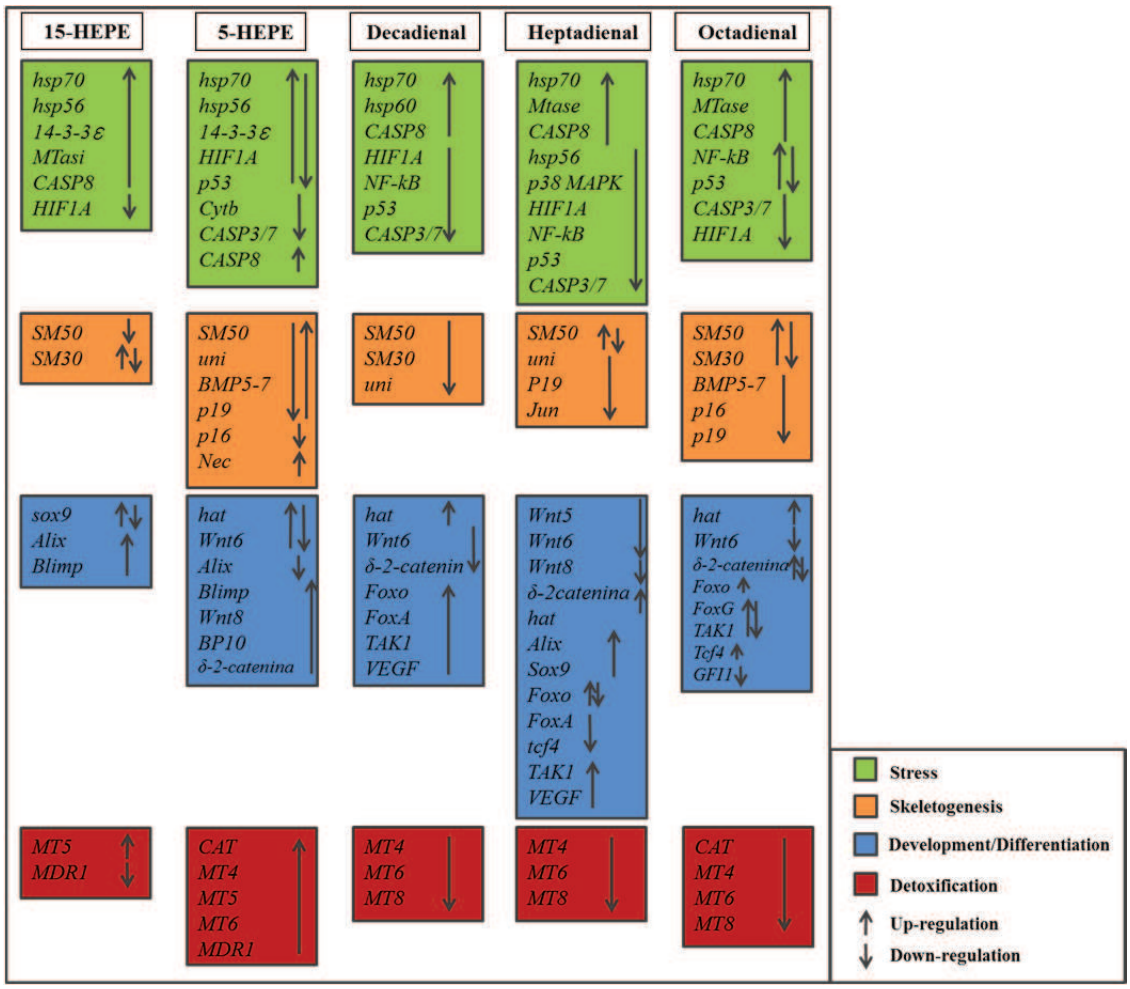


Figure 1.16. Schematic overview of the targeted genes analyzed by Real Time *q*PCR. The genes are reported in colored boxes according to the different functional classes to which they belong. Up- and down-regulation of genes are indicated with upward and downward pointing arrows, respectively. (Modified from Varrella et al., 2016b).

On the other hand, treatments at higher PUAs (decadienal 3.3 µM, heptadienal 9.0 µM and octadienal 11.0 µM) and HEPEs (100 µM) concentrations induced apoptosis in *P. lividus* embryos, detected by microscopic observation and through the activation of caspase-3/7 and caspase-8 measured by luminescent assays (Ruocco et al., 2016).

Regarding the molecular effects triggered by oxylipins on marine invertebrates, gene expression analysis has also been performed on copepods after feeding on diatom

diets. Lauritano et al. (2011) showed that two days of feeding on a strong oxylipin-producing diatom, *S. marinoi*, was sufficient to inhibit a series of genes involved in aldehyde detoxification, apoptosis and cytoskeleton structure and stress response in the copepod *C. helgolandicus*. Of the eighteen transcripts analysed by *Real time qPCR* at least 50% were strongly down-regulated (aldehyde dehydrogenase 9, 8 and 6, cellular apoptosis susceptibility and inhibitor of apoptosis IAP proteins, heat shock protein 40, alpha- and beta-tubulin) compared to animals fed on a weak oxylipin-producing diet, *C. socialis*, which showed no changes in gene expression profiles. Furthermore, these Authors hypothesized that different populations of copepods may deal differently with the same oxylipin-producing diatom diet. In fact, comparative studies of expression level variations of selected genes have been provided for three *C. helgolandicus* populations (North Sea, Atlantic Ocean and Mediterranean Sea) exposed to the same strain of the oxylipin-producing diatom *S. marinoi*, using as control alga the flagellate *Rhodomonas baltica* (Lauritano et al., 2012). Expression levels of detoxification enzymes and stress proteins (e.g. glutathione S-transferase, glutathione synthase, superoxide dismutase, catalase, aldehyde dehydrogenases and heat shock proteins) and proteins involved in apoptosis regulation and cell cycle progression were analysed in copepods after both 24 and 48 hours of feeding on the diatom or on a control diet. Strong differences occurred among copepod populations, with the Mediterranean population of *C. helgolandicus* being more susceptible to the toxic diet compared to the others.

Recent studies on the congeneric species *Calanus sinicus* fed on *S. marinoi* have shown that this copepod is more resistant to diatoms compared to *C. helgolandicus* (Lauritano

et al., 2015). A significant down-regulation of different genes (beta-tubulin, aldehyde dehydrogenase 6, heat shock protein 70 and catalase) has been found after 48 hours of feeding; whereas, after five days of feeding, the expression patterns changed, with an up-regulation of aldehyde dehydrogenase 2 and 9, heat shock protein 70 and glutathione synthase genes. The different expression levels of genes involved in defence and detoxification systems highlighted the genetic and/or phenotypic flexibility of copepod species to cope with environmental constraints. Moreover, the first large-scale transcriptional profiling of *C. helgolandicus* fed for two days with toxic (*S. marinoi*) and non-toxic food (*R. baltica*) was performed. In particular, 947 ESTs have been sequenced and generated 376 unigenes of *C. helgolandicus* from two suppression subtractive hybridization (SSH) libraries, showing differences in the transcriptome between females fed with the two diatom diets. ESTs belonging to biological processes such as response to stimuli, signal transduction, and protein folding were significantly over-expressed in copepods feeding on *S. marinoi* comparing the two transcriptome libraries. On the other hand, copepods feeding on *R. baltica*, displayed higher expression of genes involved in cellular processes such as protein synthesis (ribosome biogenesis, macromolecule biosynthetic process) and energy metabolism (electron transport) (Carotenuto et al., 2014).

Despite the number of papers published in the literature on oxylipins from planktonic diatoms and their effects on grazer reproduction, there are very few data on benthic species (Jüttner et al., 2010; Scholz and Liebezeit, 2012a), probably due to difficulties to isolate, culture and grow benthic diatoms in axenic conditions (Simental et al., 2001). The microphytobenthos assemblages can be highly productive (Pinckney and

Zingmark, 1993) and benthic primary producers represent an important source of organic carbon in some littoral areas (Allen et al., 2015). The real capacity of benthic organisms to produce PUAs, as well as the effect that these compounds may have on the same benthic ecosystems remains largely unknown. The genus *Cocconeis*, epiphytic diatom living on the leaves of *P. oceanica*, has been the most studied among benthic diatoms, despite the low growth rates and strong adhesion to the substratum, which makes their isolation and manipulation difficult (Raniello et al., 2007). These diatoms were demonstrated to play an important trophic role for the marine shrimp *Hippolyte inermis*, by influencing its sex reversal. In fact, the ingestion of *Cocconeis neothumensis*, *C. scutellum parva* and *C. scutellum scutellum* triggered the apoptosis of its androgenic gland (Zupo, 2000; Zupo and Messina, 2007), followed by the complete destruction of the male gonad and a shift to the female sex. The ingestion of diatoms is helpful for natural populations since the production of young females in spring, when the abundance of *Cocconeis* sp. on *Posidonia* leaves is very high, assures higher reproductive bursts (Zupo, 1994). Therefore, *H. inermis* and the pro-apoptotic compound produced by these diatoms, acts as a spring signal for the production of young females, probably due to a long co-evolutionary process (Zupo et al., 2007). Another study demonstrated the apoptotic activity of *C. scutellum* diatoms on breast cancer cells BT20, suggesting their potential use for pharmacological applications. Interestingly, it has been shown that the richest EPA fraction of diethyl ether extract selectively induced apoptosis in a dose-dependent manner (up to 89.2% at 1 μg/well) and decreased viability in BT20 cells (Nappo et al., 2012). Moreover, odour choice experiments were conducted with several species of macroinvertebrates to test their

response to volatile organic compounds (VOCs) obtained from the extracts of the three benthic diatoms *C. scutellum parva*, *Cocconeis posidoniae* and *Diploneis* sp. and embryo-toxicity assays with the organic extract of these diatoms, revealed dose-dependent effects on the first cleavage and the normal division of sea urchin *P. lividus* embryos (Maibam et al., 2014). Chemical analyses of VOCs extracted from *C. scutellum parva* showed a large production of PUAs with chain lengths in the range between C_5 and C_{10}. Unexpectedly, C_6 compounds have also been found, represented by 3*Z*-hexenal, 2*E*-hexenal, hexanal and hexanol-1 that resemble the typical bouquet observed in higher plants (Juttner et al., 2010).

Recently, it has been demonstrated that three benthic diatoms, *Tabularia affinis*, *Proschkinia complanatoides* and *Navicula* sp. produced PUAs during the stationary and senescence phases, with cell concentrations (from 1.8 to 154.4 fmol $cell^{-1}$) quite similar to planktonic diatoms (Pezzolesi et al., 2017). From a qualitative point of view, medium-chained PUAs were the most abundant aldehydes produced by *Navicula* sp. Although hexadienal (C6:2), octadienal (C8:2), decatetraenal (C10:4), undecatetraenal (C11:4), undecapentenal (C11:5) and tridecatetraenal (C13:4) were also detected, octenal (C8:1) was the most representative compound (93.8%). PUAs composition of the other two species was more variable, but results were similar between the two diatoms: hexadienal (C6:2) contributed up to 50.56% and 39.04%, for *T. affinis* and for *P. complanatoides*, respectively. Decatetraenal (C10:4) reached a maximum value of 0.09 ± 0.01 pg $cell^{-1}$ for *T. affinis* and 0.13 ± 0.04 pg $cell^{-1}$ at different times of the curve growth (**Figure 1.17**; Pezzolesi et al., 2017).

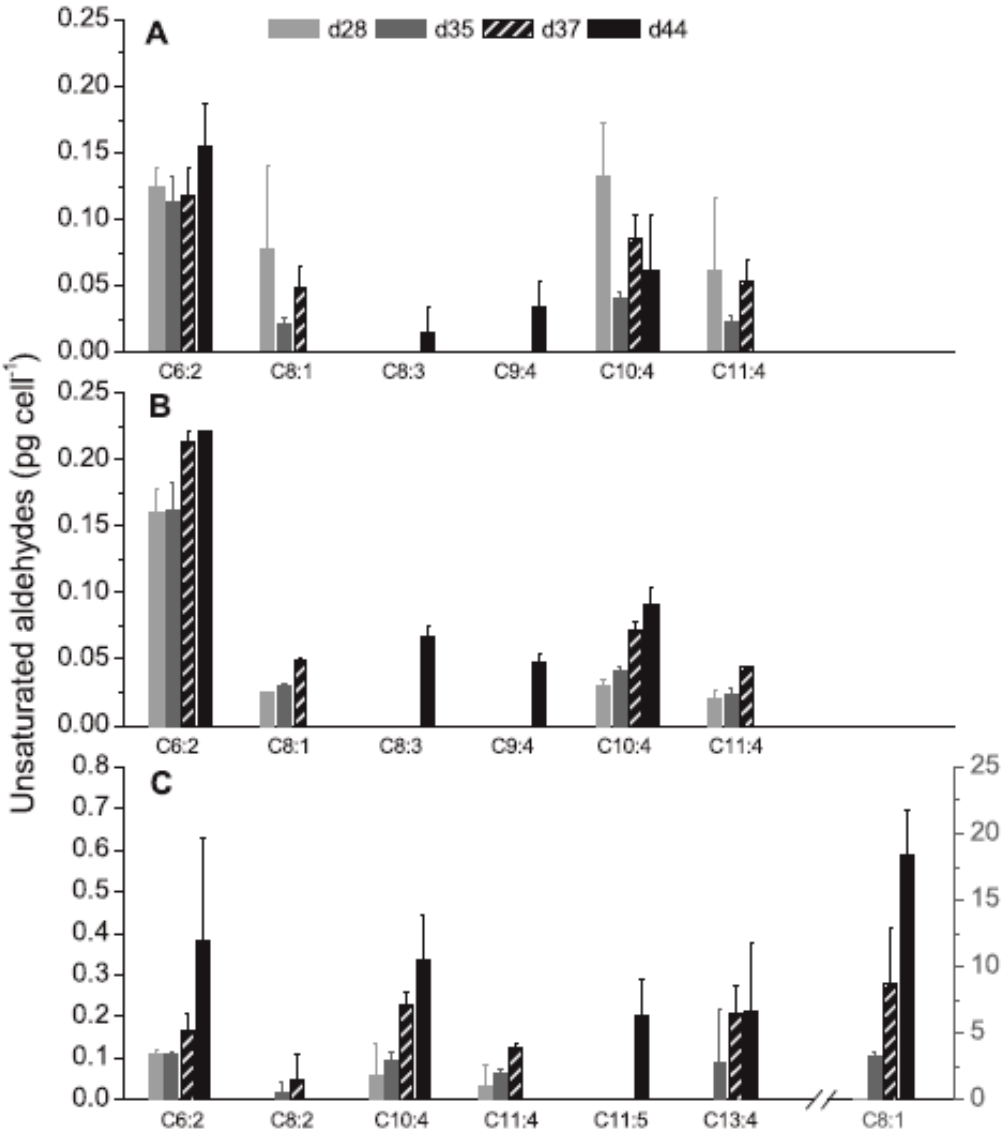
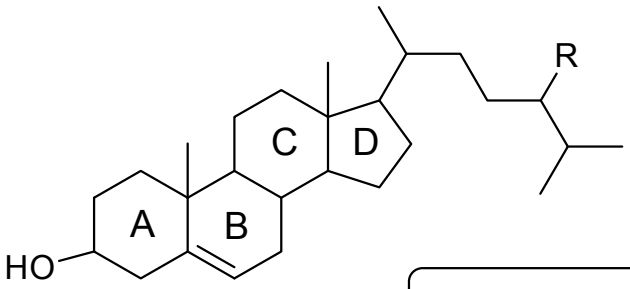


Figure 1.17. Mono- and polyunsaturated aldehydes production in (A) *P. complanatoides*, (B) *T. affinis* and (C) *Navicula* sp. at different days (28, 35, 37, 44). Right Y axis in *Navicula* sp. refers only to C8:1. (From Pezzolesi et al., 2017).

These results underlined the importance of designing studies to understand the interactions which occur between chemical signals and various organisms, especially in benthic biofilms, which are particular micro-environments where species are exposed to more concentrated chemicals exuded by neighbouring cells (Allen et al., 2015).

1.2.2 Sterol sulfates (StS)

Sterols are amphipathic lipids synthesized from acetyl-coenzyme A via the mevalonate pathway. They have a tetracyclic structure with the –OH group in the equatorial position on its first A ring and possess a short aliphatic chain (–R group), with two ending methyl groups, branched on the D pentenic ring. The hydroxyl group on the A ring is polar, while the rest of the aliphatic chain is non-polar (**Figure 1.18**).



Cholesterol	R = H
β-Sitosterol	R = - C_2H_5
Stigmasterol	R= - C_2H_5; Δ^{22}
Campesterol	R = - CH_3
Δ^5-Avenasterol	R = CH - CH_3
Brassicasterol	R = - CH3; Δ^{22}

Figure 1.18. Tetracyclic structure (A-B-C-D rings) of sterols and some examples differing in the aliphatic –R chain.

Sterols occur in the membranes of plants, animals, and microorganisms and are termed phytosterols, zoosterols and mycosterols, respectively. Bacteria usually lack sterols in their membranes, but yeasts accumulate considerable quantities of sterols, which may represent up to 10% of the cellular dry weight. Cholesterol is the main zoosterol, whereas sterols in plants commonly occur as mixtures with β-sitosterol, campesterol, and stigmasterol representing three of the major phytosterols. These sterols are all Δ^5-sterols, but Δ^7-sterols may also be present in small quantities.

Sterols play an essential role in modulating membrane bilayer structure and dynamics due to their high-water insolubility and amphipathic nature. Sterols are anchored to the aqueous interface via the polar –OH group, whereas the rest of the molecule is hydrophobic. The four rigid sterol rings are responsible for the sterols' major function, controlling membrane fluidity and packing free volume.

Moreover, sterols are involved in typical eukaryotic mechanisms, such as phagocytosis. For example, genes involved in sterol biosynthesis have been shown to be selectively up-regulated in the amoebozoan *Dictyostelium discoideum* during phagocytosis (Sillo et al., 2008). Eukaryotes that are not able to synthesize sterols have to obtain them from food, such as is the case, for example, of insects and of most marine invertebrates.

The diversity and nature of sterols, together with the pathways leading to these compounds, have been thoroughly studied in vertebrates, fungi, and land plants. Characterizations of sterols from other organisms have unveiled a wide variety of molecules, among which not only the same sterols already known in animals, fungi, and land plants but also other types of sterols with unsaturations at various positions of the cycle or in the side chain, and possibly alkylations or inclusion of a cyclopropane ring mostly in C_{24} or C_{22} (Volkman, 2003).

Sponges, a primitive group of multicellular organisms, represent the richest source of bizarre sterols found in nature. Most sponges have the general sterol structure found in animals, plants, and fungi, but bearing one to three extra carbon atoms at C_{24} (Giner, 1993). In fact, 24-isopropylcholesterol is abundant and characteristic (with its analogue unsaturated at C_{22}-C_{23}) of the class *Demospongiae*. In microalgae, sterols usually possess C_{27}-C_{29} skeletons, with differences in alkylation at C_{24} and double bonds in the

nucleus and side chain. Dinoflagellates are unusual in terms of steroid composition, as they often contain sterols with additional methyl groups at C_4 and C_{23}. For example, dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol) is typically found in dinoflagellates, where it is generally accepted as a reliable biomarker (Boon et al., 1979). 23-Methyl sterols have been also reported in diatoms and their sterane equivalents were also unambiguously identified in sediments and petroleum from the late Jurassic onwards (Rampen et al., 2009a,b).

In the present Ph. D. thesis, a particular attention has been given to some sterol derivatives, called sterol StS. The biosynthesis of these compounds is controlled by cytosolic sulfotransferases (SULTs) which transfer a sulfo residue ($–SO_3^-$) group from the universal donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to the hydroxyl function at C_3 of the steroid skeleton (**Figure 1.19**).

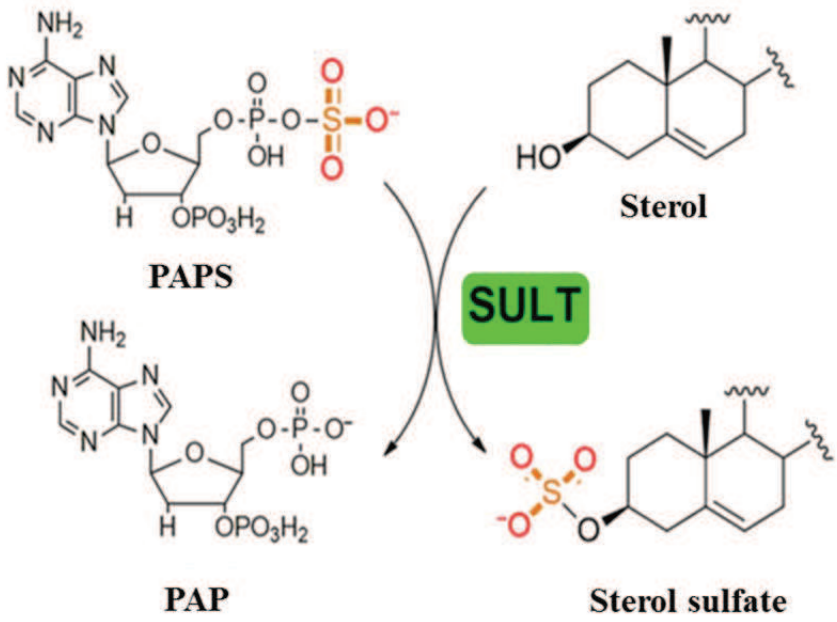


Figure 1.19. Sulfation reaction of sterols catalysed by SULTs. (From Gallo et al., 2018).

SULTs are divided in cytosolic and membrane-associated proteins, although this organization can be misleading since in most cases the subcellular localization of these enzymes is unknown. In fact, the term cytosolic refers to the ability of the enzymes to be extracted from cells in a soluble form. Membrane-associated SULTs add sulfo groups to large biomolecules such as complex polysaccharides and proteins, whereas the cytosolic forms accept, as substrate, small organic molecules including sterols, flavonoids, glucosinolates, phenols and catecholamines (Chapman et al., 2004).

After the first isolation of cholesterol sulfate (CHOS) from the starfish *Asterias rubens* (Björkman, et al., 1972; Godfellow and Goad, 1973), several studies have been described StS from a wide variety of marine organisms, particularly sponges (Prinsep et al., 1989; Fusetani et al., 1994; Aiello et al., 1999; Yang et al., 2003) and echinoderms (D'Auria et al., 1990). These compounds have also shown interesting biological activities. For example, Fusetani and co-authors have shown that the marine sponge *Topsentia* sp. (Halichondriidae) produces StS, called topsentiasterol sulfates A-E, with antimicrobial activities. A bio-assay guided fractionation performed from the same sponge, revealed additional StS with strong inhibitory effects on platelet $P2Y_{12}$ receptors (Yang et al., 2003).

Despite the large amount of sulfated compounds found in marine sponges and echinoderms, very little is known about their biosynthesis (Kornprobst et al., 1998). SULT activity has been described in cnidarians (Cormier et al., 1970), sea urchins (Malins and Roubal, 1982) and algae, microalgae, and other phototrophic organisms (Hernández-Sebastià et al., 2008). Ho et al. (2015) found 84 SULTs of the superfamily 1 and 2 by mining the genomes of green microalgae and diatoms.

Four genes coding for putative SULTs have been recently identified by transcriptomic analysis in the diatom *S. marinoi* (Gallo et al., 2017). The amino acid sequence of the highly conserved regions underlines the phylogenetic relationship of these proteins with cytosolic SULTs from different groups of organisms, including other diatoms, cyanobacteria, green algae, plants, and mammalians. Gallo and co-authors also showed that, at least, one of the sequences of *S. marinoi* encodes for a putative protein with 30% similarity with human SULT2B1, SULT2A1, and SULT1C that highly prefer cholesterol and other 3β-hydroxysteroids, as substrates.

Few studies have been published on StS from algae and diatoms, and even no data are available on benthic species. A C_{29} StS, called hymenosulphate, was isolated from the laboratory-cultured haptophyte *Hymenomonas* sp. (Kobayashi et al., 1989). This compound showed in the sarcoplasmic reticulum a calcium releasing activity ten times more potent than caffeine, which is a well-known Ca^{2+}-releaser. Moreover, the lipid composition and structural analysis of the non-photosynthetic marine diatom, *Nitzschia alba*, have revealed the presence of 24-methylenecholesterol sulfate (Anderson et al., 1978; Kates et al., 1978), whereas a 5α,8α-epidioxysterol sulfate has been isolated by Toume and Ishibashi (2002) from the cultured diatom *Odontella aurita*. Very recently, Gallo et al. (2017) have characterized StS in *S. marinoi* cells through Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) analyses. Three classes of molecules were predominantly found: β-sitosterol sulfate (βSITS), dihydrobrassicasterol sulfate (DHBS) and CHOS (**Figure 1.20**). The natural mixture and the major components DHBS and CHOS showed cytotoxic effects on *S. marinoi* cells, with EC_{50} values of 10 μM, 5 μM and 7.5 μM, respectively. The intracellular

concentrations of StS increased along with the growth curve, reaching the highest values at the declining phase (Gallo et al., 2017). This correlation between intracellular concentrations and culture aging explain an auto inhibitory mechanism, which is in agreement with previous reports that correlate aging to bloom demise in phytoplankton communities (Bidle, 2016; Bidle and Bender, 2008; Bidle and Falkowski, 2004; Chung et al., 2005).

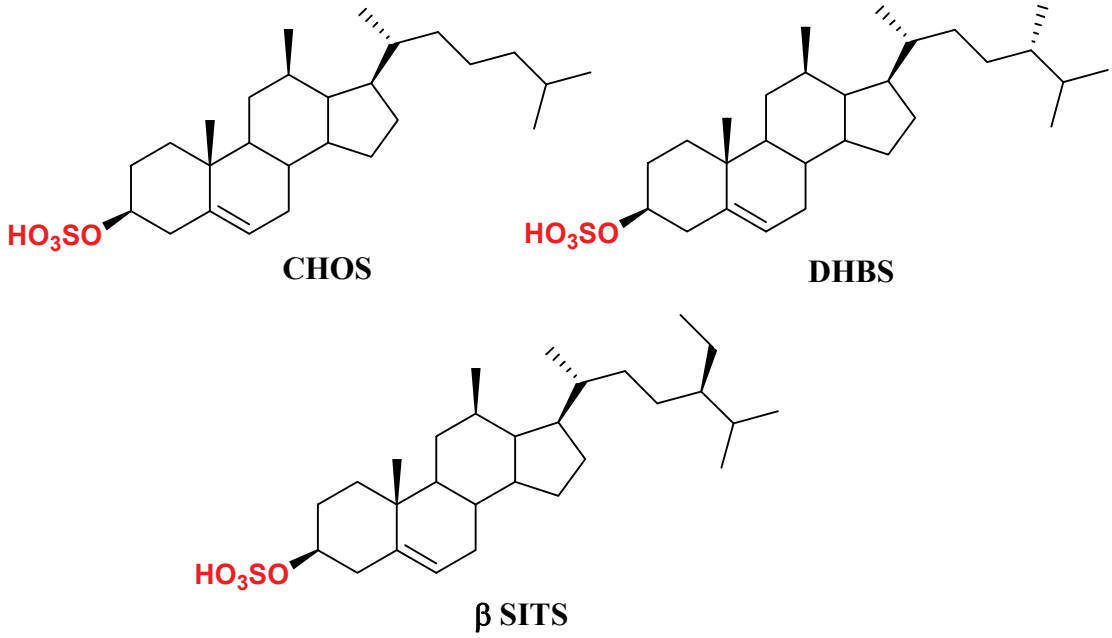


Figure 1.20. Chemical structures of cholesterol sulfate (CHOS), dihydrobrassicasterol sulfate (DHBS) and β-sitosterol sulfate (βSITS) identified by NMR and MS analyses from the marine diatom *S. marinoi*. (From Gallo et al., 2017).

The physiological process activated by StS, was also explored in *S. marinoi* cells. CHOS exposure at lethal concentrations (50 and 100 µM) induced a huge increase of ROS and nitric oxide (NO) levels. In addition, treatments with CHOS at 2.5 µM, which is lower than natural concentrations occurring during the declining phase, caused DNA fragmentation revealed by TUNEL assay.

ROS and NO mediators are involved in stress response in plants (Bozhkov et al., 2011) and have been related to aging and death in diatom cells (Vardi et al., 2006; van Creveld et al., 2015; Bidle, 2016). Furthermore, both mediators are related to overexpression of death specific proteins (DSP) in the stationary growth phase of *S. costatum* (Chung et al., 2005, 2008) and iron starved cultures of *Thalassiosira pseudonana* (Thamatrakoln et al., 2012). In fact, the detection of DNA breaks is basically considered a standard marker to distinguish apoptotic cells in cultures.

Considering these findings, Gallo and co-authors (2017) have proposed that StS could induce programmed cell death in diatoms cells through the activation of NO pathways. As shown in **Figure 1.21**, the increase of StS intracellular levels above a threshold of 0.5-0.6 pg per cell alters redox balance and induces rapid generation of NO, leading to the launch of PCD and apoptotic events. The effectors of this last process have not been identified yet but metacaspases have already been reported in *S. marinoi* and other diatoms. The synthesis of StS requires sulfonation of the sterol substrates, which can be accomplished through a *de novo* synthesis, mobilization of sterols from the membrane bilayer or up-regulation of SULT expression. Each of these processes can be triggered by environmental or physiological factors that determine the intracellular level of StS and, eventually, cell fate (Gallo et al., 2017).

These considerations indicate the involvement of StS in regulation mechanisms of growth and prompt the idea that they can act alone or in connection with other molecules (d'Ippolito et al., 2009; Fontana et al., 2007a,b; Lamari et al., 2013; Vardi et al., 2006, 2008) as intracellular mediators in signal transduction pathways.

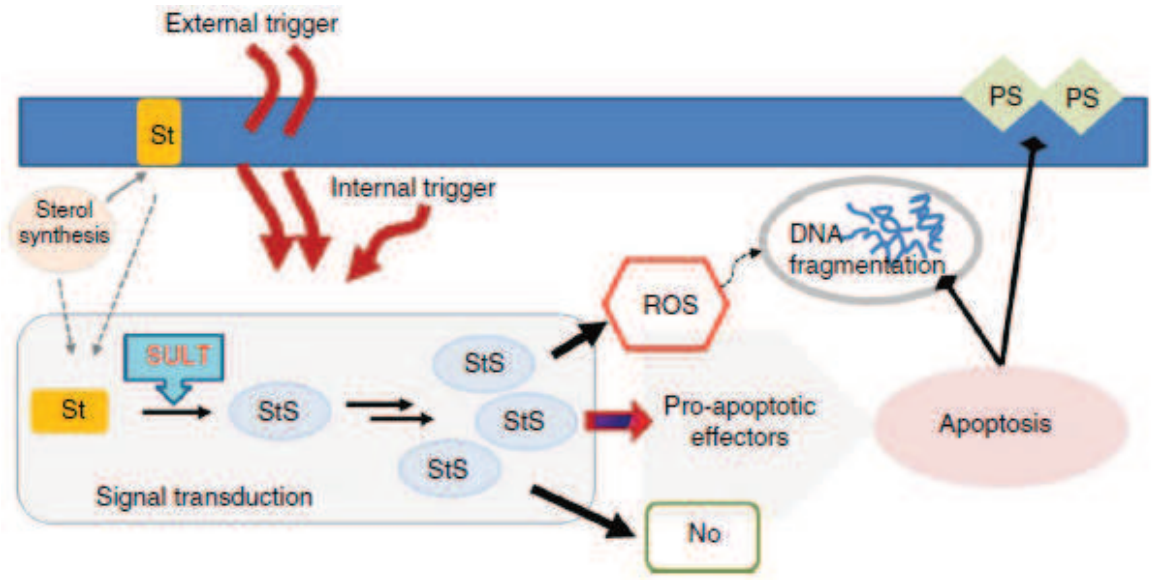


Figure 1.21. Signal transduction of StS proposed in *S. marinoi* cells. (From Gallo et al., 2017).

Looking at the future challenges opened by the discovering of these cell-signalling molecules, the manipulation of their biosynthesis could offer a new view of cellular regulation in the oceans and may find direct and general applications in the development of diatom feedstocks for biotechnological applications (Daboussi et al., 2014; d'Ippolito et al., 2015).

1.3 The model organism sea urchin *Paracentrotus lividus*

Paracentrotus lividus is a species of sea urchin in the family Parachinidae, commonly known as the purple sea urchin. Sea urchins are classified within the large phylum Echinodermata, in which five classes can be found: crinoids (feather stars), asteroids (sea stars), ophiuroids (brittle stars), echinoids (sea urchins) and holothurians (sea cucumbers; Pawson, 2007). It is a widespread species, occurring in the Mediterranean Sea and Eastern Atlantic Ocean from western Scotland and Ireland to the Azores, Canary Islands and Morocco (Boudouresque and Verlaque, 2001). *P. lividus* has

a circular, flattened greenish test with a diameter of up to 7.5 cm (Boudouresque et al., 1989; Lozano et al., 1995). The test is densely clothed in long and sharply pointed spines that are usually purple but are occasionally also of other colors, including dark brown, light brown and olive green (Koehler, 1883; Mortensen, 1943; Tortonese, 1965; Gamble, 1966; **Figure 1.22**).

A

Kingdom:	**Animalia**
Phylum:	Echinodermata
Class:	Echinoidea
Superorder:	Echinacea
Order:	Camarodonta
Family:	Parechinidae
Genus:	*Paracentrotus*
Species:	***P. lividus***

B

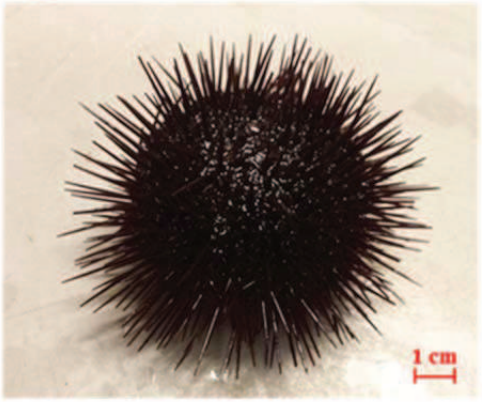


Figure 1.22. Scientific classification of the sea urchin *P. lividus* (A), specimen of *P. lividus* in the animal facility of the Stazione Zoologica Anton Dohrn. Bar, 1 cm (B).

P. lividus is usually found just below low water mark at depths down to 20 m and sometimes also in rock pools (Gamble, 1965; Tortonese, 1965; Allain, 1978; Harmelin et al., 1980; Crook et al., 2000). It is found on rocks and boulders, and in sea-grass meadows of *Zostera marina* and *P. oceanica*. Although *Cymodocea nodosa* is a preferred food item, it is seldom found in meadows of this seagrass, perhaps because the shifting sand substrate does not suit it or because of pressure from predators (Traer, 1980; Fernandez and Boudouresque, 1997; Marco-Méndez et al., 2015). In fact it avoids soft substrates and can sometimes be found clustered on stones or shell "islands" surrounded by sand (Zavodnik, 1980).

Sea urchins are deuterostomes as they develop the anus from the blastopore (or near it) and the mouth from the opposite cavity. The Echinoidea class, includes about 950 marine species widespread in all habitats throughout the world and living up to 5000 meters in depth. Among these, 26 species live in the Mediterranean Sea, including 11 with a regular shape and 15 irregularly shaped. The so-called regular Echinoids, the classic sea urchins, have globular or ovoid shapes and are covered with robust spines that are evenly distributed over the entire body. Sea urchin spines are formed by calcium carbonate mixed with organic matter and their length differs according to the genus (Smith, 1999; Su et al., 2000; **Figure 1.23**).

The adult body has pentamerous symmetry (the body is divided in 5 regions arranged around a central plate) with a size typically ranging from 6 to 12 cm, although large species can reach up to 36 cm. The body is divided equatorially into two hemispheres. The side facing the substrate is known as the oral hemisphere.

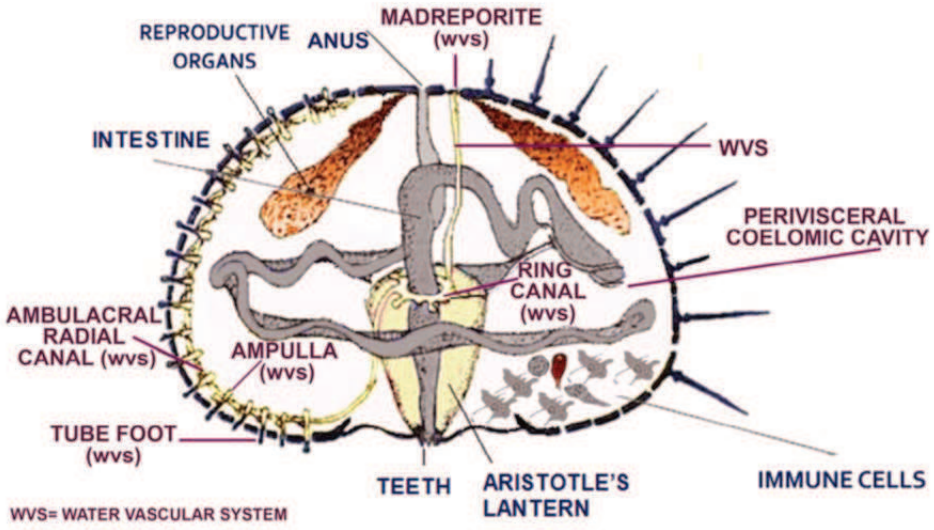


Figure 1.23. Schematic representation of sea urchin morphology. The complex open water vascular system (WVS) is shown in purple. (From Pinsino and Matranga, 2015).

The mouth is situated in the middle of this area and can be recognized by the presence of a structure used to chew (the 5 teeth lantern of Aristotle; **Figure 1.24**), surrounded by a membranous layer called the peristomal membrane. The upper part is called aboral hemisphere, formed by a ring with 10 plates, where the anal region is localized (called periprocto).

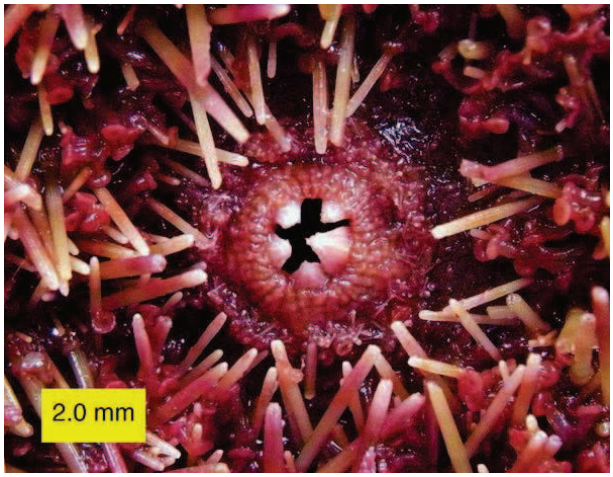


Figure 1.24. Oral surface of sea urchin, showing teeth of Aristotle's lantern, spines and tube feet. (From https://www.myrokan.com/2013/12/keunikan-hidupan-laut-landak-laut.html).

A structure known as madreporite or madreporic plate is also present on the aboral surface, through which the liquid of the aquifer system is in connection with the external environment. This is connected to the ring canal, which in turn is joined with the radial canals. Sea water is brought into the tube feet allowing the sea urchin movements on the substrate.

At the basis of sea urchin spines are the pedicellariae, small wrench- or claw-shaped appendages with different functions, including the pedicellaria ending with a suction-cup, used for movement or holding objects through which some species of sea urchins inject poison in their enemies.

Gas exchanges occur mainly through the ambulacral pedicels, where oxygen enters the body. Most sea urchins possess also five pairs of external gills, located around their mouths. These thin-walled projections of the body cavity are the main organs of respiration in those urchins that possess them. Fluids can be pumped through the gill interiors by muscles associated with the lantern, but this is not continuous, and occurs only when the animal is low on oxygen. Tube feet can also act as respiratory organs, and are the primary sites of gas exchange in heart urchins and sand dollars, both of which lack gills.

All external appendages of the sea urchin (spines, tube foot, pedicellaria) are able to quickly regenerate, as well as the wounds of the shell that are repaired, regenerating the skeleton.

The reproductive system consists of five gonads joined together by mesenteric filaments at the inner surface of the inter-ambulacral areas (**Figure 1.25**). When mature they are large and orange. Gonads extend from the aboral hemisphere, where they communicate with the external environment, almost to the lantern of Aristotle (Lawrence, 2013).

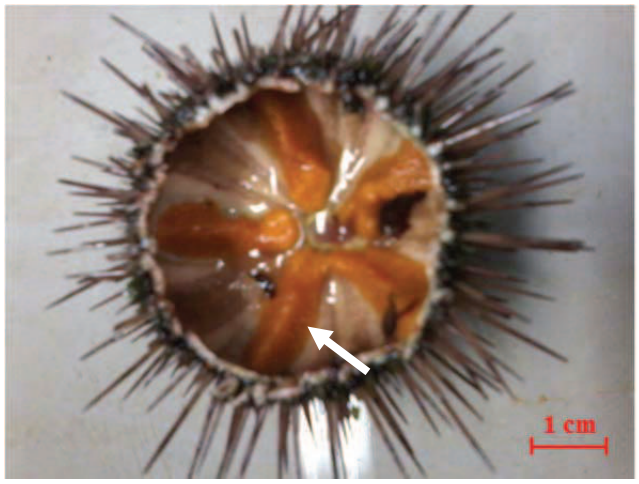


Figure 1.25. Picture of *P. lividus* gonads (indicated with white arrow). Scale bar, 1 cm.

P. lividus is a gonochorsitic species. They aggregate for spawning and release gametes into the water column. The larvae form part of the zooplankton for about 28 days before settling and undergoing metamorphosis.

The spawning season of *P. lividus* starts in January (although mature individuals can occur from October-November), and ends in May (Byrne, 1990). The eggs released by a good mature female are usually ready to be fertilized immediately. Both meiotic divisions and maturation occur in the ovary, after which the polar bodies are lost. Sometimes, females may also release immature eggs, which are easily recognizable by the large nuclei present in the primary oocytes (germinal vesicle). The eggs of *P. lividus* have a diameter of about 90-100 μ. The mature egg shows a transparent gelatinous layer with a thickness of about 30 μ. The vitelline membrane is situated under the gelatinous layer, too thin to be visible on the surface of the egg, but visible when it is lifted from the surface at the time of fertilization. The cortex is below the vitelline membrane, which contains characteristic granules with a diameter of 0.5-0.8 μm. The cytoplasm contains spherules of an oily substance, mitochondria and yolk granules. Generally, the rate of cleavage and development are temperature-dependent. At 20° C, zygotes of *P. lividus* reach the first division in about an hour. **Figure 1.26** reports in detail the first cell divisions and following morphogenetic movements, leading to the formation of the pluteus (Ahmed et al., 2016).

Although sea urchins are often primarily herbivorous, they basically are omnivores and can consume a great variety of foods. Ingestion is an important aspect of nutrition (Zupi and Fresi, 1984). The amount and frequency of food ingestion by sea

urchins is affected by their physical and chemical characteristics, the physiological state of the individuals, and also abiotic environmental conditions.

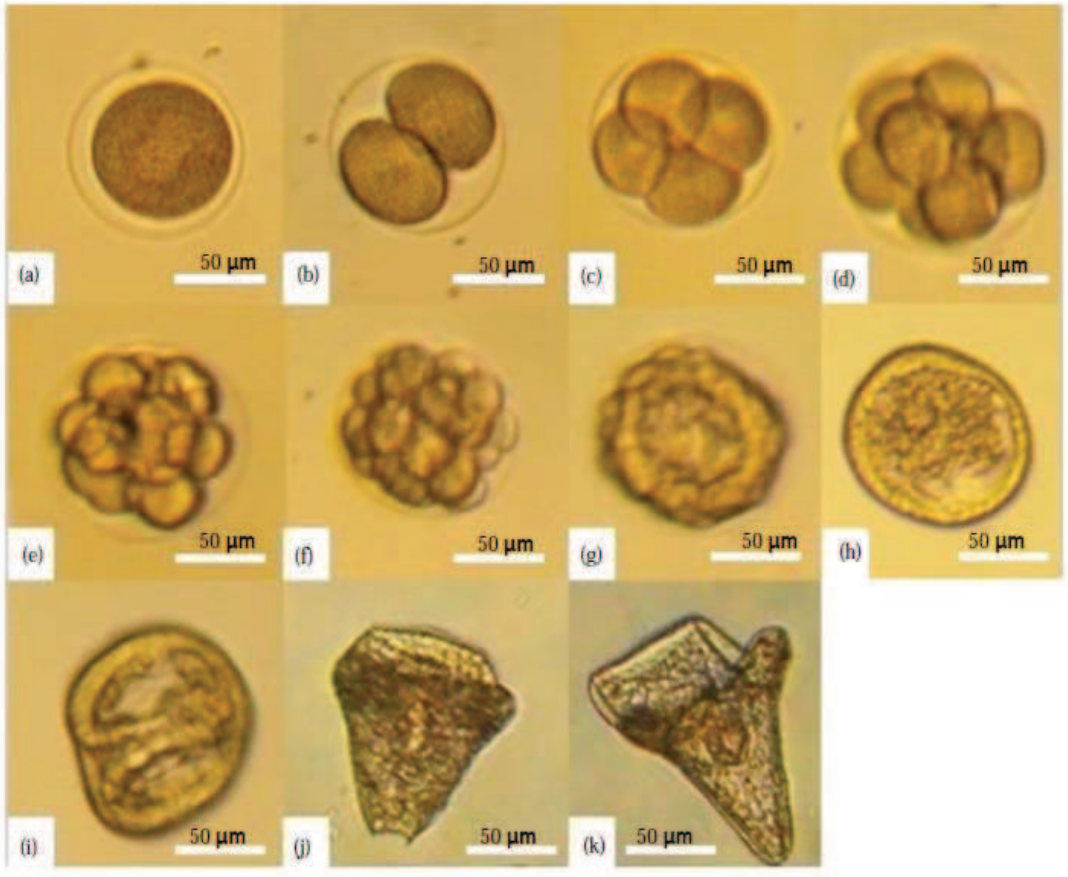


Figure 1.26. Series of developmental images from fertilized egg until full rudiment formation: (a) fertilized egg, (b) 2-cell stage, (c) 4-cells stage, (d) 8-cells stage, (e) 16-cells stage, (f) 32-cells stage, (g) morula stage, (h) blastula, (i) gastrula stage, (j) prism stage and (k) pluteus stage. Scale bar, 50 µm (From Ahmed et al., 2016).

The amount and frequency of food ingestion by sea urchins is affected by their physical and chemical characteristics, the physiological state of the individuals, and also abiotic environmental conditions. The response of animals to the chemical composition of food that results in consumption is complex and has several stages (Lindstedt, 1971).

An animal may be attracted to food but not stimulated to eat it or may be incited to eat

food but deterred from ingestion. These stages are not always explicitly recognized in studies on feeding in sea urchins. Attraction and ingestion are the usual means of evaluating the response of sea urchins to food. Klinger and Lawrence (1984) suggested that differences in consumption of foods by *Lytechinus variegatus* are determined primarily by degree of persistence in feeding and not by selection of food.

Most interest has focused on feeding deterrents as chemical defences of plants to sea urchins (Hay, 1988; Hay and Fenical, 1992). Extracts of the seagrass *P. oceanica*, which contains phenol, deterred feeding in *Sphaerechinus granularis* and also, to a lesser extent, in *P.lividus* and *Arbacia lixula* (Vergés et al., 2007a). Vergés et al. (2011) reported that these phenolic compounds are more concentrated in young leaves of *P. oceanica*. In analogy, the more apical and younger areas of the thallus of the alga *Avrainvillea elliottii* have greater deterrence to consumption by *Lytechinus variegatus* (Lima et al., 2008).

Deterrent compounds do not necessarily prevent ingestion. Irvine (1973) reported *Strongylocentrotus droebachiensis* and *Strongylocentrotus franciscanus* consumed *Nereocystis luetkeana* and *Agarum* spp. but not the sulfuric acid-containing alga *Desmarestia viridis* when all algae were provided in the laboratory. When *D. viridis* was the only alga provided, *S. franciscanus* never consumed it but *S. droebachiensis* began consuming it after several days and had eroded teeth after several months.

Food consumption is related to reproductive state. Fernandez and Boudouresque (1997) noted the inverse relation between the repletion index (amount of food in the gut) and the gonad index they found for *P. lividus* contrasted to Régis (1979) report of a parallel pattern of seasonal variation in gut contents and gonad size. Schlosser et al. (2005) also

found an inverse ratio between the size of the gonad and the number of algae consumed by *P. lividus*.

The stomach (first loop) is the primary site of production of digestive enzymes and the intestine (second loop) is the primary site of uptake of nutrients. Almost all studies on digestive enzymes in sea urchins concern carbohydrates, such as amylase, agarase, glycogenase activities (Lawrence et al., 2013). Klinger (1984) concluded that sea urchins have carbohydrases that are broadly appropriate for herbivorous diets. This does not include a great capacity for digestion of structural carbohydrates, since they display a minimal cellulose activity on native cellulose. Certainly, they can hydrolyse water-soluble polysaccharides and oligosaccharides, but it is possible these must be made available by mechanical disruption of cell walls or other hydrolytic origin. Fewer data exist for proteases and lipases in sea urchins. Trenzado et al. (2012) found that trypsin, lipase and total and acid proteolytic activity was higher in *Arbacia lixula* than in *P. lividus* and *Sphaerechinus granularis* while chymotrypsin activity did not differ among the three species. Their conclusion was that this profile was correlated with the primarily animal diet of *A. lixula* and that nutritional condition has an effect on enzymatic activity.

For more than a century sea urchins have been widely used as model organisms for scientific research to understand many biological processes. In fact, it represents one of the main systems on which the history of developmental biology was built. The first discoveries on fertilization and parthenogenesis are described by Hertwig in 1875. Starting with those studies a wide array of new knowledge on fertilization, molecular, physiological and cellular biology mechanisms have been acquired, using the sea urchin. These processes included gamete formation, recognition and fusion, reinitiation of the

egg's metabolism, blockage of polyspermy, and cytoskeletal and motility events, as well as the changes in the pronuclei which permit syngamy and the activation of new gene expression (Ernst, 2011; Sugni et al., 2013). Furthermore, sea urchins represent an optimal model from the ecological perspective, because they are a key herbivore for their major role in structuring and controlling macroalgae assemblages, and thus in shaping the benthic seascape (Boudouresque and Verlaque, 2001; Uthicke et al., 2009; Sala et al., 2012). These organisms also represent valuable bioindicators for detecting environmental perturbations (Dinnel et al., 1988; Sconzo et al., 1995; Morale et al., 1998; Matranga et al., 2000). Among the echinoderms, the sea urchin *P. lividus* is considered a suitable organism to study the eco-toxicological response of marine invertebrates to environmental pollutants. It represents a prime candidate for model toxicological test organisms in the marine ecosystem for several reasons: it is an important component of benthic marine communities in the Mediterranean Sea and Atlantic Ocean; the extraction and maintenance of gametes are easy; the embryos grow rapidly and synchronously (pluteus stage at 48 hours post fertilization); the embryos are transparent and therefore suitable for microscopic detection of sub-lethal effects of pollutants on normal development; they have a long reproductive period (from October to May). For these reasons the sea urchin represents a good model for eco-toxicological studies on the response of marine invertebrates to environmental pollutants, such as physical and chemical xenobiotics (Zito et al., 2005; Kobayashi and Okamura 2005; Bellas 2008; Bošnjak et al., 2010; Pinsino et al., 2010; Bonaventura et al., 2011).

1.4 Aims of the thesis

Diatoms are unicellular algae playing a key role as photosynthetic organisms in the world's ocean food webs. As extensively reported in Paragraph 1.2, many planktonic diatoms have been discovered to produce a wide range of oxygenated fatty acid derivatives (called oxylipins; Fontana et al., 2007a,b; Cutignano et al., 2011), affecting diatom growth (d'Ippolito et al., 2009; Gallina et al., 2014) and marine invertebrates reproduction and development, such as copepods (Miralto et al., 1999; Ianora et al., 2004; Fontana et al., 2007b; Lauritano et al., 2012, 2015; 2016), sea urchins (Romano et al., 2010, 2011; Varrella et al., 2014, 2016a,b; Ruocco et al., 2016), sea stars (Caldwell et al., 2009), polychaete worms (Simon et al., 2010) and ascidians (Caldwell et al., 2009; Pohnert and Boland, 2002). In addition, very recently, it has been demonstrated that a planktonic diatom, *S. marinoi*, releases StS capable to induce apoptosis in diatom cells (Gallo et al., 2017). Contrary to the well-documented chemical ecology of planktonic diatoms, few studies have reported on the chemical ecology of benthic species (Juttner et al., 2010; Pezzolesi et al., 2017), due to difficulties in their collection, quantification and massive culturing (Simental et al., 2001).

In this context, the present Ph. D. project aims to shed light on the possible toxigenic effects of benthic diatoms upon grazing activity. In particular, this research project represents a new initiative, because for the first time the effects of four benthic diatoms have been studied on the reproductive success of the sea urchin *P. lividus* (used as model organisms) by using multidisciplinary approaches. Firstly, four benthic diatoms have been isolated from the leaves of the seagrass *P. oceanica* (which represents a common food item for this species), in collection sites where they usually

live as epiphytes. Then adult sea urchins were fed on these diatoms and the effects of these diets have been studied from the morphological, metabolomic and molecular points of view. In addition, since there is very little information available on chemical compounds from benthic diatoms, further studies were necessary to identify putative compounds responsible for the possible toxic effects on sea urchins. The project focuses on the following issues:

1) Morphological effects of feeding experiments with benthic diatoms on adults and on developing embryos of the sea urchin *P. lividus*.

i) Setting up of microcosms for feeding experiments.

An open (flow-through) system was set up in the Animal Facility of the Stazione Zoologica Anton Dohrn, in which sea water was directly pumped from the sea (at 250 m off the coast and at a depth of 12 m). Feeding experiments in these microcosms had the aim to bring ecologically relevant components of the natural environment under controlled conditions (Watts and Bigg, 2001).

ii) Definition of daily feeding rate of the sea urchins *P. lividus*.

Adults of the sea urchin *P. lividus* were fed for three months on the green macroalga *Ulva rigida*, the seagrass *P. oceanica* and a fish pellet used in aquaculture. This investigation aimed at defining the feeding rates of *P. lividus* reared in test tanks, according to the feeds used, and to measure the effects of feeds on its body growth and the maturation of gonads, as compared to the well-known patterns observed in the environment. These data were useful to define the quantity of benthic diatoms to culture in the laboratory for feeding experiments.

iii) Feeding experiments with benthic diatoms and morphological analyses.

Four benthic diatoms were firstly characterized by SEM observations and rRNA 18S PCR analysis: two abundantly occurring benthic diatoms, *Nanofrustulum shiloi* and *Cylindrotheca closterium* and two slow-growing species *Cocconeis scutellum* and *Diploneis* sp. These diatoms have been considered quite interesting from the ecological point of view because they display different behaviours in the marine environment. In fact, *N. shiloi* and *C. closterium*, also called meroplanktonic diatoms, exploit a half-planktonic and half-benthic existence since they are readily transported by wave energy into the water column where they survive and then they come back to the sediments under calm conditions. On the contrary, *C. scutellum* and *Diploneis* sp. are strictly attached to the substrate and may dominate benthic environments in the case of high nutrient abundance and optimal light.

For feeding experiments, benthic diatoms were grown until the end of the exponential phase, using 2% agar as substrate. Adults of the sea urchin *P. lividus* were also fed on the fresh green alga *U. rigida*, which represent natural dietetic items and are characteristic of environments populated by *P. lividus*. After one month of feeding performed in the open (flow-through) system described before, morphological analysis (by microscopic observation) on embryos spawned from adults fed on these benthic diatoms has been performed in comparison with those fed on *U. rigida*.

2) Metabolomic effects on gonads from adult sea urchins fed for one month on four benthic diatoms

Gonad tissues were collected from five adult sea urchins after one month of feeding with the four benthic diatoms and the control *U. rigida*. Gonads were re-suspended in methanol and sonicated, and then the aqueous (polar) and lipophilic (apolar) phases were collected separately and analysed using Nuclear Magnetic Resonance (NMR). This analysis revealed the possible changes in gonad metabolites induced by benthic diatom feeding, such as lipids and amino acids.

3) Molecular analyses on embryos from adult sea urchins fed diatom diets

Since the genome of the sea urchin *P. lividus* is still not available, high-throughput *Next Generation Sequencing* (NGS) studies have been applied to identify target genes affected by these benthic diatoms. In particular, an *RNA sequencing* (RNA-Seq) approach has been used to monitor differentially expressed genes induced by feeding experiments. In the RNA-Seq method, complementary DNAs (cDNAs) generated from the RNA of interest are directly sequenced using next-generation sequencing technologies. The high-quality reads obtained have been aligned in order to construct a whole-genome transcriptome map, providing a far more precise measurement of levels of transcripts and their isoforms than other methods. Moreover, this sequencing technology and assembly algorithms have facilitated the reconstruction of the entire transcriptome even without a reference genome. To this aim, eggs and sperms have been collected from sea urchins fed with benthic diatoms under analysis, and then fertilized. Samples were collected at the pluteus stage

(embryos at 48 hours post fertilization). Different RNA extraction protocols have been tested and compared to obtain a high-quality RNA extraction for high-throughput RNA-Sequencing Then *de novo* transcriptome analysis has been performed on sea urchin plutei from adult sea urchins fed on the analysed benthic diatoms in comparison with the control. Furthermore, the variation in expression levels of fifty genes has been followed by *Real Time qPCR*: these genes have key roles in different functional processes (such as stress, development, differentiation, skeletogenesis and detoxification), previously identified and analysed in response to oxylipin treatments.

4) Chemical analysis of benthic diatoms.

Since there is very little information available on chemical compounds from benthic diatoms, further studies were necessary to identify possible putative compounds responsible for the toxic effects on sea urchins. Preliminary investigations have been performed to test if these diatoms, as in the case of planktonic species, produce PUAs and/or other oxylipins through GC-MS (Gas Chromatography-Mass Spectrometry) and LC-MS (Liquid Chromatography Mass Spectrometry), respectively. Moreover, StS content has also been evaluated through LC-MS.

Concluding, this study has added new information on the effects of benthic epiphytic diatoms on their grazes, such as sea urchins. Moreover, this Ph. D. project has also advanced current knowledge to better understand chemical defence mechanisms that benthic species employ in response to predators living at the bottom of the sea.

All Ph. D. results also open the door to new challenges by looking at the potential of benthic diatom-derived compounds as possible sources of new marine natural compounds for biotechnological applications in pharmacological, cosmeceutical and nutraceutical fields.

2. Setting up of a microcosm for feeding experiments and definition of daily feeding rate of the sea urchin *Paracentrotus lividus*

2.1 Introduction

The first part of this Ph. D. project was focused on experimentally defining the feeding rates of the sea urchin *Paracentrotus lividus*, used as a model organism, and the best diet to use as a control in comparison with the tested benthic diatoms. In order to reach this aim, the first step consisted in the setting-up of a microcosm, in which feeding experiments were performed on the sea urchin *P. lividus*. In particular, this system had the aim to mimic natural conditions bringing ecologically relevant components of the natural environment under controlled conditions. Furthermore, the trophic ecology of the sea urchin *P. lividus*, a key species in several shallow benthic communities, has been intensively studied, but the role of various foods in the processes of growth and gonadal maturation is still scarcely understood. This research assessed the effects of two fundamental food items for wild specimens of the sea urchin *P. lividus*, the tissues of the seagrass *Posidonia oceanica* and of the green alga *Ulva rigida*, compared to the effect of a commercial compound feed on the somatic growth, gonad development and maturation, fertilization success and post-embryonic development, as compared to the well-known patterns observed in the environment (Vadas et al., 2000). More specifically, fresh *U. rigida* and *P. oceanica* represent natural dietetic items, also because both these plants characterize environments usually populated by *P. lividus*. In fact, this sea urchin is one of the major macro-herbivores in the Mediterranean Sea

eating a range of red, green and brown algae in addition to seagrass (Boudouresque and Verlaque, 2001). *U. rigida* is considered a control food in several feeding experiments on fish and invertebrates (Valente et al., 2006) and it is included in various diets for sea urchins (Frantzis and Grémare, 1993), demonstrating that it affected the growth rate of *P. lividus* (Frantzis et al., 1992). Moreover, *P. lividus* represents the dominant grazer for *P. oceanica*, choosing this seagrass because of the greater availability of shelter and food in the seagrass (Pinna et al., 2012). It prefers leaves covered with epibiota and adult, thicker leaves (Vergés et al., 2011), consuming all parts of the seagrass as a "preferred" item for feeding during spring and summer (Boudouresque and Verlaque, 2001). In fact, the sea urchin *P. lividus* is one of the main consumers of *P. oceanica* (Verlaque, 1987), avoiding other species that synthesize toxic or repellent secondary metabolites (Guerriero et al., 1992; Lemée et al., 1996; Tejada et al., 2013). Moreover, *P. lividus* is a key species that controls the dynamics of seaweeds and seagrasses, by eliminating, when at high densities, the erect stratum of algae and seagrasses (Sala and Zabala, 1996). Sea urchins were also fed with a pre-hydrated pelletized formulated feed (Classic K®; Hendrix SpA, Mozzecane-VR, Italy). Previous data demonstrated that these commercial pellets provided rapid fattening of gonads (Fabbrocini and D'Adamo, 2010, 2011; Fabbrocini et al., 2012), representing a positive control on the size and quality of gonads. It is also inexpensive, available on the market and producing negligible amounts of wastes (Fabbrocini et al., 2012).

This preliminary test can be considered useful for the definition of optimal diets for the production of mature broodstocks of an ecologically important marine model organism.

All the results reported in this Chapter have been published in Ruocco et al., 2018b (Appendix B) and Zupo et al., 2018 (Appendix B).

2.2 Materials and methods

2.2.1 Ethics Statement

Wild individuals of *P. lividus* (Lamarck) were collected from a site in the Bay of Naples that is not privately-owned or protected in any way, according to the Italian legislation (DPR 1639/68, 09/19/1980 confirmed on 01/10/2000). Field studies did not include endangered or protected species. All experimental procedures on animals were in compliance with the welfare guidelines of the European Union (Directive 609/86).

2.2.2 Sea urchin collection

Adult sea urchin *P. lividus* were collected in January (corresponding to the beginning of the reproductive cycle; Byrne, 1990) by scuba-divers in the site "Rocce Verdi" in the Gulf of Naples, Italy. Adult individuals of an average weight of 40 g were collected and immediately transported to the laboratory, using a thermically insulated box containing seawater. Further, they were transferred to plastic tanks with recirculating seawater prior to starting the feeding experiments. Sea urchins were individually measured using a calliper, to record the maximum horizontal diameter of thecae; adult specimens with a diameter between 4 and 5 cm (excluding the spines), that is a typical diameter for mature adults, were selected for the experiments.

2.2.3 Experimental rearing apparatus

A continuous open flow-through (35 L per hour) system (**Figure 2.1-2.2**) was set up, consisting of rectangular glass tanks (three tanks for each diet used; chamber size 30x35x40 with 35 L of sea water). In this system, seawater is taken from the sea (in the Gulf of Naples, just in front of the Stazione Zoologica Anton Dohrn, at a distance of 250 m and depth of 12 m) and collected in an outdoor basin (capacity = 140 m^3). Water is then filtered twice on gauze filters (200 µm) and moved to an indoor basin (capacity = 700 L), filtered again by means of a protein skimmer, a UV sterilizer, a refrigerator and a mechanical filter, and then pumped to the experimental tanks. The aeration in the tanks was provided by airstones. Waste water was released through outflow tubes from each tank at a rate of one full change per hour.

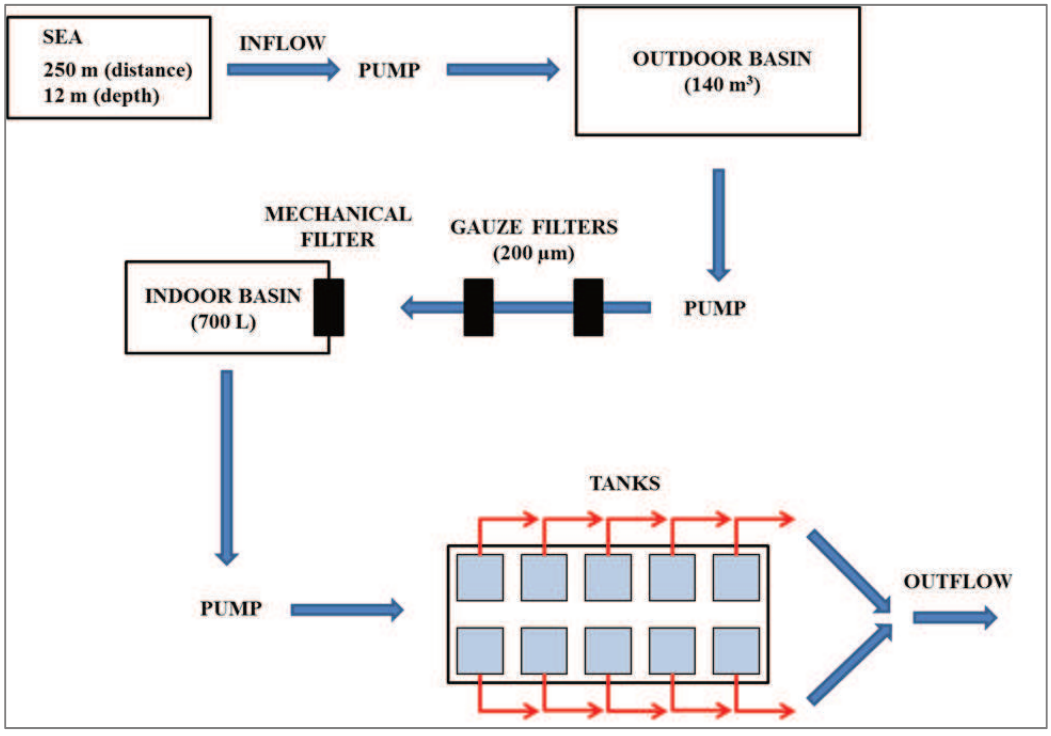


Figure 2.1. Schematic representation of the open flow-through system.

The main abiotic parameters were recorded three times a week using a multiparametric probe (YSI 85, YSI, Incorporated) and kept constant using water chillers, circulation pumps and filters (temperature: 18 ± 1° C; salinity: 38 ± 1; dissolved O_2: 7 mg/L; pH: 8.1). The internal surface of the tanks was manually cleaned of their epiphytes and fouling organisms three times a week, using synthetic sponges and scrapers.

Figure 2.2. Picture of the experimental tanks in the SZN (Stazione Zoologica of Naples) Animal Facility.

2.2.4 Feeding experiments

Preliminary experiments were performed to define the daily feeding rates of adult sea urchins. Ten adults of *P. lividus* (ten females and ten males for each diet treatment were identified according to the morphology of the apical system of females and the different genital plates between females and males under the dissecting microscope; Philip and Foster, 1971; Jeffery and Emlet, 2003) were reared in each tank of the continuous open flow-through system, and then fed with 10, 20 and 30 g wet weight (ww) per day of the green macroalgae *U. rigida* (basically used as control feed for sea urchins laboratory experiments) to define their daily feeding rate. *U. rigida* was chosen since it is

considered among the most palatable and nutritionally suitable feeds for *P. lividus* (Hiratsuka and Uehara, 2007; Cyrus et al., 2015) and therefore, its consumption is close to the maximum feeding rates for this species. Additional feeds, *P. oceanica* and pre-hydrated formulated feed (pre-hydrated 'Classic' formulated feed, HENDRIX SpA, Mozzecane, Verona, Italy), were provided at the same rate and the presence of small residuals of all the feeds was checked prior to starting the experiments. Food consumption was measured after 24, 48 and 72 hours to check that residuals were still present, for all the items, after 1 day and that they could be still consumed in further days. Doses were set in order to assure that the residual food was maintained in low abundance, to avoid water pollution and lixiviation (Sartori and Gaion, 2016). Once this initial dose was determined, guaranteeing an ad libitum consumption in 24 h on all items considered, feeding experiments were initiated.

Twenty adult *P. lividus* (ten females and ten males for each diet treatment) were reared in each experimental tank and fed, alternatively, with 20 g per day of a) fresh *U. rigida*, b) fresh *P. oceanica* leaves (including both brown and green tissues) and c) pelletized (2.5 x 2.5 x 5 mm) pre-hydrated formulated feed (Classic K; HENDRIX SpA, Mozzecane, Verona, Italy), a commercial food characterized by a high protein content 465 g/kg, proteins of animal origin accounting for < 50 g/kg) as suggested by Fabbrocini and D'Adamo (2010). Before starting experiments, a three-day time frame of starvation was considered. The short acclimation and starvation time was chosen because the aim was to study the effects of three different feeds on gonads maturation and GI, starting from the gonadic state of experimental sea urchins at the moment of the collection. A longer starvation time would produce auto-digestion of gonadic tissues to sustain the

metabolism of starved animals (Sartori et al., 2015) and this was in contrast with the experimental purposes.

The residual amount of food in each tank was weighed (fresh weight) every day to calculate the individual daily food consumption. Since the feeds were replaced daily and were mainly ingested by sea urchins in the first hours after the administration, the influence of lixiviation processes in the formulated feeds was excluded. Excess moisture was removed from *U. rigida* by blotting the leaves on paper towels before weighing. The total food daily ingested was calculated as the difference between the introduced feed and that removed in each tank. Food intake was calculated in mg of feeds (dry weight) consumed per animal, per day. Since adult sea urchins were grouped in a tank for each diet, an average consumption was also evaluated.

2.2.5 Carbon and nitrogen measurements in feeds and faecal pellets

Additional samples of algae and seagrass tissues were collected and stored for chemical analyses, in order to define the quality of the fresh feeds provided. *U. rigida* thalli and *P. oceanica* leaves were collected in the Gulf of Naples by scuba-divers, transferred to the laboratory and stored at -20° C. Samples were subsequently dried at 65° C for three days up to constant weight. *P. lividus* faecal pellets were also collected in the experimental tanks. Since a short starvation period has been used (as described above) and considering that faecal material is still produced for a large number of days after food intake has ceased, faecal pellets were collected fifteen days after the beginning of the feeding experiments, in order to be sure that gut retention time would not affect this analysis. These faecal pellets were dried up to constant weight, as described above. Dry

samples were homogenized in a grinder in order to obtain a thin dust. About 3 mg for each sample were loaded into a CN (Carbon and nitrogen) Analyzer (FlashEA 1112 Automatic Elemental Analyzer, Thermo Scientific Waltham, MA, USA), following the procedure described by Hedges and Stern (1984). Acetanilide was used as standard. Carbon and nitrogen contents, measured by CN Analyzer, allowed the calculation of C/N ratios. Analyses were conducted in triplicates.

2.2.6 Gonadal index and histological preparation

Evaluations of GI were performed on twenty field-collected adult specimens of the sea urchin *P. lividus* (t_0) and compared with twenty specimens fed for one month and three months on each of the three feeds, *U. rigida*, *P. oceanica* and formulated pellets. These sea urchins were weighed, sacrificed and dissected; their gonads were extracted and weighed for the evaluation of the gonadal index (GI):

GI = gonadal wet weight (g)/sea urchin wet weight (g) × 100 (Sánchez-España et al., 2004; Fabbrocini and D'Adamo, 2011; Keshavarz et al., 2017).

The gonads of three males and three females for each treatment were dissected, fixed in Bouin, included in paraffin blocks, sliced and stained by hematoxylin, to evaluate the histological structure of tissues (Byrne, 1990). The slices, after staining, were enclosed into permanent mountings and observed under the optical microscope.

2.2.7 Gamete collection, embryo culture and morphological analysis

After three months of feeding, adult *P. lividus*, reared in the experimental tanks, were injected with 2 mL of 2M KCl through the peribuccal membrane to trigger the release of

gametes. Eggs were immediately washed with filtered seawater (FSW) and kept in FSW until use. Concentrated sperm was collected and kept at 4° C until use. Eggs were fertilized in FSW (400 eggs in 3 mL of FSW), utilizing sperm-to-egg ratios of 100:1 (Romano et al., 2011).

Fertilized eggs were incubated at 20° C in a thermostatic chamber with 12:12h light:dark cycle. These experiments were conducted in triplicates.

Percentages of fertilization, first cleavage (at about 1 hour post-fertilization, hpf) and normal and malformed plutei (48 hpf) were evaluated for at least 200 plutei from each female (fixed in formaldehyde 4% in FSW) using a light microscope (Zeiss Axiovert 135TV; Carl Zeiss, Jena, Germany).

2.2.8 Statistical analyses

The statistical significance of differences among daily feeding rates recorded according to the three feeds, and C and N concentrations in feeds and faecal pellets were evaluated by the average and variation of the data reported as 'mean ± standard deviation (SD)'. Standard deviation bars were plotted in order to allow an immediate perception of the intervals of superimposition of our replicates. Statistical significance of differences between individual treatments was evaluated using unpaired *t*-tests (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com). P values < 0.05 were considered statistically significant.

2.3 Results

2.3.1 Daily feeding rate

Preliminary experiments were performed to define the daily feeding rates of adult sea urchins. Ten adults of sea urchins *P. lividus* were reared in each tank of the continuous open flow-through system and then fed with 10, 20 and 30 g (ww) per day of the green macroalga *U. rigida* to approximately determine the daily sea urchin feeding rate. Food consumption was measured after 24, 48 and 72 h. Food consumption was higher in the first 24 h with significant differences between 10 g group versus 20 g (p value = 0.047) and 30 g (p value = 0.0012) groups (**Figure 2.3**).

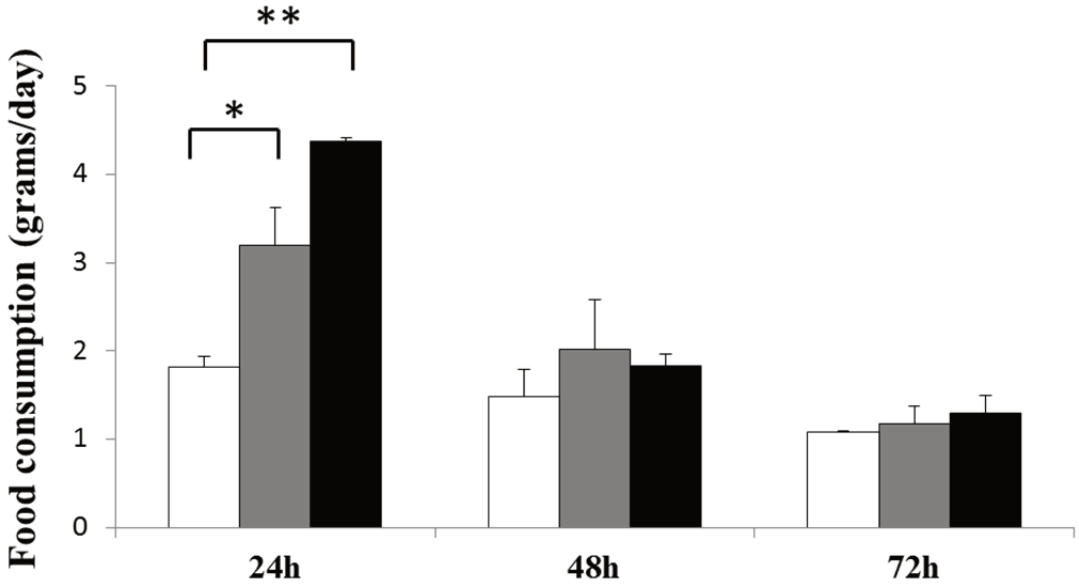


Figure 2.3. Ten adults of sea urchins *P. lividus* were fed for three days with 10 g (white bars), 20 g (grey bars) and 30 g (black bars) (ww) per day of the green macroalga *U. rigida*. Data are reported as means ± SD (N=3). Significant differences were detected between 10 g versus 20 g and 30 g; *p<0.05, **p<0.01, ***p<0.001 (Unpaired *t*-tests).

At 48 h the food consumption decreased, but differences were not significant among the three quantities; at 72 h, the daily feeding rates stabilized on an average of about 1 g (ww) per sea urchin, independently of the initial quantity of *U. rigida* administered.

According to these preliminary experiments and in order to compare the daily consumption among different feeds, sea urchins were fed with 20 g (ww) per day of *U. rigida* and *P. oceanica*. In the case of formulated pellets, ad libitum feeding was 1.8 gr/day per twenty sea urchins, about 0.1 g/animal (dry weight, dw).

After three months of treatment, the daily feeding rate (as dw) per animal corresponded to 0.16, 0.15 and 0.10 g for *U. rigida*, *P. oceanica* and formulated pellets, respectively (**Figure 2.4**).

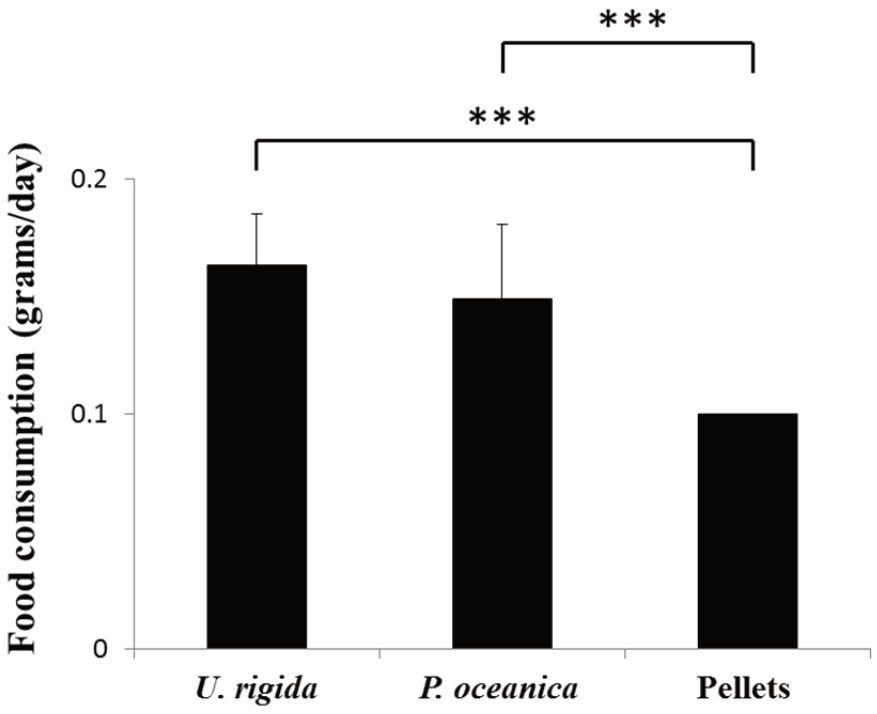


Figure 2.4. Daily feeding rate. The histogram shows the daily feeding rate per animal after feeding for one month with *U. rigida*, *P. oceanica* and the pre-hydrated 'Classic' formulated feed. Data are reported as means ± SD (N=3). Differences were considered statistically significant between formulated pellets versus *U. rigida* and *P. oceanica*; *p<0.05, **p<0.01, ***p<0.001 (Unpaired *t*-tests).

Differences in individual daily feeding rates were not significant between treatments *U. rigida* and *P. oceanica* (p value = 0.06). In contrast, the daily feeding rates measured for formulated pellets were significantly lower in comparison to *U. rigida* and *P. oceanica* (p value < 0.001).

2.3.2 Carbon and nitrogen contents of feeds and faecal pellets

The amounts of carbon (C) and nitrogen (N) measured by CN Analyzer in the three feeds (namely, *U. rigida*, *P. oceanica* and formulated pellets), were compared with those measured in sea urchin faecal pellets (**Table 2.1**).

Table 2.1. Composition in percentage (%) of nitrogen (N) and carbon (C) of the three feeds, *U. rigida*, *P. oceanica* and formulated pellets and the faecal pellets (indicated as FP) collected from sea urchins fed with the three corresponding feeds. C/N ratio has also been reported both in the three feeds and in the correspondent fecal pellets. Percentage data are reported as means ± SD (N=3).

	Composition (%)				C/N ratio	
	Feed		FP		Feed	FP
	N	C	N	C		
U. rigida	2.19 ± 0.07	26.00 ± 0.99	1.54 ± 0.06	15.87 ± 0.65	11.85	10.27
P. oceanica	2.40 ± 0.009	36.64 ± 0.12	1.02 ± 0.04	30.94 ± 0.18	15.23	30.09
Pellet	5.35 ± 0.1	44.10 ± 0.1	1.82 ± 0.05	22.39 ± 0.6	8.67	12.25

The C/N ratio was highest in *P. oceanica* (15.2) respect *U. rigida* (11.9) and formulated pellets (8.7) (**Figure 2.5A**). Statistically significant differences in the C/N ratios were found between *U. rigida* and *P. oceanica* (p value < 0.05), as well as between *U. rigida*

and formulated pellets (p value < 0.05). The difference is highly significant between *P. oceanica* and pellets (p value < 0.001).

Concerning faecal pellets, *P. oceanica* exhibited a higher C/N ratio (30.1) with respect to *U. rigida*. This value decreased in the pellets (12.2) reaching the lowest value in *U. rigida* (10.3) (**Figure 2.5B**).

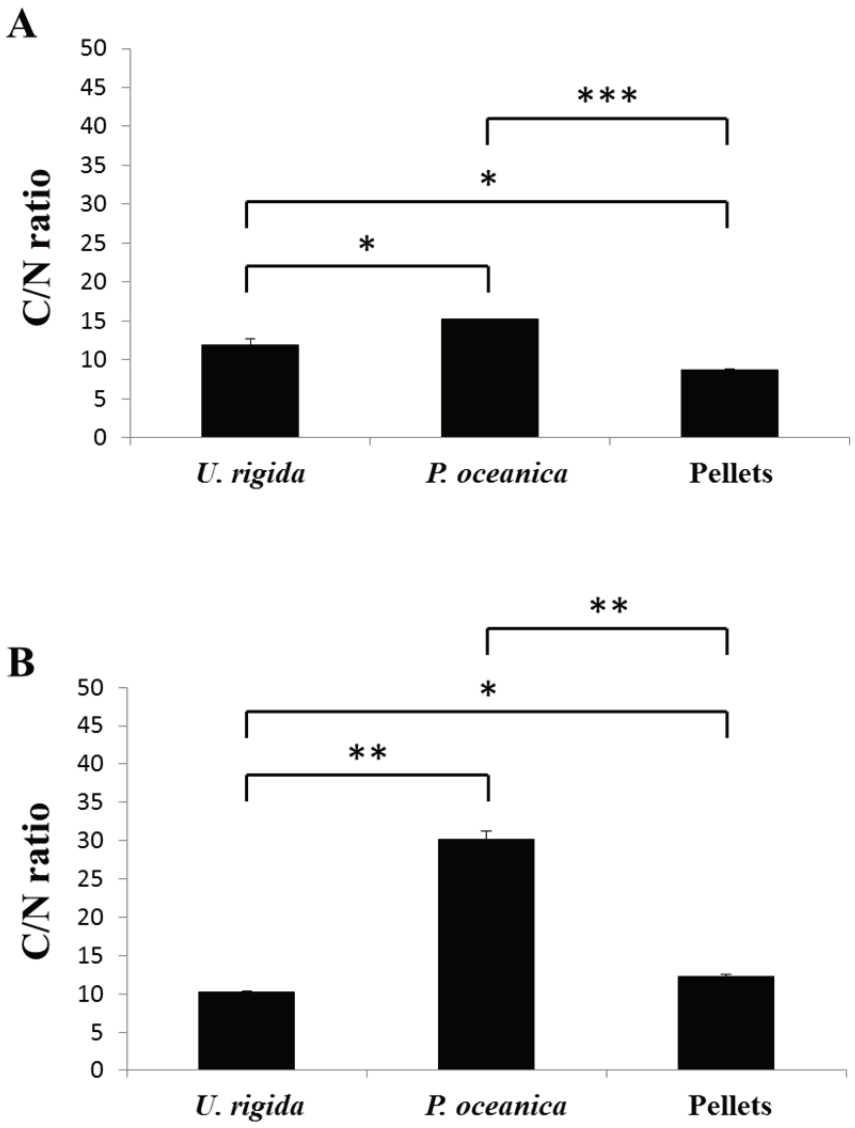


Figure 2.5. C/N ratios measured by CN Analyzer in the three feeds (A) and faecal pellets (B) collected from fed sea urchins. Data are reported as means ± SD (N=3). Statistically differences between the groups were evaluated by Unpaired *t*-test analyses (*p<0.05, **p<0.01, ***p<0.001).

The differences in C/N ratios were significant between *U. rigida* and both *P. oceanica* (p value = 0.03) and pellets (p value = 0.0274). Moreover, the difference is highly significant between *P. oceanica* and pellets (p value < 0.001).

2.3.3 Adult growth and gonadal index (GI)

No significant differences in growth rates were found among adult sea urchins fed for one month with *U. rigida*, *P. oceanica* and pellets in comparison to adults collected in the field at the beginning of the experiment (t_0; **Table 2.2**).

Table 2.2. Adult sizes (mm) and GI measures from adult sea urchins *P. lividus* collected in the field at the beginning (t_0) and after one/three months of feeding experiments with *U. rigida*, *P. oceanica* and formulated pellets (p value > 0.05 for adult sizes after one/ three months of feeding, and GI after one month of feeding; p value < 0.01 for GI after three months of feeding in sea urchins fed with formulated pellets, in comparison with those fed with *U. rigida* and *P. oceanica* (Unpaired *t*-tests). Data are reported as means ± SD, N = 20/group.

		U. rigida	*P. oceanica*	Pellet
Adult size				
t_0	40.2 ± 2.26			
1 month		41.3 ± 2.17	39.9 ± 2.39	40.5 ± 2.65
3 months		41.9 ± 2.45	40.6 ± 1.90	40.9 ± 2.93
GI				
t_0	4.0 ± 0.51			
1 month		3.9 ± 0.39	4.2 ± 0.49	4.0 ± 0.60
3 months		4.2 ± 0.44	4.4 ± 0.47	14.7 ± 1.13

Moreover, no significant differences were found among the GI values of sea urchins fed for one month on *U. rigida*, *P. oceanica* and pellets in comparison with those collected in the field at the beginning of the experiments (p value > 0.05). On the contrary,

different results have been detected after three months of feeding. In fact, we observed a high significant increase in the GI values in sea urchins fed with formulated pellets, in comparison with those fed with *U. rigida* and *P. oceanica* (p value = 0.005). In **Figure 2.6** has been reported an example of gonad from sea urchins fed with formulated pellets in comparison with those from adults fed with *U. rigida* after three months of feeding experiments.

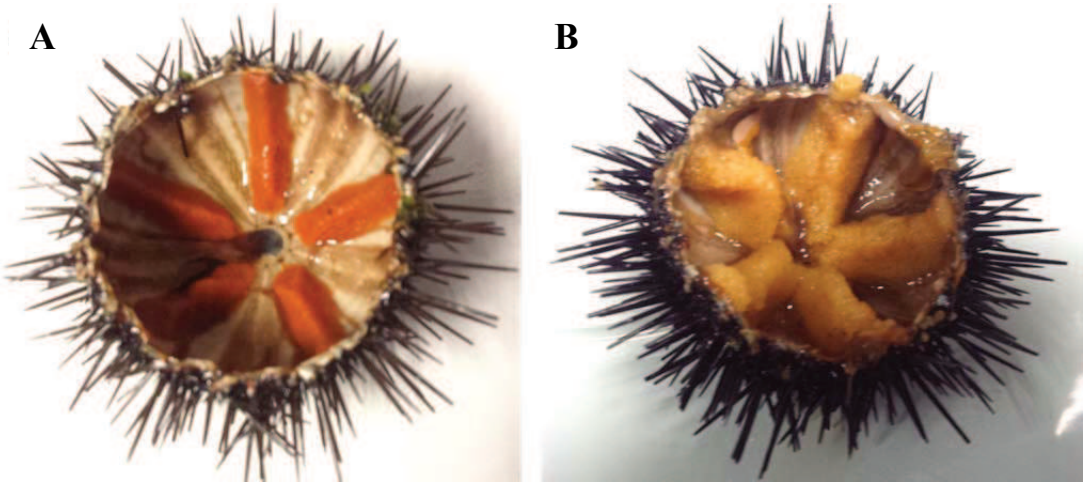


Figure 2.6. Sea urchin *P. lividus* gonads from an individual (reported as example) fed with *U. rigida* (A) and formulated pellet (B).

2.3.4 Fertility of sea urchins

Gametes were collected only from those sea urchins in the replicates fed on *U. rigida* and *P. oceanica* (see paragraph 2.2.7). In the case of sea urchins fed on formulated pellets, after one month of feeding gametes were present only in five individuals (three females and two males); after three months gametes were absent in all the individuals under this treatment. As soon as the fertilization occurred, was measured the fertilization success and the first mitotic cleavage (1 hpf) to obtain two blastomeres. Then the embryonic development has been followed until the pluteus stage. Fertilization and first

cleavage were obtained in 100% of gametes produced by individuals under treatments with *U. rigida* and *P. oceanica*. Morphological observations of the only three females producing gametes revealed that a one month feeding on formulated pellets induced an increase in the percentage of abnormal embryos (20%) with respect to *U. rigida*, *P. oceanica* and t_0 (about 10%, p value = 0.04, unpaired *t*-test).

Histological observations of the gonads for treatments with *U. rigida*, *P. oceanica* and formulated pellets demonstrated a stage quite close to maturity. In particular, *U. rigida* and *P. oceanica* sections were quite similar, displaying, at the end of the treatment, ovaries in pre-mature recovering stage (**Figure 2.7A**), with oocytes still filling the center of follicular masses.

The commercial pellet treatment exhibited pre-mature ovaries as well, with a lower abundance of oocytes in the center of follicles and a few mature eggs in their peripheral areas (**Figure 2.7B**). However, in this case the gonadic tissues appeared hypertrophic and vacuolated in the cortex.

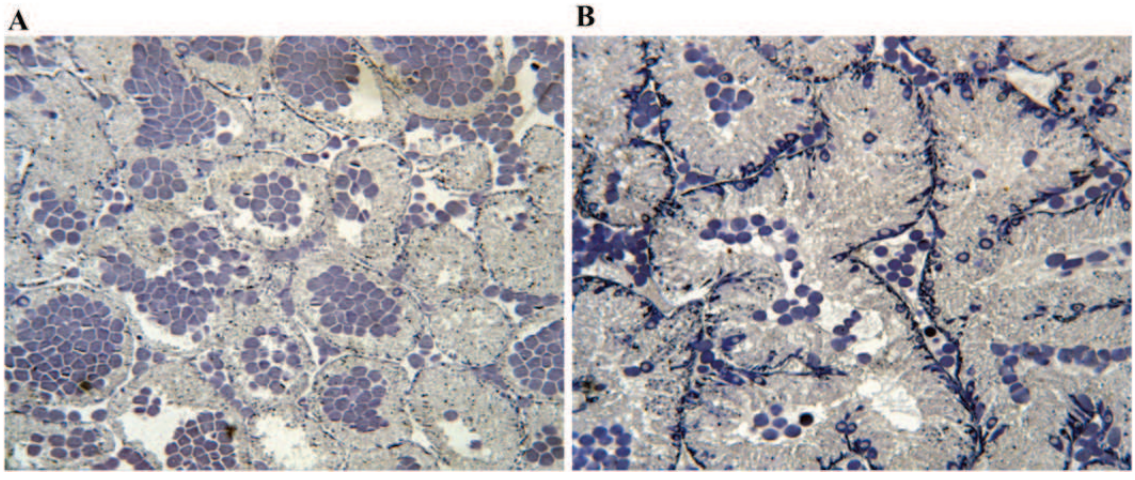


Figure 2.7. Histological sections of sea urchin *P. lividus* gonad from adult fed with *U. rigida* (A) and formulated pellets (B).

2.4 Discussion

The effect of feeding on physiological and reproductive conditions of sea urchins is essential to understand their biology and ecology and to develop novel feeds for echinoculture. However, it is interesting to observe that the feeds ingested could vary according to their quality and this study represents a confirmation of this regulation of their feeding activity. Although considered as an herbivorous species, *P. lividus* has often been classified as an opportunistic omnivore taking advantage of various food sources (Zupo and Fresi, 1984; Boudouresque and Verlaque, 2001). However, they do have preferences when presented with choice, determined by the chemical and physical characteristic of feeds. Information about nutrition, digestion and digestibility is still limited. Production of sea urchins is the result of ingestion, digestion and absorption, which have important implications for their nutrition (Boudouresque and Verlaque, 2013). Even if food is abundantly and continuously available in their own environment, sea urchins do not necessarily feed continuously. In fact, the consumption is high when food is supplied after starvation and it decreases in conditions of feeding ad libitum (Bonsdorff, 1983). Moreover, food consumption is related to the reproductive stages. Concerning the digestion system, the stomach is the primary site for digestive enzymes production, whereas the part corresponding to intestine is the leading site of nutrients uptake, although the gut is not structurally differentiated into a stomach and an intestine, but into a long digestive tube (Lawrence et al., 2013). Regional differences in digestive enzymatic activity are consistent with regional differences along the intestine tracts. For example, the “stomach” is much higher in amylase activity than the “intestine”. Almost all studies on digestive enzymes in sea urchins concern carbohydrates. In fact, many

studies have shown cellulase activity on the linear, soluble carboxymethylcellulose with minimal cellulase activity on native cellulose. Amylase also occurs in sea urchins, as well as glycogenase and agarase.

Sea urchins play an important role in shaping some coastal shallow benthic communities thanks to their grazing activity in rocky bottoms. They are recognized as being able to transform communities dominated by macroalgae into barren areas thereby reducing biodiversity, altering ecosystem functioning and regulating sea urchin population dynamics (Palacín et al., 1998; Sala et al., 1998; Barnes and Crook, 2001; Prado et al., 2007; Hereu et al., 2012). Moreover, *P. lividus* plays a central role by directly removing plant biomass (both green and brown tissues), improving nutrient export, and modifying plant production in ecosystems dominated by the seagrass *P. oceanica* (Tomas et al., 2005; Prado et al., 2007; Ruiz et al., 2009; Planes et al., 2011). At the same time, *P. lividus* is intensively exploited in many Mediterranean areas because male and female gonads are considered a delicacy (Guidetti et al., 2004; Furesi et al., 2014).

The present data show that *P. lividus* daily ingests about the same quantities of *U. rigida* and *P. oceanica* and significantly lower amounts of artificial pellets. This difference in daily feeding rates could be due to a diverse nutritional value based on a high protein and lipid content (Fabbrocini and D'Adamo, 2010). In particular, protein content of *U. rigida* is 6.64%, whereas carbohydrate is 22% and total lipid contents 12%, phenol 23% and moisture content 76%, total free amino acid 8.9%, chlorophyll a 13%, chlorophyll b 7.5% and carotenoids 4.5% (Saptati and Pal, 2011). In *P. oceanica* the total carbohydrates, proteins and lipids are, respectively, 28.98 mg/g, 607.50 mg/g and 40.50 mg/g (El Din and El-Sherif, 2013). The composition of the formulated pellets consists of

crude protein 46.5%, crude fat 10.5%, crude fiber 2.4% and ashes 9.5%, proteins of animal origin accounting for less than 5% (Mihranyan, 2010).

C/N data revealed that *P. oceanica* had the highest C/N ratio and the faecal pellets of individuals fed on its tissues are as well characterized by high C content. We may hypothesize that these relationships are due to a higher abundance of structural carbohydrates (cellulose) characterizing seagrass tissues. Cellulose is a complex carbohydrate, representing the structural component of cell walls in both green plants and algae (Baldan et al., 2001). In fact, several green algae have walls containing cellulose content up to 70% of their dry weight (Ott and Maurer, 1977). According to the high carbon content, sea urchins could discharge the excess of carbon due to cellulose through the faecal pellets, as well has been demonstrated in various studies on invertebrate consumers of *P. oceanica* (Kawamata, 1997).

To date, several studies have defined the daily feeding rates of different sea urchins in the field. In the case of *Strongylocentrotus nudus* the amount of kelp eaten has been estimated through mathematical models, using the brown algae *Laminaria* spp., *Eisenia bicyclis* and *Undaria pinnatifida* living in the same sea urchin habitats (Hiratsuka and Uehara, 2007). The predicted feeding rate was 0.5 g wet mass/day per animal, considering adults of about 40-50 mm. Feeding rates of four sea urchin species, *Echinometra* sp. A, *Echinometra mathaei*, *Echinometra* sp. C and *Echinometra oblonga*, were investigated after feeding on diets prepared from turf algae and agar for a 7-day period (Scheibling and Anthony, 2001). In that case, the feeding rates differed significantly among the four species of sea urchins, being between 0.14 g and 0.29 g for sea urchins of 30-35 mm. Furthermore, adult *S. droebachiensis* were fed on two diets,

the invasive green alga *Codium fragile* and the brown alga *Laminaria* sp. (Cyrus et al., 2015). The feeding rates declined from June to July and remained low (about 0.1 g dw per sea urchin of 38-52 mm per day) through September. These data are comparable with the present results, showing that the daily feeding rates for a sea urchin correspond to 0.16, 0.15 and 0.1 g (dw) for *U. rigida*, *P. oceanica* and formulated pellets, respectively.

Interestingly, the three different diets did not produce size increments of adult sea urchins, but affected their gonad growth and reproduction performance. In fact, we demonstrated that, at the end of feeding experiments (after three months), *U. rigida* and *P. oceanica* did not produce effects on gonad growth. Differently, after three months the formulated pellets affected the gonadal index, resulting in a significant growth of the gonads probably due to the high content of crude proteins of this food (as reported above). Our data confirmed its contribution in the production of large gonads (Sartori and Gaion, 2016), which represents the major aim of echinoculture practices (Fabbrocini and D'Adamo, 2010). Despite the large volume increase of their gonads, surprisingly they were not capable of producing gametes. This could be probably correlated to the color and texture of their gonads, which were very different from those normally observed in sea urchins collected from the field and/or fed with the other two feeds used in this work (see **Figure 2.6**). However, *U. rigida* and *P. oceanica* produced a slower maturation and enlargement of ovaries and a good oocytes production, while the commercial pellets produced a hypertrophy of the follicular tissues, a diffused vacuolization, and the maturation of few eggs, which led finally to a low fertilization success with a compromised larval development to pluteus. It is well known in the

literature that the nutrient composition of a diet has a significant effect on growth (Marsh and Watts, 2007). For this reason, optimizing a feed for the best production of gonads in a sea urchin requires a consideration to balance the energy demands of increased protein metabolism with the energy availability in the diet. On this line, in the last twenty years, technology for sea urchin culturing, including reproduction and diet formulation, has improved for the supply of sea urchins (Watts et al., 2013). On this line, our data demonstrate that there is strong relationship between gonadal index and dietary contents of proteins, as in the case of commercialized pellets. These data are in agreement with other investigation reported by Pearce et al. (2002c), demonstrating a positive effect of proteins on gonadal increase of *S. droebachiensis* fed with artificial diets at increasing protein levels. Comparable results have been reported by de Jong-Westman et al. (1995) for adults of *S. droebachiensis* fed with prepared diets with high levels of proteins.

In conclusion, this study firstly represents an important step for this experimental Ph. D. project, helping to better define the quantity of benthic diatoms to be cultivated in order to conduct sea urchin feeding for one month (see Chapter 3). Furthermore, these results have been useful to define an optimal control diet, producing mature bloodstocks with high quality eggs and larvae. In fact, among the three diets tested, *U. rigida* was chosen as control, revealing the most balanced C/N ratios and inducing complete oocyte maturation and a good larval production. More in general, it represents an additional attempt to correlate the daily feeding rate of adult *P. lividus* with the composition of feeds and their effects of gonadal growth and reproductive success, especially for the industrial cultivation of this ecologically important marine model organism.

3. RNA extraction tests

3.1 Introduction

Next Generation Sequencing (NGS) technologies have found broad applications in functional genomic research, including gene expression profiling, genome annotation, small ncRNA discovery and profiling, determination of DNA sequences associated with epigenetic modifications of histones and DNA, and profiling DNA methylations (Morozova and Marra, 2008; Shendure and Ji, 2008; Wang et al., 2009; Metzker, 2010; Martin and Wang, 2011; Liu et al., 2012).

"Omic" approaches are powerful instruments not only for genomic studies but also for many other research fields. For example starting from 2007, ecologists used this approach to address several important ecological questions, introducing the new discipline of "ecological genomics" and/or "molecular ecology" (Tautz et al., 2010). Advances in DNA technologies have had a very strong impact on molecular ecology studies, also providing new tools for understanding the response of organisms to environmental stress (Kassahn et al., 2009; Ekblom and Galindo, 2011; Andrew et al., 2013). *Paracentrotus lividus* is also an excellent animal model for toxicology, physiology and biology investigations having been used for more than a century as a model for embryological studies with synchronously developing embryos which are easy to manipulate and analyze for morphological aberrations (as extensively reported in Chapter 1, paragraph 1.3). Despite its importance for the scientific community, the complete genome is still not fully annotated. To date, only a few molecular tools and NGS studies have been performed. Thanks to NGS approaches, it would be possible to

fill this genome gap by increasing the amount of molecular information on the sea urchin *P. lividus*. However, high-throughput NGS of genomes and transcriptomes requires high-quality, clean, and concentrated DNA/RNA.

This study compares five RNA extraction protocols using embryos of sea urchin *P. lividus* to define the best method for obtaining high-quality RNA for NGS applications. Four pools of eggs were fertilized and RNA extractions were performed on sea urchin plutei (at 48 hours post-fertilization, hpf) preserved in RNA*later*®, using five different protocols: a guanidinium-thiocyanate-phenol-chloroform (GTPC) extraction protocol with TRIzol®, and four widely-used Silica Membrane kits, namely GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), RNAqueous® Micro Kit (Ambion from Life Technologies), RNeasy® Micro Kit (Qiagen) and Aurum™ Total RNA Mini Kit (Biorad). The quantity and quality of isolated RNA was evaluated taking into account the purity, measured as A260/A280 and A260/A230 ratios, and the integrity, measured as RNA Integrity Number (RIN).

All the data showed in this chapter have been published in Ruocco et al., 2017a (Appendix B).

3.2 Materials and methods

3.2.1 Fertilization, sample collection and preservation

Fertilization and embryo culture were performed according to the experimental procedures described in Chapter 2, paragraph 2.2.7.

Different quantities of eggs (500, 1000, 2500 and 5000) from five females were fertilized, using sperm-to-egg ratios of 100:1. Embryos were then collected at the

pluteus stage (48 hpf) by centrifugation at 3000 g for 10 min in a swing out rotor at 4° C. Immediately after harvesting, the embryos were placed in at least 10 volumes of the RNA*later*®, an RNA Stabilization Reagent (Qiagen, Hilden, Germany), and then frozen in liquid nitrogen and kept at -80° C.

3.2.2 RNA extraction methods and determination of quantity/quality

Five methods of RNA extraction were compared.

i) TRIzol® RNA extraction method

Total RNA was extracted using TRIzol (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, homogenizing with TissueLyser (Qiagen, Austin, TX, US) with 3 mm sterile aluminum beads at 20.1 Hertz (Hz) for 5 minutes. Extractions with chloroform/isoamyl alcohol (24:1) were performed, followed then by RNA precipitation by adding glycogen and isopropyl alcohol. Total RNA was suspended in 0.1% v/v diethylpyrocarbonate (DEPC)-treated water. Contaminating DNA was degraded by treating each sample with a DNase RNase-free kit (Roche, Milan, Italy) according to the manufacturer's instructions. The samples were then stored at -80° C.

ii) GenElute™ Mammalian Total RNA Miniprep Kit

Embryo lysis was performed in 500 µL Lysis Solution/β-ME (2-mercaptoethanol) mixture and then RNA was extracted following the manufacturer's protocol. An additional step with DNase RNase-free kit (Roche, Milan, Italy) was used to remove contaminating DNA. Finally, RNA was eluted with 50 µL Elution Solution provided by the manufacturer. Samples were then stored at -80° C.

iii) RNeasy® Micro Kit

Embryos were lysed with 350 µL Buffer RLT/β-ME (10 µL β-ME for each mL of Buffer RLT) and homogenized with TissueLyser (Qiagen, Austin, TX, US) using 3 mm sterile aluminum beads at 20.1 Hz for 5 min. RNA was extracted following the manufacturer's protocol. DNA contaminations were avoided using the RNase-Free DNase Set, provided by the kit. RNA was eluted with 14 µL RNase-free water. The samples were then stored at -80° C.

iv) RNAqueous® Micro Kit

Pellets were suspended in 500 µL Lysis Solution by vortexing vigorously. Elution was performed in two steps, adding 10 µL Elution Solution each time, preheated at 75° C. Finally a DNase treatment was performed adding 1/10 volume 10X DNase I Buffer and 1 µL of DNase I to the sample. DNase was finally blocked using a DNase Inactivation Reagent (1/10 of total volume). The samples were then stored at -80° C.

v) Aurum™ Total RNA Mini Kit

Samples were disrupted with 350 µL Lysis Solution (already supplemented with 1% β-ME), pipetting up and down several times to lyse cells thoroughly. After treatment with 80 µL of diluted DNase I (75 µL DNase Dilution Solution and 5 µL DNase I), RNA was eluted with 40 µL of Elution Solution. Samples were then stored at -80° C.

The amount of total RNA extracted with the five methods was estimated by measuring the absorbance at 260 nm and the purity as A260/A280 and A260/A230 nm ratios, by a Nanodrop (ND-1000 UV-Vis Spectrophotometer; NanoDrop Technologies), to exclude the presence of proteins, phenol and other contaminants (Riesgo et al., 2012a). The integrity measurements of RNA were finally assessed by running 100-200 ng of RNA

samples in each line of a 6000 Nano LabChip in an Agilent Bioanalyzer 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, US). RNA integrity was measured using the RIN value, which is calculated based on the comparison of the areas of 18S rRNA and 28S rRNA (Schroeder et al., 2006). RIN values over a threshold of 8 were considered to indicate non-degraded RNA extraction methods.

3.2.3 Statistical analyses

The total number of sample extractions from four different amounts of embryos is reported in **Table 3.1**. RNA quantity and quality (A260/A280 and A260/A230 ratios, and RIN values) were evaluated using One-way Variance Analysis (ANOVA). Firstly, RNA quality, absorbance ratios, A260/A230 and A260/A280, and RIN values, and degree of correlation were measured using a nonparametric Spearman's correlation coefficient. Moreover, differences between the groups were evaluated statistically using unpaired *t*-tests. P values < 0.05 were considered significant. Distribution of RIN values was graphically represented by boxplots for different treatments of sea urchin embryos. Statistical analyses were performed using GraphPad Prism Software (version 6.00 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com).

3.3 Results

3.3.1 RNA extraction protocol tests

Increasing amounts of sea urchin *P. lividus* eggs (500, 1000, 2500 and 5000) were fertilized and embryonic development was followed until the pluteus stage, which is

reached at about 48 hpf. The results of different RNA extractions are summarized in **Table 3.1**.

Table 3.1. Number of extractions, embryo amount, RNA quantity (µg), purity (A260/280 and A260/230) and integrity (RIN values) from different number of *P. lividus* embryos using five different extraction methods: TRIzol, GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), RNAqueous® Micro Kit (Ambion from Life Technologies), RNeasy® Micro Kit (Qiagen) and Aurum™ Total RNA Mini Kit (Biorad). Values reported as mean ± SD (N=4/7). N/A indicates samples in which no values are assigned.

Method	Number	Embryos	Quantity (µg)	A260/230	A260/280	RIN
TRIzol	4	500	2.4 ± 0.23	1.96 ± 0.44	1.81 ± 0.24	N/A
		1000	3.5 ± 0.08	1.68 ± 0.18	1.42 ± 0.37	4.20 ±1.13
		2500	8.1 ± 0.16	1.85 ± 0.51	1.91 ± 0.42	4.60 ± 0.89
		5000	8.9 ± 0.09	1.83 ± 0.67	1.86 ± 0.30	3.90 ± 0.67
GenElute	7	500	3.2 ± 0.56	0.86 ± 0.87	2.00 ± 0.03	8.10 ± 0.03
		1000	4.2 ± 2.05	1.21 ± 0.05	1.98 ± 0.05	7.80 ± 0.05
		2500	12.8 ± 8.16	1.83 ± 0.03	1.93 ± 0.03	7.95 ± 0.35
		5000	25.9 ± 17.96	1.97 ± 0.03	1.96 ± 0.03	7.95 ± 0.07
RNeasy	7	500	1.0 ± 0.27	0.50 ± 0.41	2.15 ± 0.25	9.20 ± 0.01
		1000	1.8 ± 0.14	0.78 ± 0.61	1.92 ± 0.26	9.45 ± 0.49
		2500	8.9 ± 0.05	2.35 ± 0.07	1.97 ± 0.01	9.30 ± 0.14
		5000	18.7 ± 7.91	2.17 ± 0.08	1.98 ± 0.05	9.20 ± 0.15
RNAqueous	7	500	1.6 ± 2.1	0.46 ± 0.71	1.90 ± 0.34	7.90 ± 0.08
		1000	2.9 ± 3.6	0.72 ± 1.07	1.88 ± 0.48	8.10 ± 0.27
		2500	3.0 ± 3.9	2.37 ± 0.29	1.83 ± 0.28	10.0 ± 0.17
		5000	4.2 ± 5.3	2.35 ± 0.17	1.93 ± 0.14	8.90 ± 0.09
Aurum	7	500	1.6 ± 0.71	1.96 ± 1.14	2.04 ± 0.25	9.65 ± 0.50
		1000	5.4 ±1.90	1.93 ± 0.36	2.13 ± 0.08	9.45 ± 0.35
		2500	13.3 ± 2.49	1.65 ± 1.18	2.08 ± 0.07	9.95 ± 0.07
		5000	23.0 ± 1.47	1.94 ± 0.75	2.05 ± 0.07	9.85 ± 0.07

Concerning RNA quantity, similar results were obtained using TRIzol, RNeasy and RNAqueous methods for 500 and 1000 embryos. In the case of 2500 and 5000 embryos significantly higher quantity of RNA was extracted using GenElute, RNeasy and Aurum methods (One way ANOVA, p value < 0.0001), whereas no significant differences were

detected between 2500 and 5000 embryos versus 500 and 1000 embryos with the RNAqueous method (p value > 0.05). All extraction procedures from 2500 and 5000 embryos yielded sufficient amounts of total RNA as requested for NGS approaches (~ 2 ± 3 µg of total RNA). No significant differences have been detected in RNA purity (A260/A230 and A260/A280 ratios) using the five considered methods. The Spearman's coefficients did not reveal a correlation between RNA quality variables based on absorbance ratios (A260/A280 and A260/A230) and RIN values (ρ ranged from 0.40 to 0.52; p values from 0.0833 to 1.0; for further details see **Table 3.2**). These results were also confirmed by *t*-test, showing that RNA quality and RIN trends were statistically different (p < 0.0001).

Table 3.2. Correlation between RNA quality based on absorbance ratios (A260/280 and A260/230) and RIN values by Spearman's correlation coefficient and evaluation of their statistical difference by *t*-test, using the five different RNA extraction protocols for *P. lividus* embryos. Statistically significant p values < 0.05.

	Spearman's correlation			*t*-test		
		RIN vs A260/A280	**RIN vs A260/A230**		**RIN vs A260/A280**	**RIN vs A260/A230**
TRIzol	*ρ*	0.5	0.5			
	p value	1.0	1.0	p value	< 0.0001	< 0.0001
GenElute	*ρ*	0.51	0.167			
	p value	0.19	0.703	p value	< 0.0001	< 0.0001
RNAqueous	*ρ*	0.4	1.0			
	p value	0.75	0.08	p value	< 0.0001	< 0.0001
Rneasy	*ρ*	0.39	0.012			
	p value	0.34	0.958	p value	< 0.0001	< 0.0001
Aurum	*ρ*	0.15	0.554			
	p value	0.19	0.1506	p value	< 0.0001	< 0.0001

Representative Bioanalyzer Agilent electrophoresis runs showed low or high quality of total RNA extracted from *P. lividus* embryos (**Figure 3.1**).

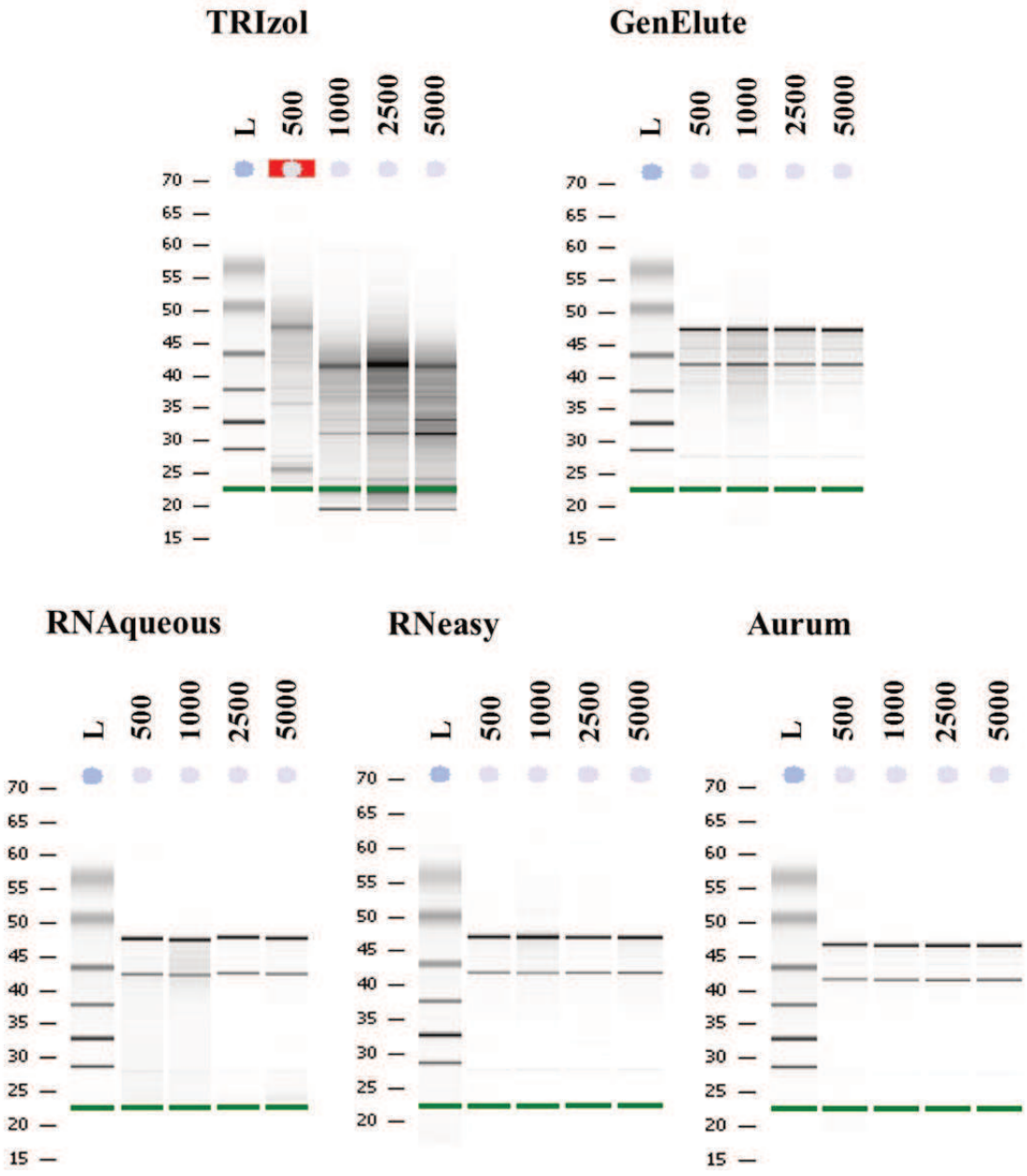


Figure 3.1. Bioanalyzer Agilent electrophoresis runs. Examples of representative Bioanalyzer Agilent electrophoresis runs for the five different methods applied for RNA extractions from *P. lividus* embryos: TRIzol, GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), RNAqueous® Micro Kit (Ambion from Life Technologies), RNeasy® Micro Kit (Qiagen) and Aurum™ Total RNA Mini Kit (Biorad). Four different numerical amounts of embryos were used for RNA extraction: 500, 1000, 2500 and 5000 embryos. The ladder (L) is reported in the first lane of each run.

The methods used for total RNA extractions consistently showed two bands, corresponding to 28S and 18S rRNA, with the only exception of the TRIzol method, showing degraded RNA with several bands. These results are also confirmed by the representative Bioanalyzer Agilent profiles showing electropherogram with low or high quality of total RNA extracted from different numerical amounts of *P. lividus* embryos along with corresponding RIN values (**Figure 3.2**).

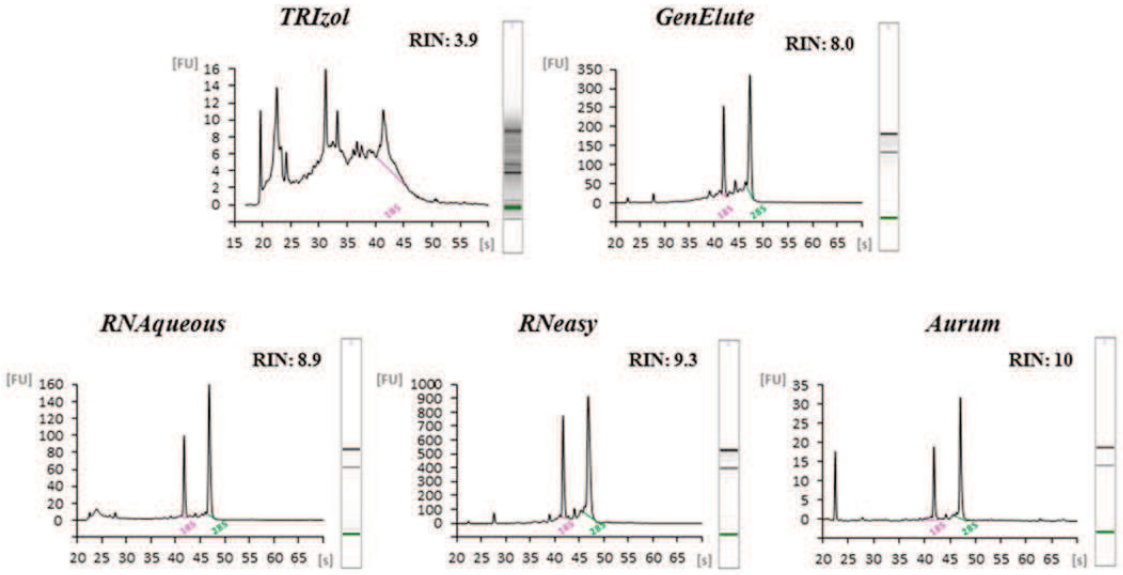


Figure 3.2. Agilent Bioanlyzer electropherograms. Examples of representative Agilent Bioanlyzer electropherograms of *P. lividus* RNA. Relative Fluorescent Unit (FU) and seconds of migration (s) of RNA samples isolated according to the five different extraction methods. RIN values are also reported.

Total RNA extracted with TRIzol showed a single peak corresponding to the 18S at 42 s, absence of a 28S peak, a high amount of small size RNA occurring between 25 s and 42 s and RNA degradation with a very low RIN value (RIN = 3.90). The integrity of *P. lividus* plutei RNA significantly improved with the use of Silica Membrane methods. In fact, in all four commercial kits applied for RNA extraction, the electropherograms

showed two peaks corresponding to 28S (42s) and 18S (47s) with very high RIN values ranging from 8.0 for GenElute, 8.9 for RNAqueous, increasing to 9.3 for RNeasy and reaching a maximum value with the Aurum kit (RIN = 10.0). Such high RIN values were considered suitable for NGS technologies.

In samples exhibiting A260/A280 and A260/A230 ratios lower than 1.8 or over 2.0 (**Table 3.1**), RIN values higher than 8 were observed. These samples also showed intact 28S and 18S. The distributions of RIN were also reported in the boxplot of RIN values (**Figure 3.3**).

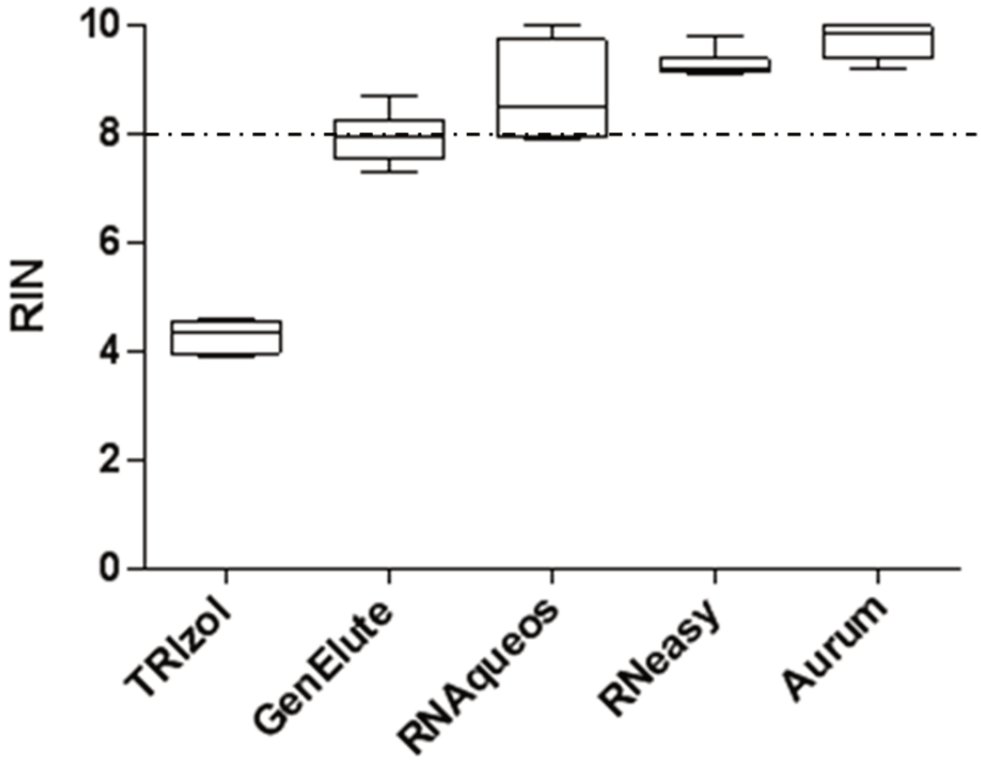


Figure 3.3. Boxplot of RIN values obtained with the five different methods of RNA extraction. The boxes extend from the 25th to the 75th percentile, and the line in the middle is the median. The error bars extend down to the lowest value and up to the highest. Dashed line at the RIN value of 8 is reported, because higher values than 8 are considered suitable for NGS analysis.

Extraction by RNAqueous resulted to be the most inconstant method based on RIN values (from 7.90 to 10.0). Moreover, even if GenElute and Aurum methods yielded comparable results producing higher quantities of RNA (**Table 3.1**), the boxplot very clearly showed that they were not comparable for the RNA integrity. In fact, the GenElute method produced RNA with RIN values lower than those obtained by the Aurum method.

3.4 Discussion

This work represents the first attempt to standardize a method for RNA extraction from sea urchin embryos at the pluteus stage with the aim to obtain sufficient quantities of high-quality RNA for NGS technologies. Firstly, RNA was extracted from different amount of sea urchin embryos at the pluteus stage, applying five different protocols. Results revealed that all extraction procedures from 500 and 1000 embryos did not yield sufficient amounts of total RNA as requested for NGS approaches (~ 2-3 μg of total RNA). Sufficient RNA quantity was obtained from 2500 and 5000 embryos. The most efficient procedures to produce such quantities were obtained with GenElute, RNeasy and Aurum, producing more than 10 and 20 μg of RNA, using 2500 and 5000 embryos, respectively.

Once the number of embryos necessary for NGS was established, RNA quality was assessed by measuring different features: A260/A280 and A260/A230 ratios and estimation of RIN values (Pinsino et al., 2008; Gayral et al., 2011; Hillyard and Clark, 2012; Riesgo et al., 2012a). The most efficient strategies for assessing RNA integrity was represented by the estimation of the RIN value using Agilent Bioanalyzer chips.

The Bioanalyzer profiles showed that GenElute, RNeasy and Aurum procedures were only comparable for RNA quantity, not for RNA integrity. In fact, RIN values using the GenElute procedure were the lowest in comparison to the other Silica Membrane procedures applied. RNAqueous was the most variable method whereas the most efficient for RNA integrity were RNeasy and Aurum.

Interestingly the present data demonstrated no significant correlations between A260/A280 and A260/A230 ratios and RNA integrity. A possible explanation for this result may be due to the different stability of the RNAs. In fact, ribosomal RNA is more stable than mRNA (Houseley and Tollervey, 2009; Simister et al., 2011; Riesgo et al., 2012a,b; Pérez-Portela and Riesgo, 2013). Moreover, the A260/A280 ratio could have values of about two, because of the intact ribosomal RNA, even if the mRNA was degraded. This is, for example, the case of RNA samples after TRIzol extraction, suggesting that the method of preservation of our embryo samples represents a very important step to obtain high-quality RNA. Preservation in RNA*later* ensured greater stability for our samples since this a nontoxic storage reagent able to permeate cells and/or tissues and to stabilize and protect cellular RNA. This step minimizes the need for immediate processing of the samples, without jeopardizing the quality or quantity of RNA obtained after subsequent RNA isolation. RNA extraction from fresh cell/tissues has been used in many cases with success (Matranga et al., 2000; Simister et al., 2011; Pérez-Portela and Riesgo, 2013), but several laboratory conditions or field experiments do not allow for direct extraction upon collection. There are several indications that RNA*later* is a reliable preservative for RNA in a wide array of tissues improving the yield of total RNA (Riesgo et al., 2012a; Pinsino et al., 2008).

This study fills a gap considering that no comparative studies for RNA extractions are available in the literature for sea urchin *P. lividus* plutei. A similar methodological analysis was performed by Pérez-Portela and Riesgo (Pérez-Portela and Riesgo, 2013), although they did not use embryos at the pluteus stage (as in this case) from *P. lividus*, but tissues from another sea urchin, *Arbacia lixula*. In fact, their study aimed to optimize a preservation protocol for the isolation of high-quality RNA from three different *A. lixula* tissues: gonad, oesophagus and coelomocytes. Extractions of total RNA were performed with a modified TRIzol protocol for all tissues, applying four preservation treatments. The results showed high values of RNA quantity and quality for all tissues, indicating non-significant differences among samples. Insufficient RNA amount and great variability in RNA integrity were found in coelomocytes in RNA*later*. The most efficient system to stabilize RNA was the TRIzol method that produced high RNA quality and quantity. These data are comparable with the present results (see above), because no correlation was found between RNA integrity and absorbance ratios, as in our experiments. Furthermore, the evaluation of RIN values by Agilent Bioanalyzer chips was the best approach to evaluate the RNA integrity.

The extraction of high-quality RNA represents a fundamental step, considering that transcriptomic information and NGS approaches represent significant tools to acquire information regarding several biological processes. Numerous attempts, mainly for non-well-established model organisms, were recently made to establish methods for high-quality RNA extractions, because NGS represents the only way to address specific ecological and evolutionary questions (Riesgo et al., 2012b; Asai et al., 2014).

Asai et al. (2014) compared three commonly-used methods: TRIzol, Aurum Total RNA Mini Kit and Qiagen RNeasy Micro Kit, in combination with preservation reagents TRIzol or RNA*later*, to obtain high-quality and large quantities of RNA from the copepod *Calanus helgolandicus*. Their results confirmed that the preservation of copepods in RNA*later*® and the extraction with Qiagen RNeasy Micro Kit were the optimal isolation method for high-quality and quantity of RNA for NGS studies of *C. helgolandicus*.

In conclusion, all these data provide a standard protocol for isolating high-quality RNA from sea urchin plutei for high-throughput NGS approaches that have been applied during the Ph. D project. In fact, it has been used to study the effects of benthic diatoms on embryos at the pluteus stage, deriving from adults fed on these diatoms, by *de novo* transcriptome assembly (see Chapter 4).

4. Feeding experiments with benthic diatoms

4.1 Introduction

Once *Ulva rigida* was defined as the control diet with known daily feeding rates for adult sea urchins *P. lividus* (see Chapter 2), feeding experiments with the benthic diatoms of interests were performed. Using morphological, metabolomic and *de novo* transcriptomic approaches the effects of four benthic diatoms, *Cylindrotheca closterium*, *Nanofrustulum shiloi*, *Cocconeis scutellum* and *Dilponeis* sp., isolated from the leaves of the seagrass *Posidonia oceanica*, were investigated on the reproductive success of the sea urchin *P. lividus*. Since *C. closterium* and *N. shiloi* are both abundant and fast-growing diatoms, whereas *C. scutellum* and *Diploneis* sp. are slow-growing species, feeding experiments were done separately to avoid experimental difficulties.

Sea urchins were fed one month on these four benthic diatoms in microcosms (see Chapter 2, paragraph 2.2.3), using the green alga *U. rigida* as a control feed. The development of embryos produced after feeding on these diets was followed until the pluteus stage (48 hours post-fertilization, hpf). In addition, gonadic tissues from these adults were analyzed by ^{1}H-NMR.

Finally, molecular approaches were applied to investigate the toxic effects of benthic diatoms, by generating a *de novo* transcriptome (RNA-seq) assembly and annotation of *P. lividus* to identify differentially expressed genes. In addition, fifty genes, belonging to different functional classes and previously analyzed in response to planktonic diatom-derived oxylipins, were followed using *Real Time quantitative PCR* (*qPCR*) to detect the expression level that was modulated by feeding on the benthic diatoms.

Data from experiments with *C. closterium* and *N. shiloi* reported in this Chapter have been published in Ruocco et al., 2018a (Appendix B), while all the results obtained from *C. scutellum* and *Diploneis* sp., have been submitted and are still under review.

4.2 Materials and methods

4.2.1 Isolation of benthic diatoms

Epiphytes were isolated from leaves of *P. oceanica*, collected in Ischia, Naples (Italy) using a sterilized scalpel. Individual diatoms were aspirated by means of a Narishige syringe Syr-12, taking advantage of a Leica micromanipulator under an inverted microscopy and transferred into multiwell plates in sterile seawater. The strains were collected and transferred daily to clean *f/2* medium until monoclonal cultures of the two benthic diatoms were obtained. Mother cultures were grown in 6/12-multiwell plates in *f/2* medium (Sigma Guillard's) in a thermostatic chamber at 18±1° C and 140 μmol (photons) m^{-2} s^{-1}, with a 12:12 h (light:dark) photoperiod.

4.2.2 Morphological and molecular characterization

C. closterium, *N. shiloi* and *Diploneis* sp. were characterized from the morphological and molecular points of view, whereas *C. scutellum*, previously identified and studied by Zupo (2000), was only processed for morphological observations. Diatom samples from the mother cultures were collected, fixed with 2.5% glutaraldehyde, filtered on cellulose Millipore filters and mounted on aluminum stubs for Scanning Electron Microscopy (SEM, Zeiss EVO MA LS). After three washings and treatment with osmium (1%), samples were dehydrated with increasing concentrations of ethanol (25,

50, 75 and 100%), mounted on stubs and coated with platinum for SEM observations and morphological identification.

Individual cell cultures were collected from the multi-well plates and pelleted by centrifugation for 20 min at 3000 g 4° C, then frozen in liquid nitrogen until use.

Cell membranes were disrupted by lysis buffer containing 2% Cetyltrimethylammonium bromide (CTAB) and 2-Mercaptoethanol (β-ME, Sigma-Aldrich) at 65° C for 45 min. RNAse was then added (final concentration 200 µg/mL) and digestion was performed at 37° C for 1 h. Extraction with chloroform/isoamyl alcohol (24:1) was performed two times and 1 volume of ice-cold isopropanol (100%) was then added to the aqueous phase with glycogen for DNA precipitation at -20° C overnight. After centrifugation, DNA was washed with 75% ethanol, centrifuged for 15 min, and air-dried. DNA was suspended in 20 µL sterile water. The amount of total DNA extracted was estimated by measuring the absorbance at 260 nm; purity was calculated by A260/A280 and A260/A230 nm ratios, using a NanoDrop spectrophotometer (ND-1000 UV-vis Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). The integrity of DNA was evaluated by agarose gel electrophoresis. PCRs were performed with specific primers for 18S rRNA (528F/1055R; **Table 4.1**; Elwood et al., 1985; Kooistra et al., 2003). Sequences were aligned with Basic Local Alignment Search Tool (Nucleotide Blast available at https://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al., 1990) database in order to identify the heat with best matches. In addition, PCR fragments were aligned to the whole 18S sequences found using the software MultiAlin (available at http://multalin.toulouse.inra.fr/multalin/; Corpet, 1988).

Table 4.1. Primers sequences (5'→3'), primers and amplicon length.

Name	Sequence (5'→3')	Primers Length	Amplicon Length	Reference
528F	GCG GTA ATT CCA GCT CCA A	19 bp	600-700 base pair (bp)	Elwood, 1985
1055R	ACG GCC ATG CAC CAC CAC CCA T	22 bp		Kooistra et al., 2003

4.2.3 Preparation of diatom cultures

To produce sufficient biomass for experimental purposes, intermediate cultures were prepared (using as starting material the monoclonal cultures) in PYREX® Crystallizing Dishes (70 x 50 mm) and grown until the end of the exponential phase (one week for fast-growing species and three weeks for slow-growing species) at temperature, photon flux and photoperiod described above (see paragraph 4.2.1). These cultures were then collected by aspiration using a sterile Pasteur pipette, and used to prepare massive cultures on a vegetal substrate of 2% agar, which is harmless for sea urchins (**Figure 4.1**; Fabbrocini et al., 2012).

Massive cultures were grown in a thermostatic chamber (same conditions reported in paragraph 4.2.1), up to the end of exponential growth phase to facilitate a complete spreading of the diatoms on the whole substrate.

When agar massive cultures were inoculated, three glass slides were also deployed on the surface of the agar substratum to count the number of cells on the glass slides under an inverted microscope with an ocular micrometer (magnification: 20x, area: 0.0025 mm^2). Based on these counts, the biomass of diatoms was then calculated as logC

(quantity of intracellular carbon in pg) = -0.541+0.811 x logV (cell volume expressed as μm^3; Menden-Deuer and Lessard, 2000). Cell length, width and depth were calculated by SEM images (twenty replicates) through ImageJ Software (Schneider et al., 2012).

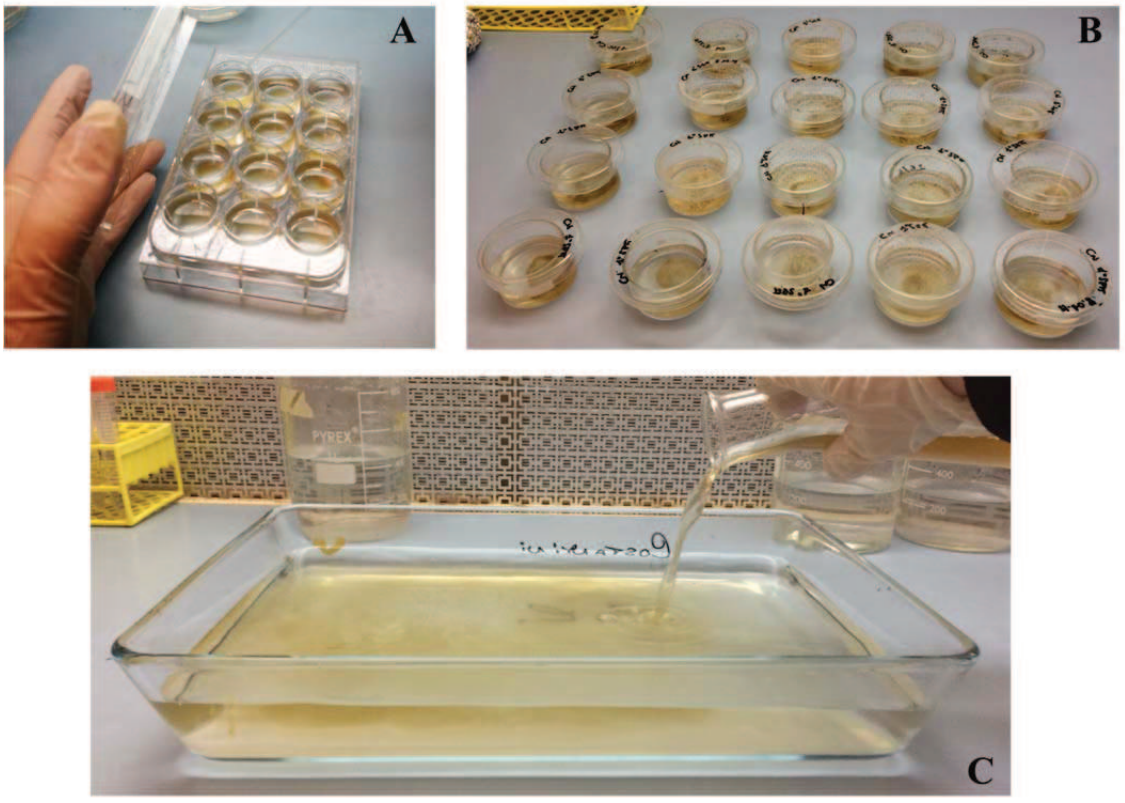


Figure 4.1. Monoclonal cultures (A), intermediate cultures (B) and massive culture on 2% agar (C).

4.2.4 Feeding experiments, faecal pellets collection, fertilization, morphological analysis of embryos

Considering the different growth rates of the benthic diatoms under analysis (as described above), *C. scutellum* was tested with *N. shiloi* (1st feeding experiment) and *C. scutellum* with *Diploneis* sp. (2nd feeding experiment). Adult sea urchin *P. lividus* were

collected in November-December for the 1st feeding experiment and in January-February for the 2nd feeding experiment.

Twenty adult (12 females and 8 males) *P. lividus* were reared in each experimental tank of the continuous flow-through system (described in the paragraph 2.2.3) and fed with *U. rigida* (3 replicate tanks) and the four benthic diatoms under analysis (3 replicate tanks for each species). Considering the different growth rates of the benthic diatoms under analysis (as described above), *C. scutellum* was tested with *N. shiloi* and *C. scutellum* with *Diploneis* sp. The daily amount of food (both *U. rigida* and the agar substrate incorporating the diatoms) given to sea urchins was 1 gram (ww) per animal. After one month of feeding, eggs and sperms were collected. Fertilization, embryo cultures and morphological observations were performed as described in Chapter 2, paragraph 2.2.7.

Faecal pellets were collected in the tanks after fifteen day of feeding with *C. closterium* and processed for SEM observations (as previously described in paragraph 4.2.2) in order to detect and confirm (as an example) the presence of diatoms ingested.

4.2.5 RNA extraction, *de novo* transcriptome assembly (*RNA sequencing*) and *Real Time qPCR*

Since morphological observations revealed toxigenic effects of the benthic diatoms on *P. lividus* embryos with the only exception of *C. scutellum*, molecular analyses were performed on embryos deriving from adult sea urchins fed on *C. closterium*, *N. shiloi* and *Diploneis* sp.

After feeding experiments on adults, about 5000 eggs (in 50 mL of filtered sea water, FSW) from each female fed on *U. rigida* and on the benthic diatoms were collected and

fertilized. Embryos at 48 hpf were then collected by centrifugation at 3000 g 4° C for 10 min in a swing out rotor. Pellets were placed in at least 10 volumes of RNA*later*®, an RNA Stabilization Reagent (Qiagen, Hilden, Germany), and then frozen in liquid nitrogen and kept at -80° C. Total RNA was extracted using Aurum™ Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions (see paragraph 3.2.2; Ruocco et al., 2017a). For RNA-seq the amount of total RNA extracted and RNA integrity were measured according to Ruocco et al., 2017a.

RNA-seq was performed (Genomix4Life Srl, Salerno, Italy) on triplicate samples of each experimental condition. RNA samples were used for preparation of cDNA libraries (TruSeq Stranded mRNA) and paired-end sequencing in single (2x100, ~ 30.000.000 total reads/sample) on Illumina platform Hiseq 2500. Reads were obtained from ends of DNA fragments for ultra-high-throughput sequencing. Prior to further analysis, a quality check was performed on the sequencing data.

A paired-end sequencing was chosen, in which short reads were obtained from ends of DNA fragments for ultra-high-throughput sequencing. Prior to further analysis, a quality check was performed on the sequencing data. The quality of the reads was evaluated also through the FASTQC bioinformatics tool (see Supplementary Information of Ruocco et al., 2018a; Andrew, 2010). The high quality reads from all the samples were joined and then used as input to perform transcriptome assembly, with Trinity (Grabherr et al., 2011).

The full dataset of raw data has been deposited in the Sequence Read Archive (SRA database; available at https://www.ncbi.nlm.nih.gov/sra: i) accession number: SUB2817153 for the transcriptomes from embryos deriving from adults fed on *C.*

closterium and *N. shiloi*; ii) accession number: PRJNA495004 for the transcriptome from embryos deriving from adults fed on *Diploneis* sp.

The sequences of the assembled transcripts were translated into proteins with Transdecoder (http://transdecoder.github.io) (minimum length 100aa). When multiple translations were possible the priority was set in order to obtain the longest complete ORF; when a complete ORF was not detected the longest sequence was kept. A gff3 annotation file was obtained by reporting the coordinates of the CDS for each translated transcript and this was named "Paracentrotus_lividus.gff3".

To evaluate the correlation between biological replicates and different experimental conditions, the Trinity 'PtR' tool (Grabherr et al., 2011) was adopted. Starting from the counts of each associated feature, this tool performs a counts-per-million (CPM) data transformation followed by a log2 transform. All analyses were performed on *de novo* genes. Reads were mapped to assembled putative transcripts using bowtie (Langmead and Salzberg, 2012). TransDecoder (http://transdecoder.github.io/) software was used to identify candidate coding regions within fragments. ORFs that were at least 100 amino acids long were selected. Expression analysis was performed by RSEM (version 1.1.21) using default parameters, and expression values were converted to FPKM (Fragments per Kilobase of exon per Million fragments mapped; Roberts et al., 2011). Differentially expressed (DE) genes were identified by setting a log fold change on base 2 threshold $\geq$ 1.5 corresponding to a False Discovery Rate (FDR) $<$ 0.05. Functional annotation analyses were conducted on all differentially expressed genes using Blast2GO. The Gene Ontology (GO) terms were assigned based on annotation with an E-value of 10^{-6}.

In the case of *Real Time qPCR* experiments, for each RNA, 600 ng of total RNA extracted was retrotranscribed with an iScript™ cDNA Synthesis kit (Bio-Rad, Milan, Italy, following the manufacturer's instructions) without dilution in a reaction containing a final concentration of 0.3 mM for each primer and 1× FastStart SYBR Green master mix (total volume = 10 µL) (Applied Biosystems, Monza, Italy). PCR amplifications were performed in a ViiATM7 Real Time PCR System (Applied Biosystems, Monza, Italy) thermal cycler using the following thermal profile: 95° C for 10 min, one cycle for cDNA denaturation; 95° C for 15 s and 60° C for 1 min, 40 cycles for amplification; 72° C for 5 min, one cycle for final elongation; one cycle for melting curve analysis (from 60° C to 95° C) to verify the presence of a single product. The variations in gene expression were followed for fifty genes, having key roles in different functional processes (**Figure 4.2**; Romano et al., 2011; Varrella et al., 2014, 2016a; Ruocco et al., 2016, 2017b).

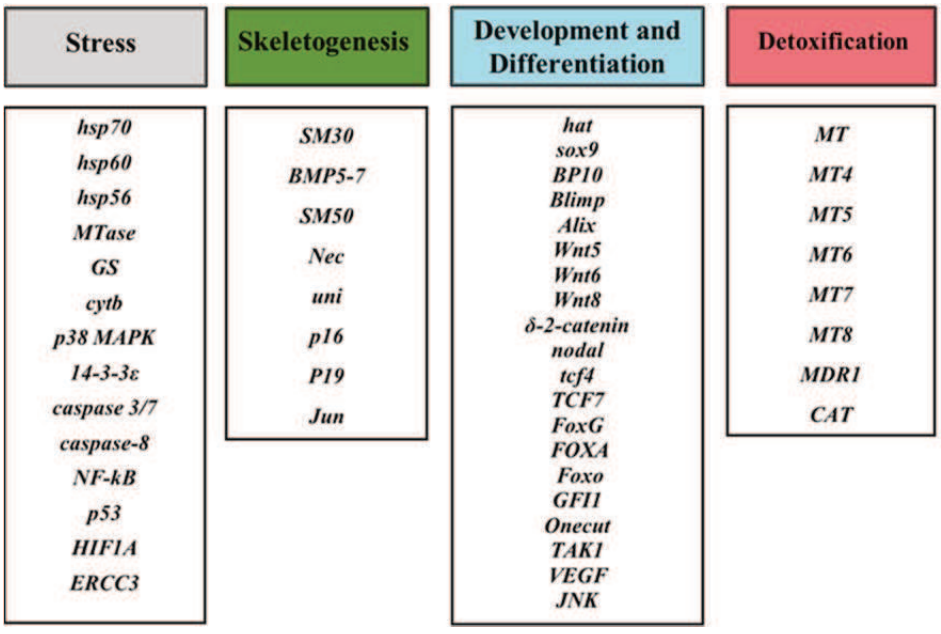


Figure 4.2. List of the genes involved in stress response, skeletogenesis, development, differentiation and detoxification processes analysed in this study.

Each assay included a no-template control for each primer pair. To capture intra-assay variability, all *Real-Time qPCR* reactions were carried out in triplicate. Fluorescence was measured using ViiATM7 software (Applied Biosystems, Monza, Italy).

The expression levels of each gene by *Real Time qPCR* were analysed and internally normalized against the control gene for *Pl-Z12-1* (Costa et al., 2012) using REST software (Relative Expression Software Tool, Weihenstephan, Germany) based on the Pfaffl method (Pfaffl, 2001; Pfaffl et al., 2002).

Variation of expression levels were calculated as relative expression ratios of the analysed genes with respect to control embryos. Only expression levels greater than ±1.5-fold with respect to controls were considered significant.

Heatmaps were generated using Heatmapper (available at http://www.heatmapper.ca; Babicki et al., 2016), a freely available web server that allows to interactively visualize the data in the form of heat maps through an easy-to-use graphical interface. Heatmapper is a versatile tool that allows users to easily create a wide variety of heat maps for many different data types and applications. In this case Heatmapper allowed generating, clustering and visualizing expression-based heat maps from transcriptomic and *Real Time qPCR* data. The hierarchical clustering was generated by average linkage calculation using Euclidean distance measurement method.

4.2.7 ^{1}H-NMR analysis of the gonads

For each treatment gonad tissues were collected from five adult sea urchins after one month of feeding and stored at -20° C. Gonads were re-suspended in 170 µL of H_2O and 700 µL of methanol and were sonicated for 30 s. Then, 350 µL of chloroform were

added and samples were mixed on an orbital shaker in ice for 10 min. 350 µL of H_2O/chloroform (1:1, v/v) were added to each cell suspension and centrifuged at 7840 g for 10 min at 4° C. Thereafter, the aqueous (polar) and lipophilic (apolar) phases were collected separately, transferred to a glass vial and dried under nitrogen flow. Samples were analyzed using ^{1}H-NMR. The polar fractions were dissolved in 630 µL of PBS-D_2O with the pH adjusted to 7.20, and 70 µL of sodium salt of 3-(trimethylsilyl)-1-propanesulfonic acid (1% in D_2O) was used as the internal standard. On the other hand the lipophilic fractions were dissolved in 700 µL of deuterated chloroform. A 600-MHz Bruker Avance DRX spectrometer with a TCI probe was used to acquire ^{1}H spectra on the cellular polar fractions. All ^{1}H-NMR spectra were acquired at 300 K with the excitation sculpting pulse sequence to suppress water resonance. A double-pulsed field gradient echo was used, with a soft square pulse of 4 ms at the water resonance frequency and with gradient pulses of 1 ms duration, adding 128 transients of 64 k complex points, with an acquisition time of 4 s/transient. Time domain data were all zero-filled to 256 k complex points and an exponential amplification of 0.6 Hz was applied prior to Fourier transformation.

The assignments were based on the comparison of chemical shifts and spin-spin couplings with reference spectra collected in the human metabolome database (HMDB; Wishart et al., 2007) and the Biological Magnetic Resonance Database (BMRB; Ulrich et al., 2008).

4.2.8 Statistical analyses

Data-set scores were compared to a normally distributed set of scores with the same mean and standard deviation through D'agostino-Pearson omnibus normality test. Statistical differences between the groups were evaluated by One-Way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons, and by Student *t*-tests. All these statistical analyses were performed using GraphPad Prism Software (version 6.00 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com).

The 0.50-8.60 ppm spectral region of the ^{1}H-NMR spectra was integrated in buckets of 0.04 ppm using the AMIX package (Bruker, Biospin GmbH, Rheinstetten, Germany). The water resonance region (4.5-5.2 ppm) was excluded during the analysis and the bucketed region was normalized to the total spectrum area using Pareto scaling. Principal Component Analysis (PCA), Orthogonal partial least squares-Discriminant Analysis (OPLS-DA) and Variable importance in projection (VIP) plots were used to compare the spectra obtained from the polar and lipid fractions.

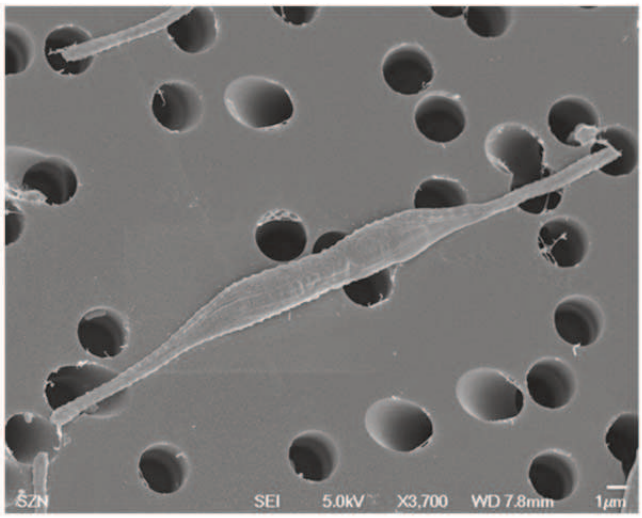
4.3 Results

4.3.1 Morphological and molecular characterization

SEM observation revealed that the first diatom isolated was needle like and thin in shape (length: 16 ± 1.1 μm, width: 2 ± 0.2 μm, depth: 1.7 ± 0.1 μm). The two ends of the cell extended far from the centre which was found to be wrapped by the raphe canal of valves (**Figure 4.3A**). According to these morphological features the strain was

identified as *C. closterium*. Molecular analysis of 18S rRNA gene amplified a 710 base pairs (bp) fragment (using the primers reported in **Table 4.1**); sequences producing significant alignments (99%) were *C. closterium* 18S strains (see **Figure 4.3B** and **Figure 4.4** showing the alignment between the fragment of 710 bp and *C. closterium* 18S, accession number: KY045848.1). The second benthic diatom was smaller (length: 4 ± 0.6 µm, width: 3 ± 0.2 µm, depth: 3 ± 0.07 µm) and characterized by rectangular frustules, forming chains linked by interlocking marginal spines characteristic of *N. shiloi* (**Figure 4.5A**). This morphological result was also confirmed by 18S rRNA gene (710 bp 18S sequence), showing 99% identity to *N. shiloi* strains (see **Figure 4.5B** and **Figure 4.6** showing the alignment between the fragment of 710 bp and *N. shiloi* 18S, accession number: AF525658.1). The third species displayed elliptical valves and with broadly rounded apices (length: 13 ± 1.3 µm, width: 5 ± 0.7 µm, depth: 3 ± 0.8 µm; **Figure 4.7A**). Its axial area is occupied almost completely by a broad raphe and the central area is small and elliptical. Striae consist of double rows of areolae, where marginal rows appeared more prominent than the adjacent row; striae were radiate throughout and extended onto the longitudinal canals. These morphological characteristics were related to *Diploneis* genera. PCRs of 18S rRNA amplified a 702 bp fragment matching by 98% to different *Diploneis* sp. strains (see **Figure 4.7B** and **Figure 4.8** showing the alignment of the fragment of 702 bp and *Diploneis* sp. 18S, accession number: KX981838.1).

A



B

Download GenBank Graphics

Cylindrotheca closterium 18S ribosomal RNA gene, partial sequence

Sequence ID: KY045848.1 Length: 1701 Number of Matches: 1

Range 1: 539 to 1248 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
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Sbjct  838   AGTTGGGGGTATTCGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTTTGAAAGACGAA  897
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```

Figure 4.3. (A) Scanning electron micrographs (SEM) of *C. closterium* cell (scale bar = 1 μm) and (B) Nucleotide Blast alignment of the 710 bp amplified fragment using the primers 528F/1055R (see **Table 4.1**; Elwood, 1985; Kooistra et al., 2003) with *C. closterium* 18S strain (accession number KY045848.1).

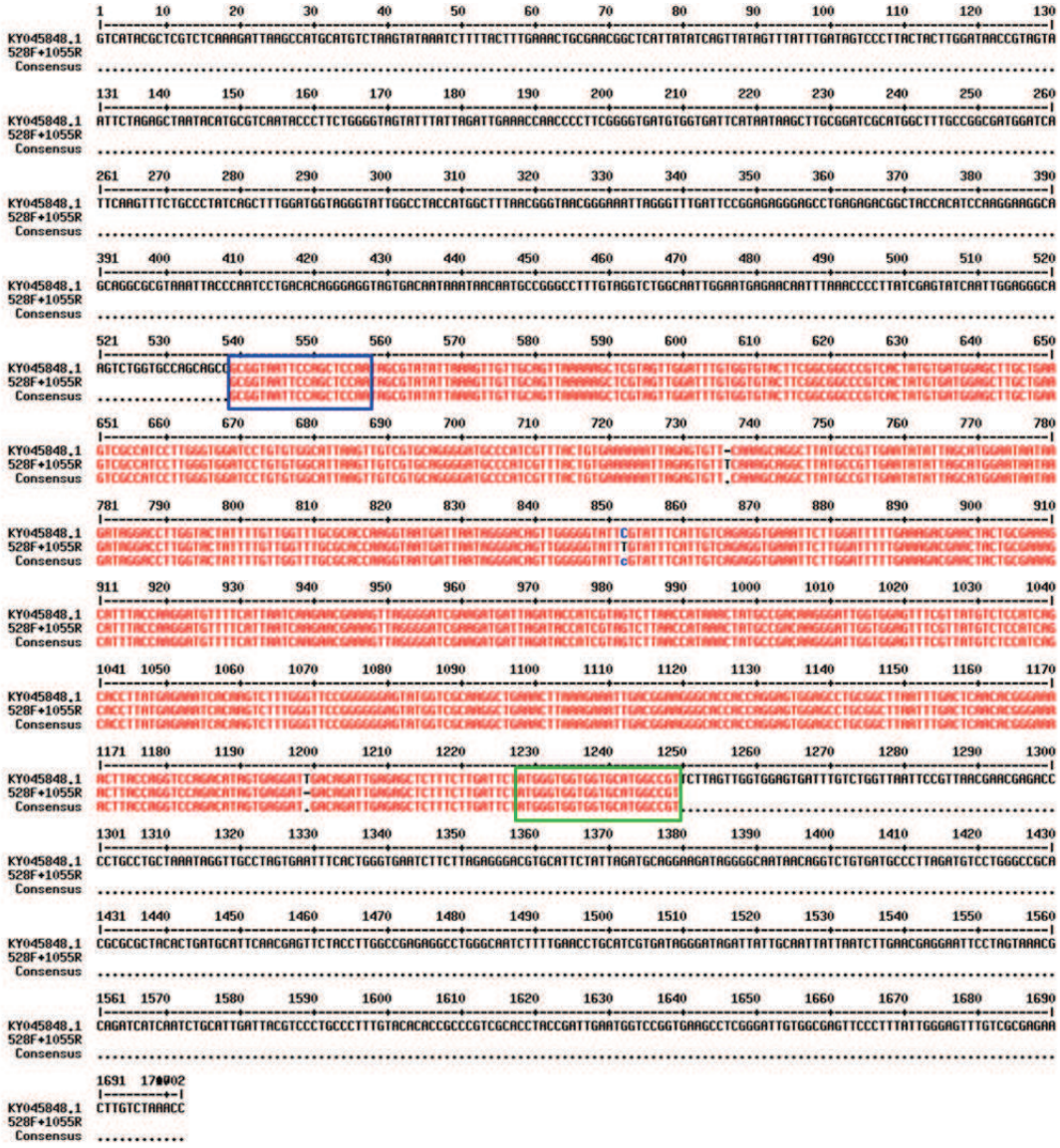
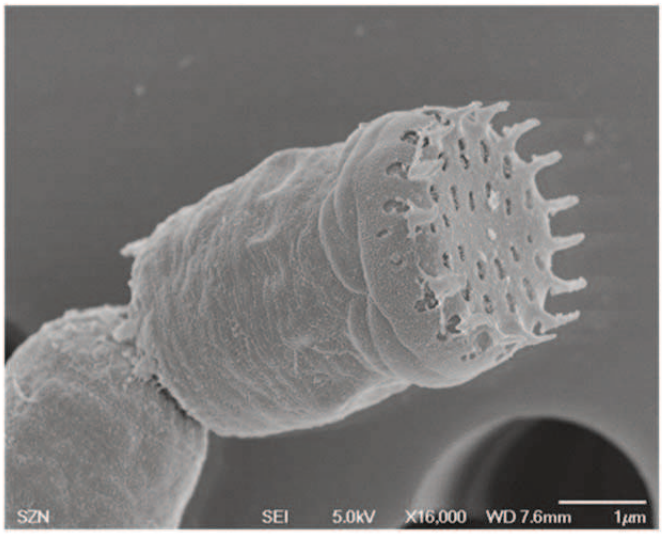


Figure 4.4. Alignment between the 710 bp fragment and *C. closterium* 18S sequence producing significant alignments in Nucleotide Blast (accession number KY045848.1), using the software MultiAlin Color code: matches, red letters; mismatches, blue/black letters; gaps, black dash; 528F primer, blue rectangle; primer 1055R, green rectangle.

A



B

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Nanofrustulum shiloi small subunit ribosomal RNA gene, partial sequence

Sequence ID: AF525658.1 Length: 1741 Number of Matches: 1

Range 1: 541 to 1250 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1225 bits(663)	0.0	695/711(98%)	2/711(0%)	Plus/Plus

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Figure 4.5. (A) Scanning electron micrographs (SEM) of *N. shiloi* cell (A; scale bar = 1 μm) and (B) Nucleotide Blast alignment of the 710 bp amplified fragment using the primers 528F/1055R (see Table 4.1) with *N. shiloi* 18S strain (accession number AF525658.1).

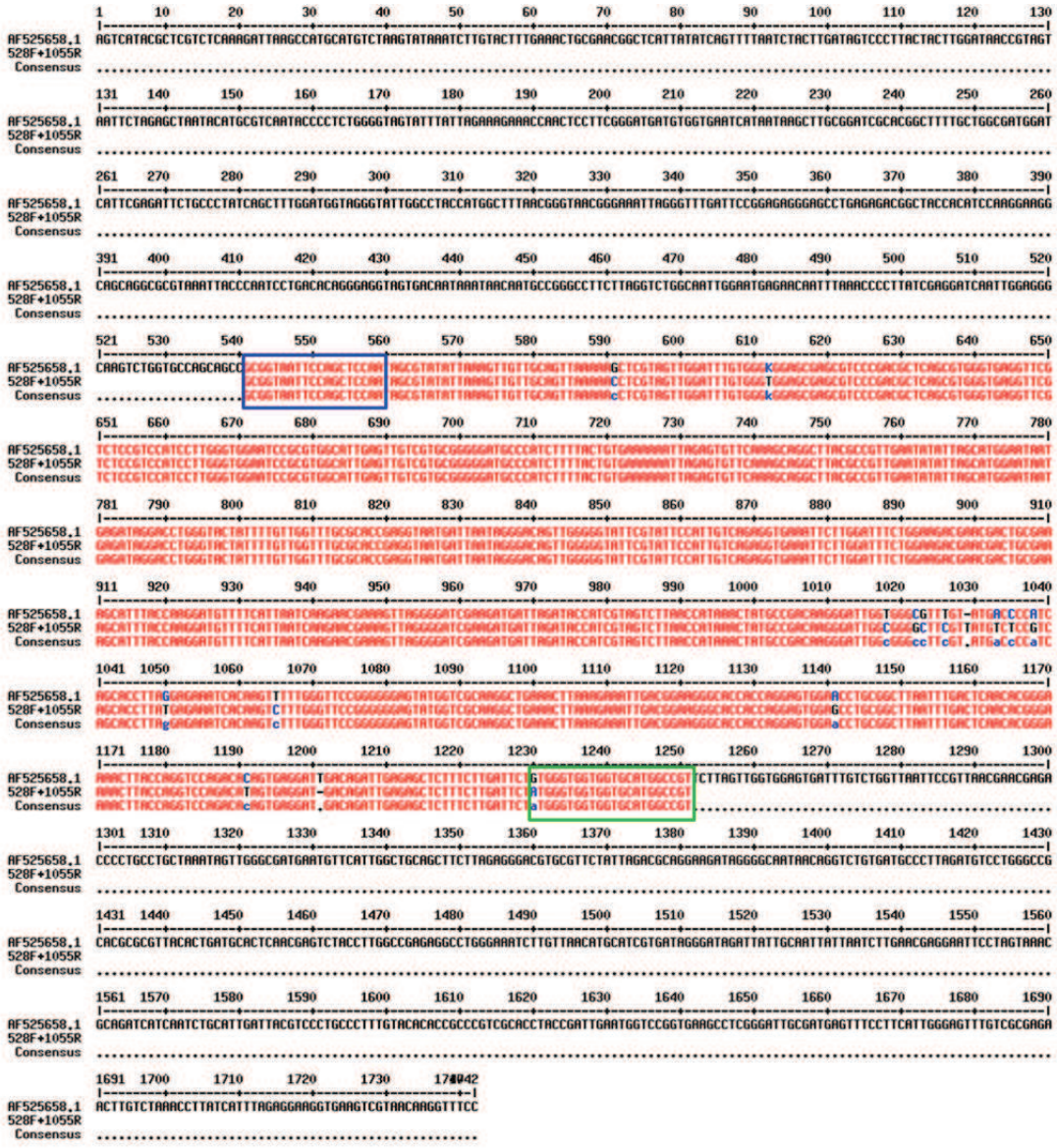
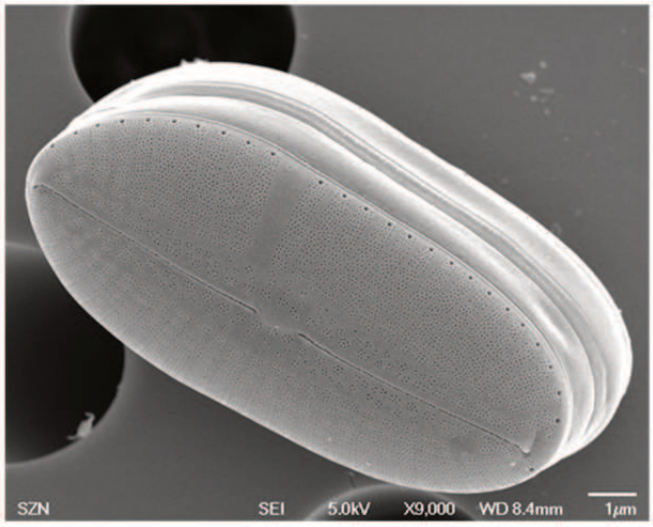


Figure 4.6. Alignment using the software MultiAlin between the 710 bp fragment and *N. shiloi* 18S sequence producing significant alignments in Nucleotide Blast (accession number AF525658.1). Color code: matches, red letters; mismatches, blue/black letters; gaps, black lines; primer 528F, blue rectangle; primer 1055R, green rectangle.

A



B

Download GenBank Graphics

Diploneis sp. isolate Coz1 cren-cp penn1 18S ribosomal RNA gene, partial sequence

Sequence ID: KX981838.1 Length: 1607 Number of Matches: 1

Range 1: 546 to 1247 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1230 bits(666)	0.0	690/702(98%)	0/702(0%)	Plus/Plus

```
Query  1     GCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTT  60
Sbjct  546   GCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTT  605
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Sbjct  606   GGATTTGTGGCAGCTCCCGAGGCCCAATTTTGGTGCCATTGGGGGAAGCCATCCTTGGGT  665
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Sbjct  666   GGAATCTGTGTGGCATTAGGTTGTCGTGCAGGGGATGCCCATCGTTTACTGTGAAAAAAT  725
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Query  241   CCTCGGTACTATTTTGTTGGTTTGCGCACTGAGGTAATGATTAATAGGGACAGTTGGGGG  300
Sbjct  786   CCTCGGTACTATTTTGTTGGTTTGCGCACTGAGGTAATGATTAATAGGGACAGTTGGGGG  845
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Query  661   GAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGT  702
Sbjct  1206  GAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGT  1247
```

Figure 4.7. (A) Scanning electron micrographs (SEM) of *Diploneis* sp. cell (A; scale bar = 1 μm) and (B) Nucleotide Blast alignment of the 702 bp amplified fragment using the primers 528F/1055R (see **Table 4.1)** with *Diploneis* sp. 18S strain (accession number KX981838.1)

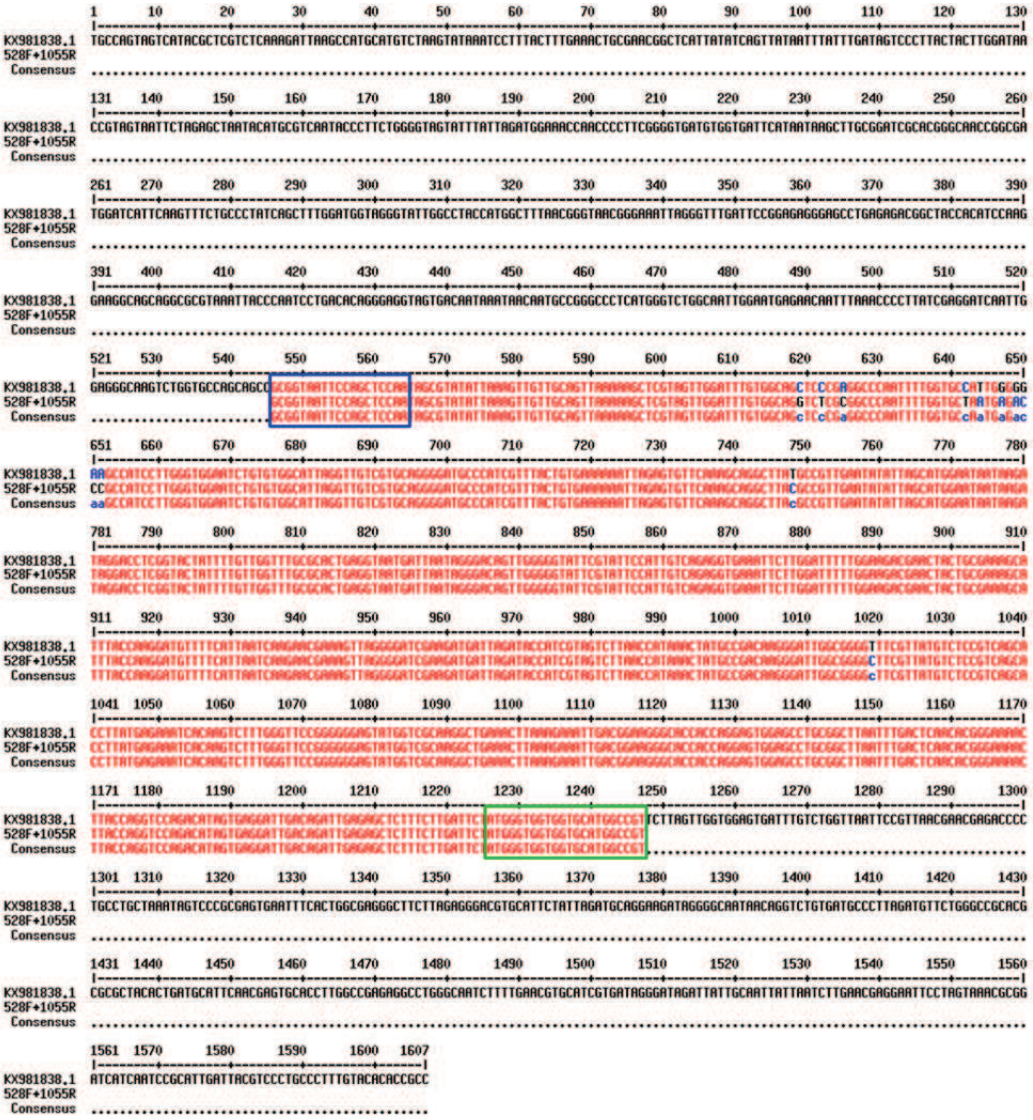


Figure 4.8. Alignment using the software MultiAlin between the 710 bp fragment and *Diploneis* sp. 18S sequence producing significant alignments in Nucleotide Blast (accession number KX981838.1). Color code: matches, red letters; mismatches, blue/black letters; gaps, black lines; primer 528F, blue rectangle; primer 1055R, green rectangle.

The fourth benthic species under analysis, *C. scutellum* (**Figure 4.9**), has already been studied and characterized by De Stefano et al., 2000. This diatom is quite similar to *Diploneis* sp. in terms of shape (oval valves) and dimension (length: 13 ± 2 µm, width: 7 ± 1 µm, depth: 2 ± 0.4 µm). The raphe is straight, thin, with coaxial proximal endings

converging in a very small central area, and distal endings terminate in reduced, submarginal areas. The striae are uniseriate in the centre of the valve face, biseriate at the margin and triseriate in the mantle, and have a radiate arrangement. Valve face areolae are arranged in an apically aligned pattern, with a sub-quadrangular shape and are internally occluded by rotate hymenes with one or two radial bars.

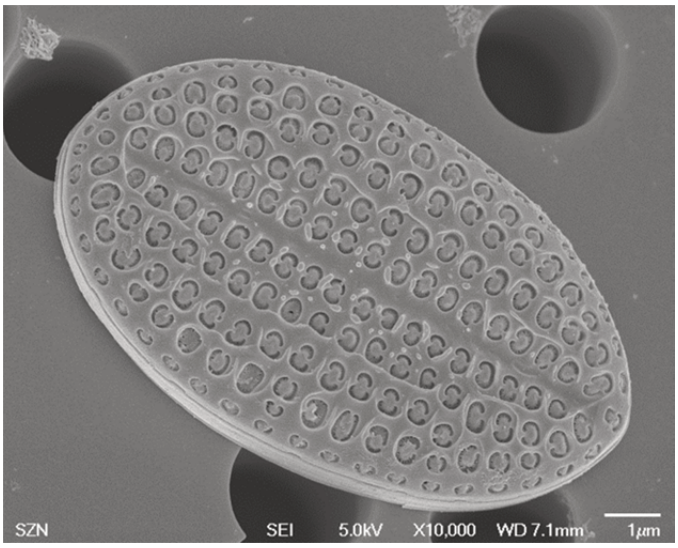


Figure 4.9. SEM image of *C. scutellum* cell (scale bar = 1 μm).

4.3.2 Feeding experiments

Sea urchins were fed for one month with *U. rigida* (control feeding) and the benthic diatoms incorporated into the agar substrate. **Figure 4.10** shows a picture of a sea urchin grazing on the agar substrate with the incorporated diatoms in the experimental tanks.

The biomass given to sea urchins was calculated as described above (see paragraph 4.2.3). The quantity of diatoms measured as pg C $cell^{-1}$ in a 0.0025 mm^2 area resulted quite similar among the four diatoms corresponding to 1.6 for *C. closterium*, 1.8 for *N. shiloi*, 1.6 for *Diploneis* sp. and 1.5 for *C. scutellum*.

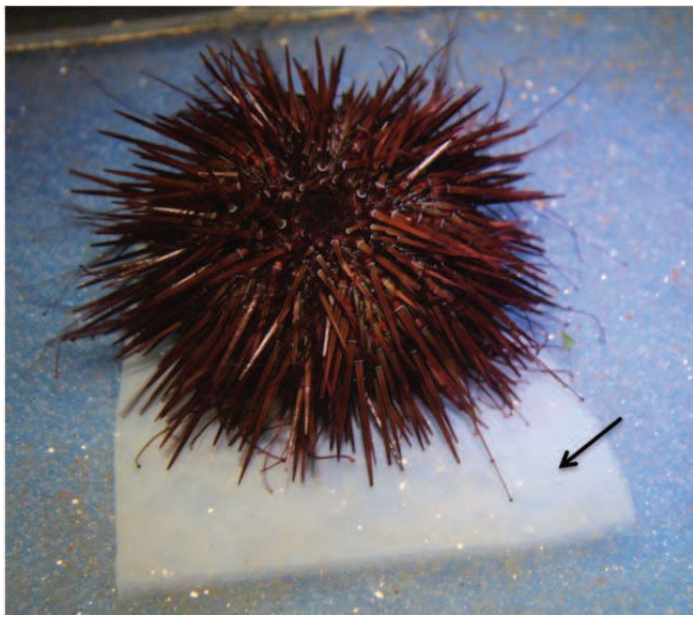


Figure 4.10. Sea urchin grazing on diatom biofilm incorporated in agar substrate (indicated by the black arrow).

After one month of feeding on these four benthic diatoms, eggs and sperms were collected from sea urchins fed with *U. rigida* and the four benthic diatoms. As soon as fertilization occurred, fertilization success and the time to reach first mitotic cleavage to obtain two blastomeres were measured in comparison with embryos deriving from sea urchins collected in the field at the beginning of the experiments (t_0). Fertilization success and first cleavage was 100% for all treatments and the control group. This data suggested that benthic diatoms under analysis did not affect the fertilization and the early stages of embryo development.

Embryonic development was then followed until the pluteus stage. Morphological observations showed that the percentage of abnormal embryos increased in sea urchins fed with *C. closterium* (39%), *N. shiloi* (55%) and *Diploneis* sp. (42%) (p value < 0.001) in comparison to *U. rigida* (control) diet (**Table 4.2**). On the contrary, feeding on *C.*

scutellum showed percentage of abnormal plutei very close to the control (p value > 0.05), with significant differences with respect to the other diatoms (p value < 0.001).

Table 4.2. The percentage of fertilization, cleavage (1 hpf), and abnormal plutei (48 hpf) in embryos from t_0 (sea urchins from the environment) and sea urchins fed with *U. rigida*, *C. closterium*, *N. shiloi*, *C. scutellum* and *Diploneis* sp. Data reported as means ± SD (N= 20).

Samples	%		
	Fertilization	Cleavage	Abnormal plutei
t_0	100	100	10 ± 1.9
U. rigida	100	100	9.4 ± 3.8
C. closterium	100	100	39 ± 10.2
N. shiloi	100	100	54.7 ± 8.1
C. scutellum	100	100	12.5 ± 4.5
***Diploneis* sp.**	100	100	47.9 ± 9.8

Of the three diatoms tested, *N. shiloi* induced the highest percentage of abnormal plutei (p value < 0.001), and was thus the most toxic benthic diatom for sea urchins (**Figure 4.11**). In particular, *C. closterium*, *N. shiloi* and *Diploneis* sp. induced the same malformations, which principally affected the arms, spicules and apices, in comparison with control embryos (**Figure 4.12**).

To confirm that sea urchins had really fed on diatoms, the content of faecal pellets collected from sea urchins fed with *C. closterium* (taken as an example for all diatoms) were also analyzed by means of SEM. These observations showed the presence of silica frustules in the faecal pellets, confirming that sea urchins had effectively eaten the diatoms (**Figure 4.13**).

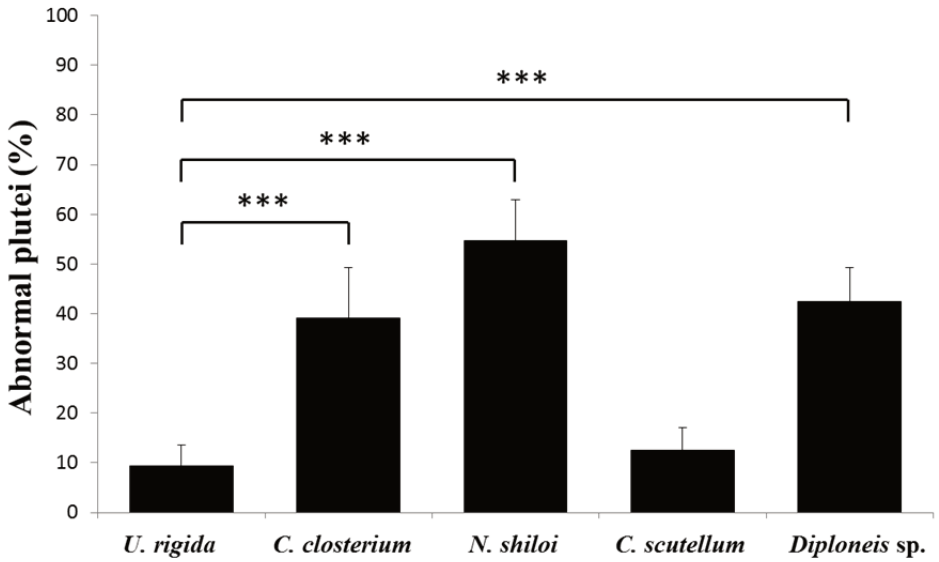


Figure 4.11. Percentage of abnormal plutei (embryos at 48 hpf) deriving from sea urchins fed with the four benthic diatoms and the control diet. Data are reported as means ± SD (N= 20/group). One-Way ANOVA, followed by Tukey's post-hoc test (*p<0.05, **p<0.01, ***p<0.001).

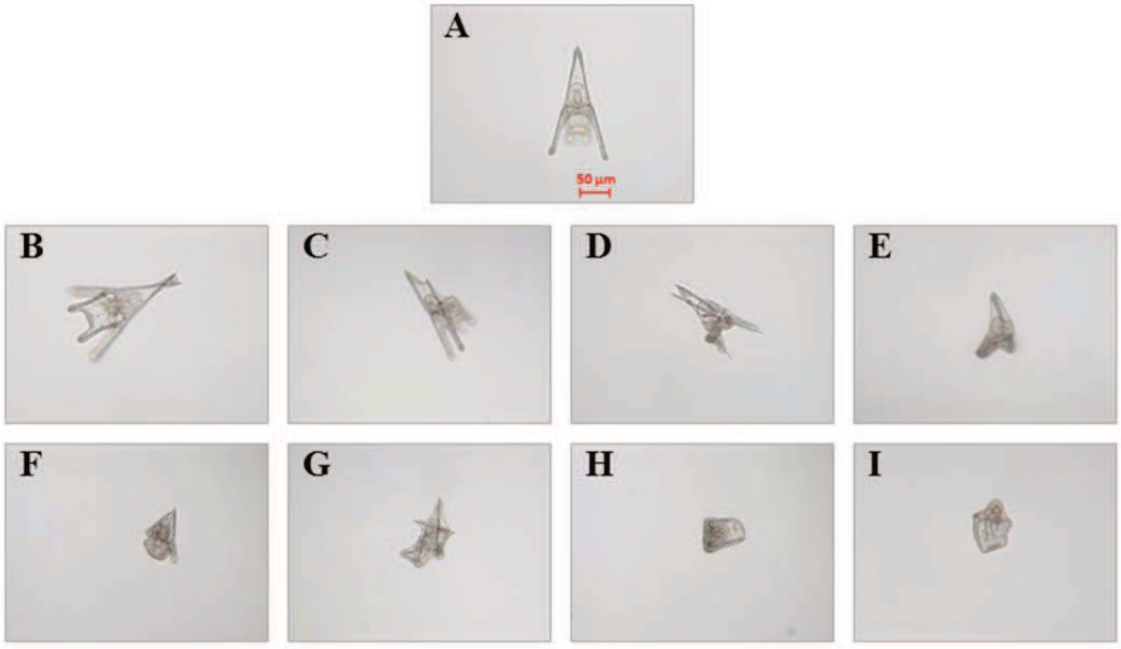


Figure 4.12. Examples of malformations observed in *P. lividus* plutei spawned from adults fed for one month with *C. closterium*, *N. shiloi* and *Diploneis* sp. (B-I) in comparison with (A) control embryos spawned from adults fed with *U. rigida*. Such plutei showed (B) a poorly-formed apex with (C-D) spicules that appeared crossed at the apex or disjoined at the tip, or with (3D) poorly-formed and degraded arms. Some embryos showed (E-G) an abnormal trunk, and (H) delayed (gastrula stage) and (I) abnormal development. Scale bar = 50 µm.

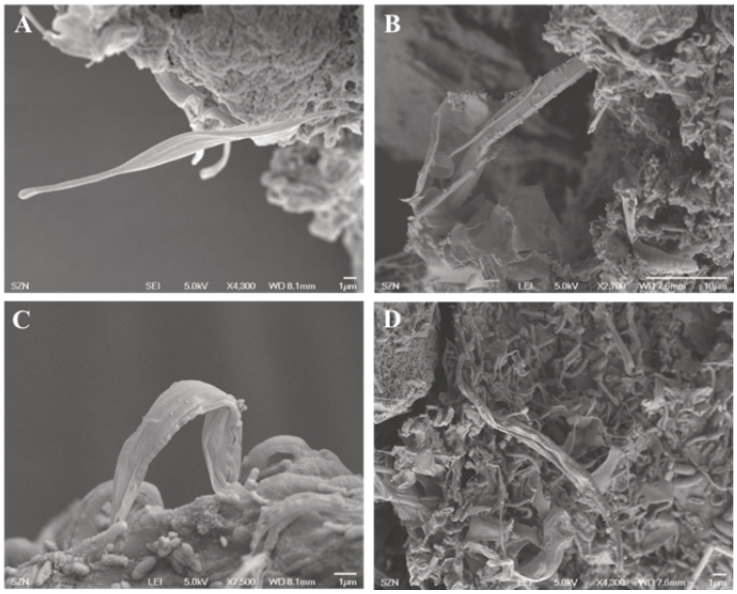


Figure 4.13. SEM micrographs of (A-D) faecal pellets from adult sea urchins fed with the benthic diatom *C. closterium*. Scale bar = 1/10 μm, depending on the magnification.

4.3.3 ^{1}H-NMR analysis of metabolites from sea urchin gonads

^{1}H-NMR spectra were obtained from aqueous extracts of gonad tissues from five adult sea urchins fed with *U. rigida* (control), *C. closterium*, *N. shiloi*, *C. scutellum* and *Diploneis* sp. The primary peaks in the spectra were assigned to individual metabolites. Since feeding experiments were performed in different times, metabolomic analysis was firstly applied on sea urchin gonads from sea urchins fed with *C. closterium* and *N. shiloi*. Some metabolite classes were identified, including acetoacetate, ATP, choline, lactate, glucose and amino acids (valine, leucine, isoleucine, alanine, threonine, arginine, lysine, glutamate, glutamine, aspartate, glycine, tyrosine, phenylalanine, tryptophan and histidine).

OPLS-DA plot (45% of the total variance) showed that the control and two treated groups clustered in separate classes, with a slight overlap between the two treated

groups (**Figure 4.14A**), suggesting the presence in these three groups of statistically different levels of metabolites (**Figure 4.14B**). In particular: i) the levels of acetoacetate and of six amino acids such as tyrosine, proline, valine, isoleucine, leucine and lysine, and acetoacetate were higher in the *N. shiloi* group when compared to the control group, and ii) their levels were even higher in the *C. closterium* group when compared to the *N. shiloi* group. On the other hand, the levels of tryptophan decreased after feeding on both benthic diatoms and were lower in the *C. closterium* group compared to the *N. shiloi* group. Finally, the levels of arginine and alanine were higher in the treated groups when compared to the control group and, even higher in the *N. shiloi* group compared to the *C. closterium* group.

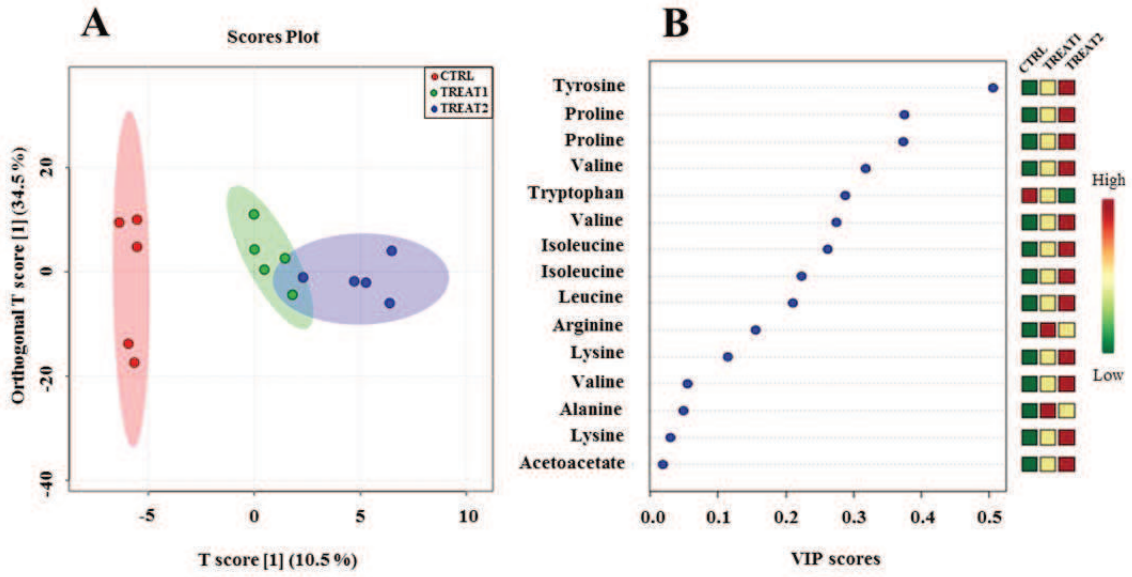


Figure 4.14. OPLS-DA (A) and VIP (B) plots (where the metabolites increased or decreased) of aqueous extracts from gonad tissues from adults sea urchin *P. lividus* after one month of feeding with *U. rigida* (feeding control, reported as CTRL), *N. shiloi* (reported as TREAT1) and *C. closterium* (reported as TREAT2).

^{1}H-NMR spectra were also obtained from lipophilic extracts of gonad tissues. Phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, linoleic acid, cholesterol and other unassigned lipids were found in the gonads of *P. lividus*.

OPLS-DA plot (38.1% of the total variance) showed that the control and two treated groups clustered in separate classes with a slight overlap between the control group and the two treated groups (**Figure 4.15A**), and a total overlapping of the *N. shiloi* and *C. closterium* groups. These data suggest the presence in these three groups of statistically different levels of metabolites between the control and the two treated groups, and metabolites with similar levels between the two treatments. In fact: i) the levels of linoleic acid and cholesterol were higher in the *N. shiloi* group when compared to the control group, and ii) their levels were even higher in the *C. closterium* group when compared to the *N. shiloi* group. The levels of phosphatidylcholine, sphingomyelin and other lipids decreased after feeding on both benthic diatoms and were lower in the *N. shiloi* group compared to the *C. closterium* group; whereas the levels of phosphatidylethanolamine and phosphatidylcholine $POCH_2$ were lower in the *N. shiloi* group compared to the *C. closterium* group (**Figure 4.15B**). Considering only fatty acids, some fatty acids such as linoleic acid and other fatty acids, were higher in the *N. shiloi* group and even higher in the *C. closterium* group when compared to the *N. shiloi* group (**Figure 4.15C**). On the other hand, other fatty acids decreased in *N. shiloi* and increased in *C. closterium* groups, with respect to the control group.

Considering slow growing species, metabolomic analysis was done separately for each species versus the control group, because they displayed different increasing/decreasing levels of metabolites in sea urchin gonads. Score plot by PCA analysis (53.7% of the

total variance) showed that the control and treated group with *Diploneis* sp. clustered in two well separated classes, suggesting the presence in these two groups of statistically different levels of metabolites (**Figure 4.16A**). In particular, the levels of trimethylamine, 2-hydroxybutyrate, ornithine, lactate, malate and the amino acids lysine, leucine/isoleucine, hydroxylysine, valine, hydroxyproline, phenylalanine were lower in the *Diploneis* sp. group when compared to the control group; only the levels of citrulline increased after feeding on this benthic diatom (**Figure 4.16B**).

Control and treated groups with *C. scutellum* also clustered in separate classes (50.5% of the total variance in score plot by PCA analysis), with a slight overlap (**Figure 4.16C**). In this case the levels of three metabolites (lactate, pyruvate and 2-hydroxybutyrate) decreased in samples treated with *C. scutellum*, together with several amino acids, such as leucine/isoleucine, lysine, hydroxylysine, valine, hydroxyproline, phenylalanine and tyrosine. On the contrary, proline and citrulline increased after feeding on this benthic diatom (**Figure 4.16D**).

^{1}H-NMR spectra were also obtained from lipophilic extracts of gonad tissues. Score plot by PCA analysis (90.4% of the total variance) showed that there was no clear separation between the control and treated group with *Diploneis* sp. (**Figure 4.17A**). In particular, feeding on this benthic diatom increased the levels of cholesterol, phosphatidylcholine, phospholipids and acyl groups of fatty acids and ω3 fatty acids in the sea urchin gonads (**Figure 4.17B**).

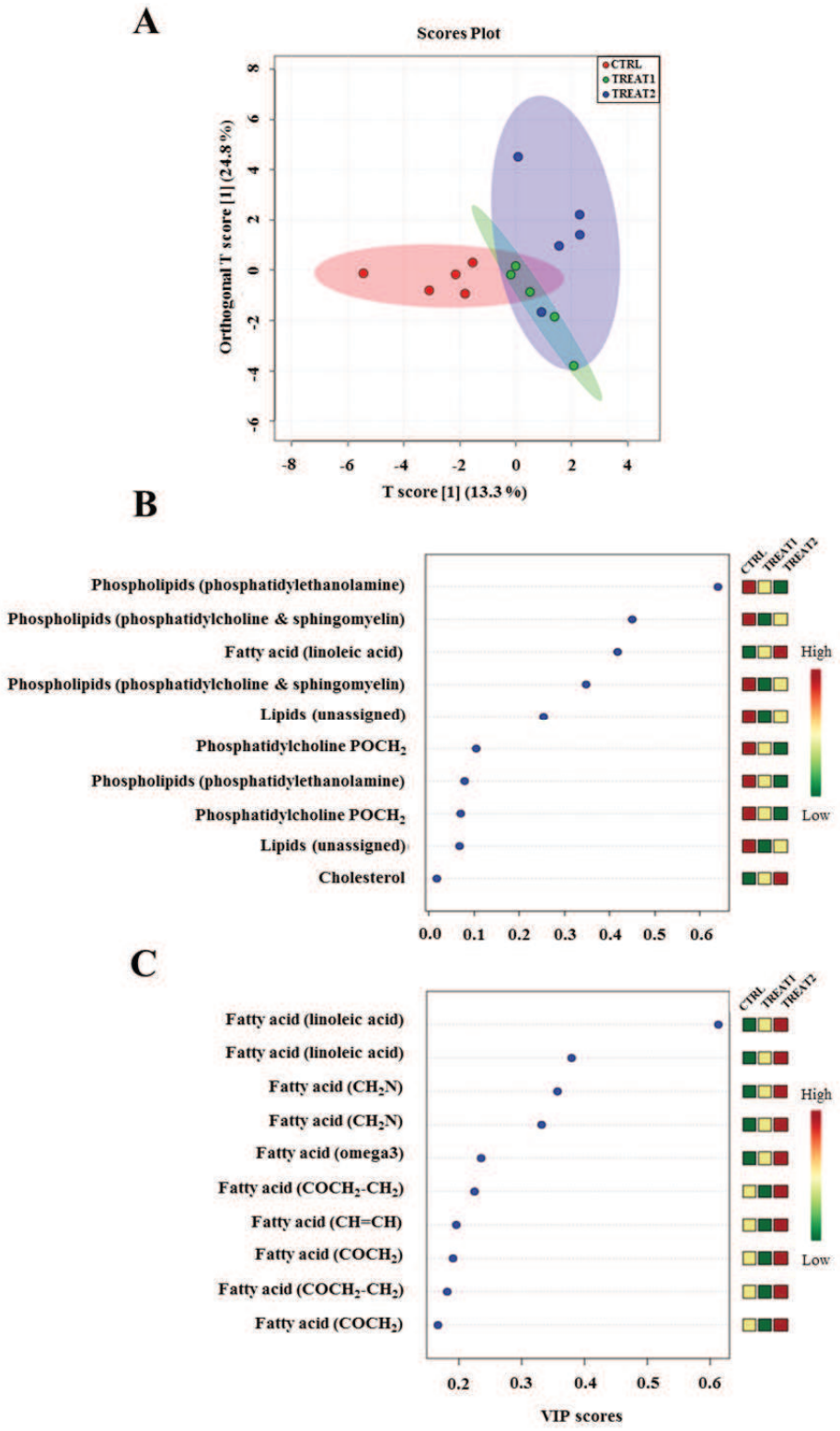


Figure 4.15. OPLS-DA (A) and VIP plots of lipids (B) and fatty acids (C) from lipophilic extracts of gonad tissues from adults sea urchin *P. lividus* after one month of feeding with *U. rigida* (feeding control, reported as CTRL), *N. shiloi* (reported as TREAT1) and *C. closterium* (reported as TREAT2).

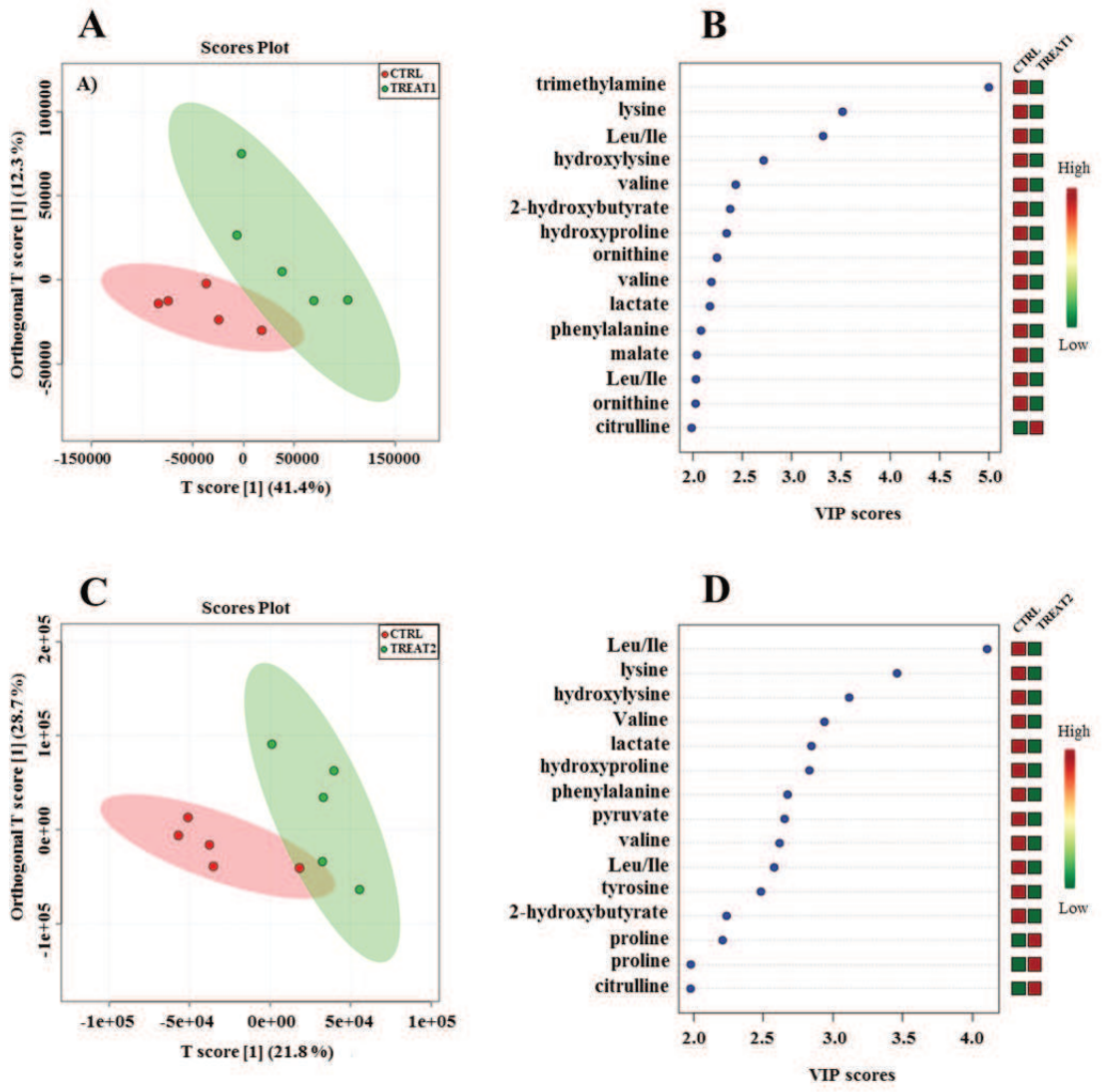


Figure 4.16. Aqueous phase. OPLS-DA (A) and VIP (B) plots from *Diploneis* sp. (reported as TREAT1) samples and OPLS-DA (C) and VIP plots (D) from *C. scutellum* (reported as TREAT2) samples with respect to *U. rigida* samples (control feeding, reported as CTRL).

When the control and treated group with *C. scutellum* were compared, OPLS-DA plot (94.0% of the total variance) showed that the control and treated group clustered in two well separated classes (**Figure 4.17C**). Almost all lipid levels increased (cholesterol, linoleic acid, triglycerides and acyl groups of fatty acids and ω3 fatty acids with the only exception of phospholipids (**Figure 4.17D**).

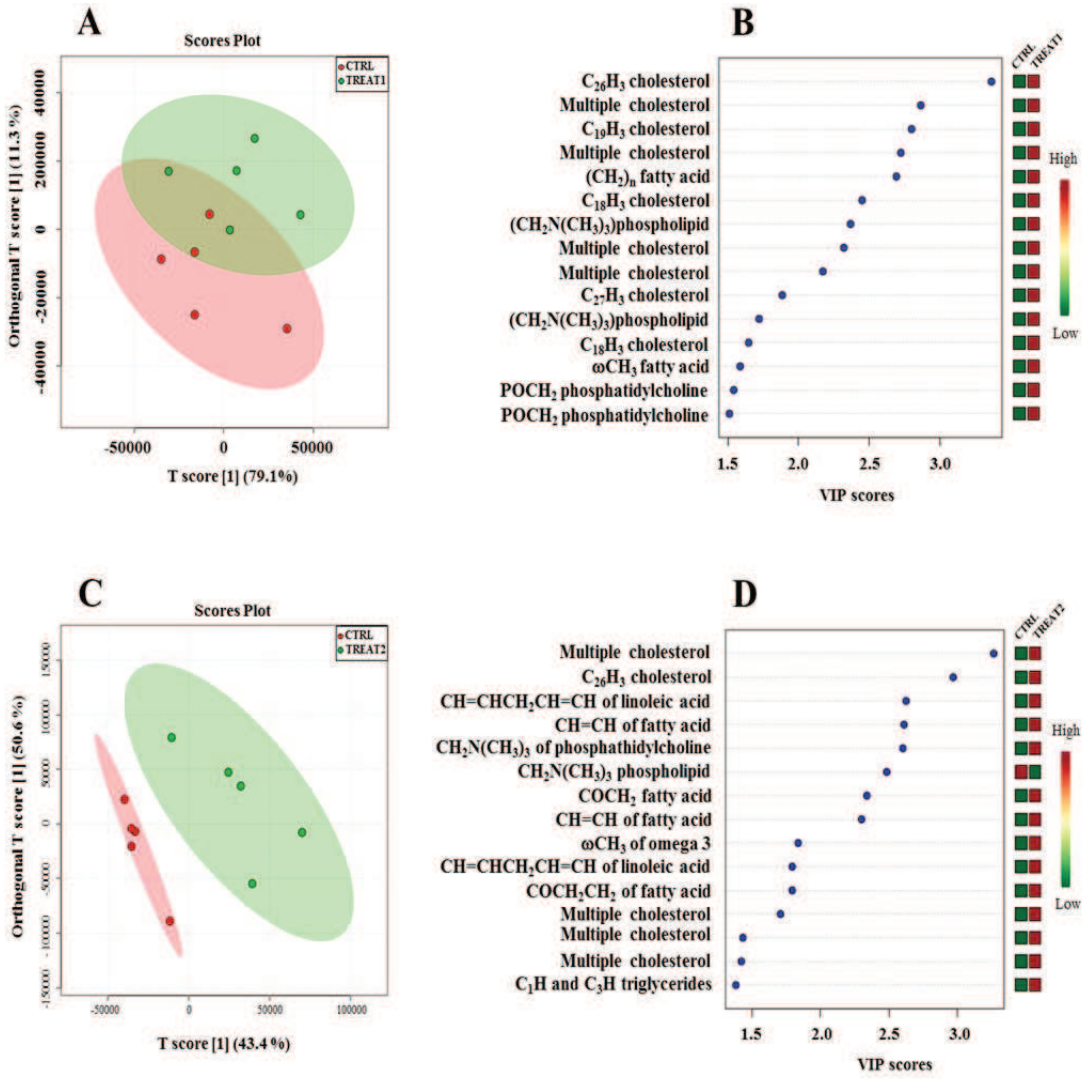


Figure 4.17. Lipophilic phase. OPLS-DA (A) and VIP (B) plots from *Diploneis* sp. (reported as TREAT1) samples and OPLS-DA (C) and VIP plots (D) from *C. scutellum* (reported as TREAT2) samples respect *U. rigida* samples (the control feeding, reported as CTRL).

4.3.4 Transcriptomic assembly

According the morphological results showing that only *C. scutellum* had no toxic effect on *P. lividus* embryonic development, *de novo* transcriptome assembly was performed on embryos at the pluteus stage deriving from adults fed on *C. closterium*, *N. shiloi* and

Diploneis sp., in comparison with the control (embryos deriving from adults fed on *U. rigida*).

The raw assembled transcriptome resulting from embryos at the pluteus stage deriving from the feeding experiment with fast growing species included almost 121 Mbp in 192493 transcripts grouped in 126941 genes. The GC mean content was 41.37%. The average and median contig length were 357 bp and 627 bp, respectively.

The transcriptome assembly using samples from adult sea urchins fed with *Diploneis* sp. included 761356 transcripts grouped in 312958 genes. The GC mean content was 37.67%. The average and median contig lengths were 402.39 bp and 301 bp, respectively (**Table 4.3**).

Table 4.3. Transcripts and genes number, GC content, contig length average and median reported for both RNA-seq experiments (1st experiment: *C. closterium* and *N. shiloi* versus *U. rigida*; 2nd experiment: *Diploneis* sp. versus *U. rigida*).

	1st experiment	2nd experiment
Transcripts number	192493	761356
Genes number	126941	312958
GC content (%)	41.37	37.67
Contig length average (bp)	357	402.39
Contig length median (bp)	627	301

All analyses were conducted on *de novo* assembled genes and the biological replicates clustered according to the experimental design.

Figure 4.18 reports the BLASTx top hit species distribution of matches for all the transcriptomes with known sequences and indicates that the majority of *P. lividus* contigs (reads) showed the highest homology with *Strongylocentrotus purpuratus* (18.6%). The other most represented species included *Exaiptasia pallida* (5.4%),

Acropora digitifera (4.2%), *Crassostrea gigas* (2.2%), *Lingula anatina* (2.2%) and *Saccoglossus kowalevskii* (2.2%). All alignments were carried out setting the E-value thresholds at ≤ 1e-5 values.

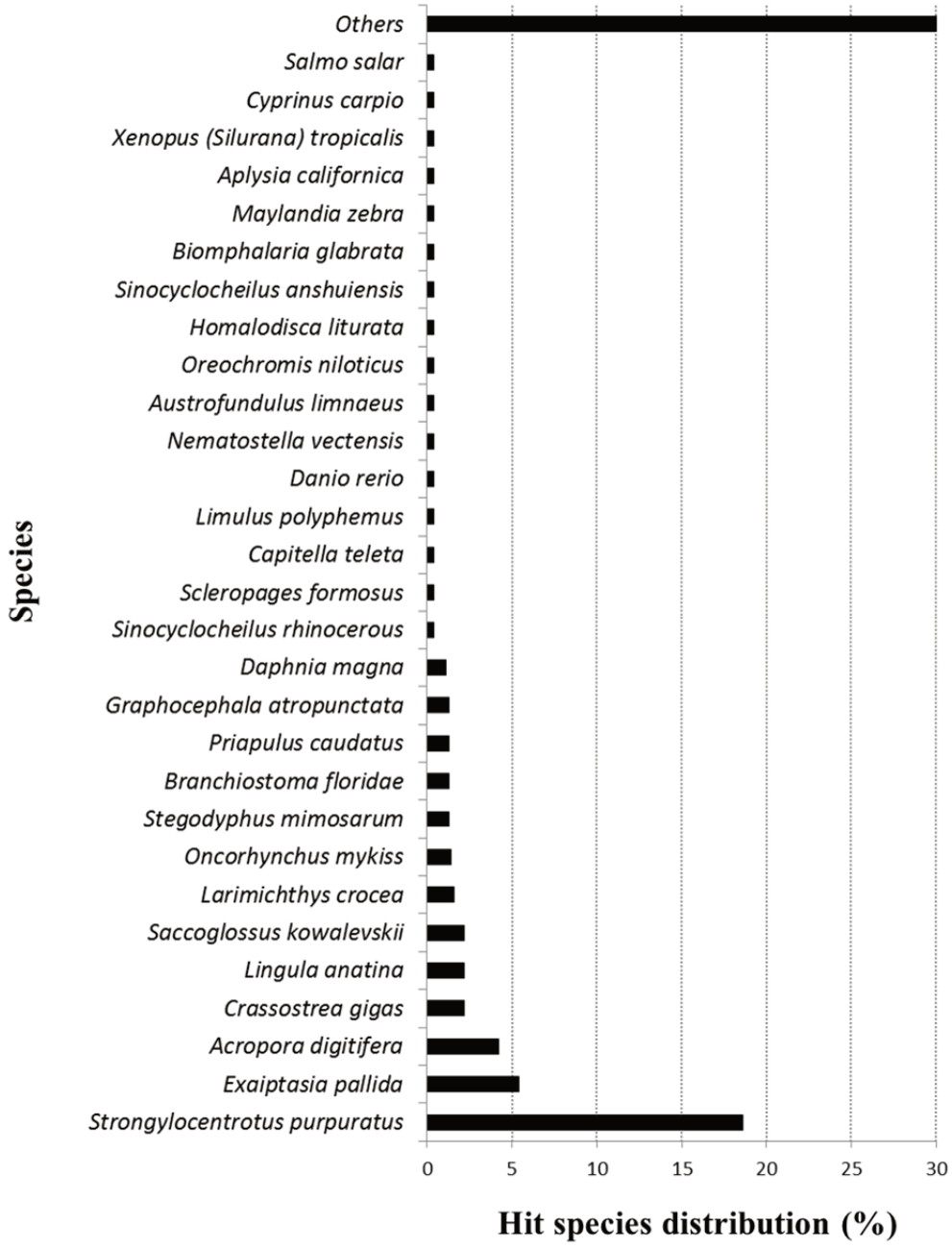


Figure 4.18. Blastx top hit species distribution of matches with known sequences.

4.3.5 Differentially expressed genes in *P. lividus* plutei after feeding experiments in RNA-seq

i) *N. shiloi* and *C. closterium* versus *U. rigida*

Differentially expressed genes were identified between the three conditions: embryos at the pluteus stage spawned by adults fed for one month with *N. shiloi* and *C. closterium*, compared to those fed with *U. rigida* as control, considering three biological replicates. Using the R Bioconductor package DESeq2, the numbers of DE genes in pairwise comparisons were counted, discriminating those that were up and down-regulated. The total number of DE genes was: 1285 between plutei from sea urchins fed with *N. shiloi* compared to controls fed with *U. rigida*; 2386 between plutei from sea urchins fed with *C. closterium* compared to control; 303 between plutei from sea urchins fed with *N. shiloi* compared with those fed with *C. closterium*. For all these genes we found the following DE annotated genes, considering FDR ≤ 0.05, fold change > 1.5 for up-regulated and fold change < -1.5 for down regulated.

In the case of plutei spawned from sea urchins fed with *N. shiloi*, 217 total genes were identified, of which 113 were up-regulated (with a range of fold-changes between 1.8 and 3.6) and 104 down-regulated (with a range of fold-changes between -1.7 and -12) compared to the control. Among these genes, some showed very high values of fold changes, such as the two down regulated genes *sperm flagellar 2* and *centrosomal of 170 kDa B isoform X7*.

Plutei from sea urchins fed with *C. closterium* displayed 670 total DE genes of which 541 transcripts were up-regulated (with a range of fold-changes between 1.7 and 20) and 129 down-regulated (with a range of fold-changes between -1.5 and -10). Also in this

case, some genes showed very high values of fold changes: the down regulated genes *vacuolar sorting-associated 13C- partial*, *centromere W* and *uncharacterized protein LOC589705* and the up regulated genes *cleavage stimulation factor subunit 1*, *fibrocystin-L* and *cAMP-responsive element-binding -like 2*.

Furthermore, comparing the two treatments, 303 DE genes were found, of which 177 up-regulated genes (with a range of fold-changes between -1.7 and -30) and 129 down-regulated (with a range of fold-changes between -1.6 and -20). Two genes were strongly down-regulated, *CD9 antigen* and *leukocyte elastase inhibitor-like isoform X3* and two strongly up-regulated, *isocitrate dehydrogenase [NADP] mitochondrial-like* (35.8-fold) and *peptidyl-prolyl cis-trans isomerase E* (**Figure 4.19**).

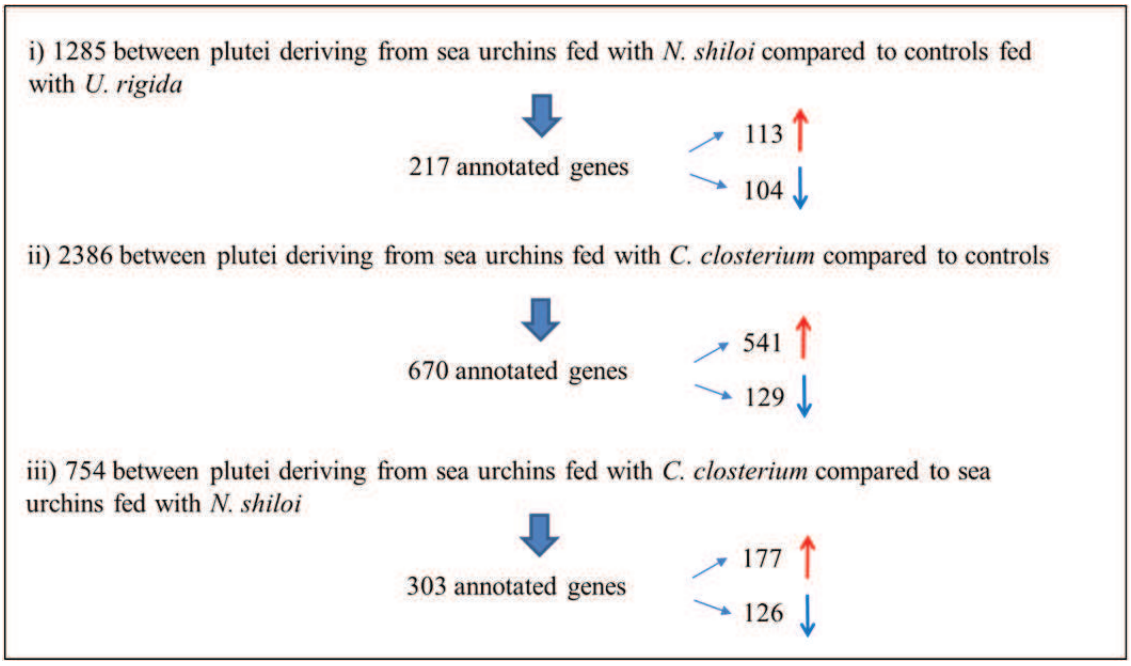


Figure 4.19. DE genes identified among the three conditions: *N. shiloi* versus *U. rigida*, *C. closterium* versus *U. rigida* and *N. shiloi* versus *C. closterium*. Red arrows: up-regulation, blue arrows: down-regulation. FDR ≤ 0.05, fold change > 1.5 for up-regulated genes and fold change < -1.5 for down regulated genes.

To identify the pathways in which these genes were involved, a GO term enrichment analysis was performed using DE genes. Twenty-seven GO terms were enriched including 12 in biological process (BP) followed by 10 in cellular component (CC) and 5 in molecular function (MF) (p value < 0.05). Overrepresented GO categories, highlighted in red (**Figure 4.20**), included binding, catalytic activity, cellular processes, metabolic processes and regulation of biological processes.

ii) *Diploneis* sp. versus *U. rigida*

Using the R Bioconductor package DESeq2, the numbers of DE genes were counted in pairwise comparisons, discriminating those that were up-/down-regulated. Transcriptomic analysis identified 101395 DE genes between embryos at the pluteus stage spawned by adults fed for one month with *Diploneis* sp., compared to those fed with *U. rigida* as control, including three biological replicates. All genes with p values greater than 0.05 were extracted. All the results showed that i) the triplicates were very similar between them in both control and treated sample; ii) there was a clear separation between control and treated sample, suggesting a great number of down- and up-regulated genes in treated sample compared to the control. In fact, as reported in **Figure 4.21**, 1393 genes were DE with FDR ≤ 0.05, of which 665 genes were up-regulated (fold change ≥ 1.5) and 728 were down-regulated (fold change ≤ 1.5), compared to the control. After the annotation 415 genes were found: 221 genes up-regulated (with a range of fold changes between 1.8 and 90) and 194 genes down-regulated (with a range of fold changes between 1.8 and 95).

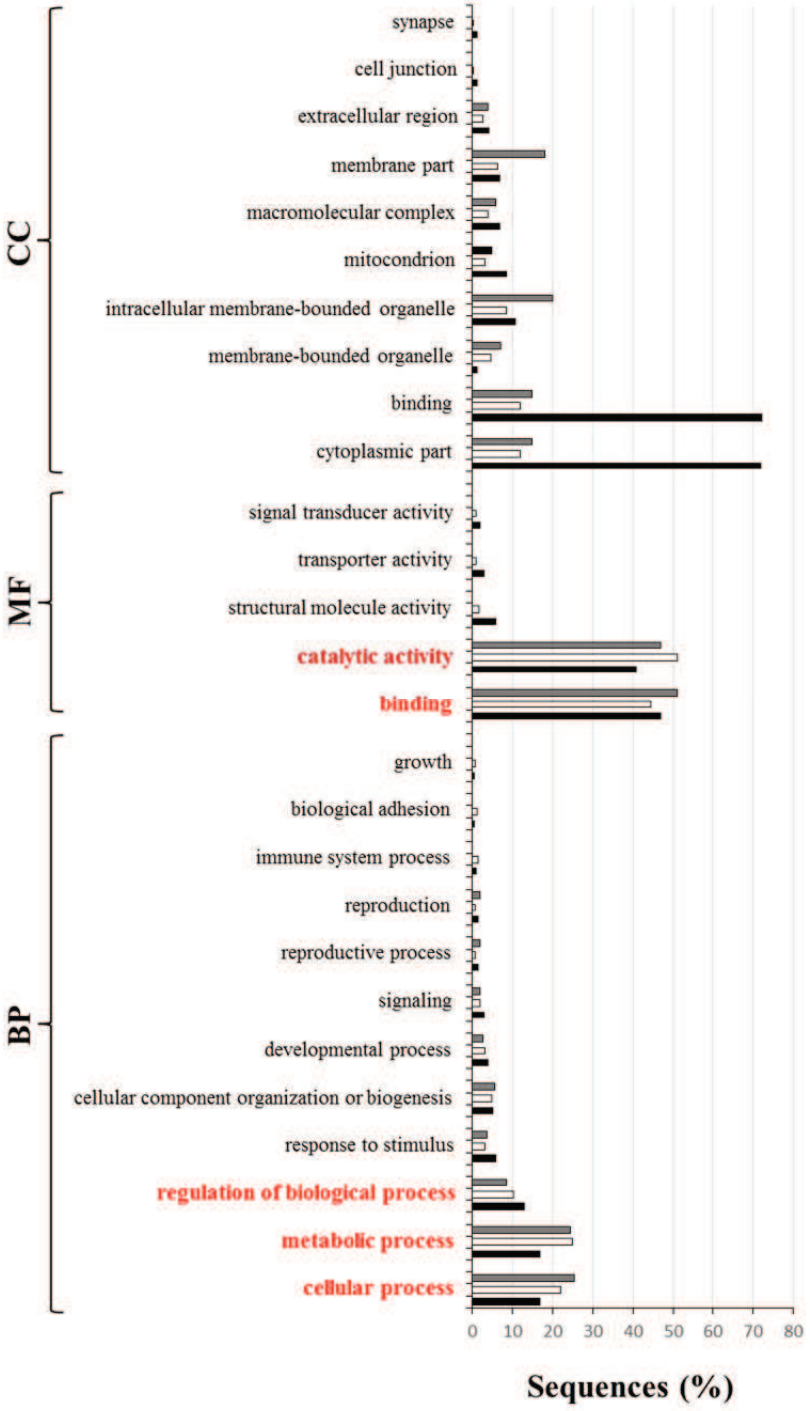


Figure 4.20. Overrepresented GO terms of sea urchin plutei after feeding experiments with the two benthic diatoms, *N. shiloi* and *C. closterium*, in comparison with *U. rigida* (feeding control), in the three major functional categories: biological process (BP), molecular function (MF) and cellular component (CC). The tree bars represent: black bar "control versus *N. shiloi*", white bar "control versus *C. closterium*" and grey bar "*N. shiloi* versus *C. closterium*". The most representative GO terms for each functional category are highlighted in red.

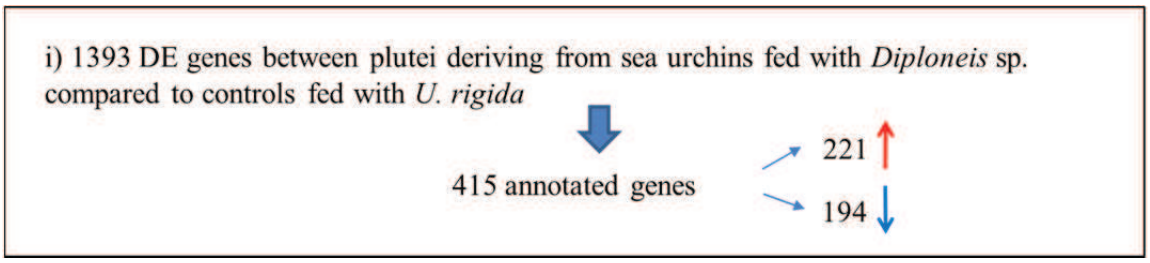


Figure 4.21. DE genes found between *Diploneis* sp. versus *U. rigida*, Red arrows: up-regulation, blue arrows: down-regulation. FDR ≤ 0.05, fold change > 1.5 for up-regulated genes and fold change < -1.5 for down regulated genes.

Some of annotated genes showed very high values of fold changes, such as the up-regulated *glutathione S-transferase theta-1*, *cytochrome oxidase subunit I*, *NADH dehydrogenase subunit 1* and *phosphatidylinositol 4-kinase beta-like* and the down-regulated genes *nuclear migration protein nudC*, *heme-binding protein 2*, *tyrosine-protein kinase Src42A-like* and *gamma-glutamyltranspeptidase 1*.

As already reported for the 1st experiment, a GO term enrichment analysis was performed to identify the pathways in which DE genes were involved (**Figure 4.22**). Seventy-seven GO terms were enriched including 29 in BP followed by 26 in MF and 22 in CC (p value < 0.05). Overrepresented GO categories included oxidation-reduction process, translation, proton and electron transmembrane transport, ATP/RNA/GTP/heme/calcium and metal ion binding, NADH dehydrogenase activity, cytochrome c oxidase. Moreover, these genes were mainly localized in mitochondrial membrane, cytoplasm, nucleus, cytosolic large ribosomal subunit and microtubule.

Figure 4.22. Overrepresented GO terms of sea urchin plutei after feeding experiments with *Diploneis* sp. in the three major functional categories: biological process (BP), molecular function (MF) and cellular component (CC). The most representative GO terms are highlighted in red.

4.3.6 Differentially expressed genes by *Real Time qPCR*

The expression levels of fifty genes (see **Figure 4.2**; Romano et al., 2011; Varrella et al., 2014, 2016a; Ruocco et al., 2016, 2017b) were followed by *Real Time qPCR*.

The histograms reported in **Figures 4.23** show the relative expression ratios of the analyzed genes with respect to control embryos in sea water without PUAs. Only expression values greater than a 1.5-fold difference with respect to the controls were considered significant. **Table 4.4** summarizes the results, highlighting up-regulated genes in red and down-regulated genes in blue.

- Stress genes

All fourteen genes were targeted by benthic diatoms with the exception of *p38MAPK*. In particular, *C. closterium* and *N. shiloi* up-regulated the expression levels of *hsp70*, *hsp60*, whereas *Diploneis* sp. down-regulated these genes, as well as *hsp56*, which is also down-regulated by *N. shiloi* and up-regulated by *C. closterium*. Three genes were equally affected by all diatoms with an up-regulation of *GS* and *cytb* and down-regulation of *HIF1A*. *C. closterium* and *N. shiloi* down-regulated *MTase* and *p53*, whereas *Diploneis* sp. up-regulated these genes. Moreover, *14-3-3ε* was up-regulated by meroplanktonic species and, on the contrary, *Diploneis* sp. down-regulated the expression levels of this stress gene. The expression level of of *NF-kB* was differentially affected: *C. closterium* down-regulated this gene, compared to *N. shiloi* and *Diploneis* sp. In which this gene was up-regulated. Furthermore, *C. closterium* and *Diploneis* sp. up-regulated *caspase-8*, whereas *N. shiloi* down-regulated *caspase 3/7* with respect to the control. Finally, *Diploneis* sp. was the only diatom to significantly affect the expression level of *ERCC3* gene.

- Genes involved in skeletogenesis

All eight genes were switched on by benthic diatom diets. The three diatoms down-regulated *uni* gene. *SM30* was down-regulated by *C. closterium* and *N. shiloi* and up-regulated by *Diploneis* sp.; the same result was found for *BMP5-7*. *C. closterium* and *N. shiloi* had other three up-regulated common targets: *Nec*, *p19* and *Jun*. Moreover, *p16* was targeted only after feeding with *Diploneis* sp. and SM50 by *C. closterium*.

- Genes involved in development and differentiation

Among the twenty genes, analyzed by *Real Time q*PCR, only *BP10* and *JNK* were not targeted by the benthic diatoms. Common molecular targets for the three benthic diatoms were *Blimp* (up-regulated gene), *δ-2-catenin* and *GFI-1* (both down-regulated genes). Several genes were significantly affected by both *C. closterium* and *N. shiloi*. In fact, *Wnt6*, *nodal*, *FoxG*, *Foxo*, *OneCut* were up-regulated, whereas *FOXA* and *VEGF* were down-regulated. On the contrary, these two diatoms differentially regulated another two genes, *TCF7* and *TAK1*. *C. closterium* and *Diploneis* sp. targeted *Wnt8*, increasing and decreasing its expression level, respectively. The gene *sox9* was up-regulated only after feeding with *C. closterium*, whereas *Diploneis* sp. up-regulatred *Alix* and *Wnt5* and down-regulated *tcf4*.

- Genes involved in detoxification

All eight analyzed genes were switched on by the benthic diatom diets. *MT*, *MT5*, *MDR1* and *CAT* were up-regulated and *MT8* was down-regulated by all the three benthic diatoms. *MT4* represented a common target for *N. shiloi* and *Diploneis* sp., which was up-regulated and down-regulated, respectively. *MT6* was also a common target for the

three diatoms: *C. closterium* and *Diploneis* sp. decreased and *N. shiloi* increased its expression levels. Moreover, *C. closterium* and *N. shiloi* up-regulated *MT7* gene.

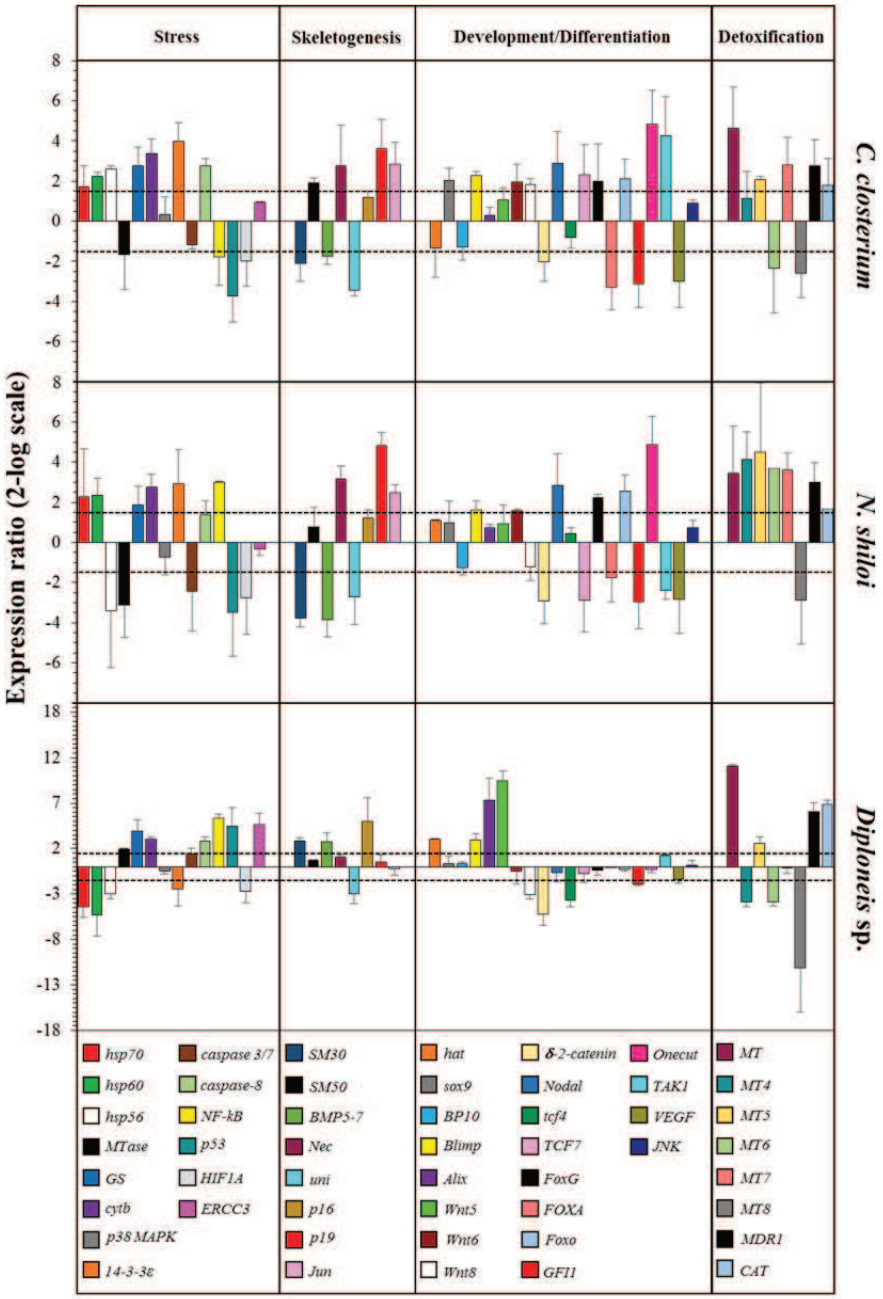


Figure 4.23. Histograms show the differences in expression levels of analyzed genes involved in different embryonic processes (see **Figure 4.2**), followed by *Real Time qPCR,* from embryos deriving from sea urchins fed with *C. closterium*, *N. shiloi* and *Diploneis* sp. with respect to the control diet, *U. rigida*. For *Diploneis* sp. data are shown with a different scale due the high values found in this case. Data are reported as a fold difference compared with control (mean ± SD, N= 3). Fold differences greater than ± 1.5 (see dotted horizontal guidelines indicating 1.5 and -1.5 values) were considered significant.

Table 4.4. Data of expression levels detected in embryos at 48 hpf deriving from sea urchins fed with *C. closterium*, *N. shiloi* and *Diploneis* sp. reported as a fold difference (in red up-regulated genes; in blue down-regulated genes) with respect to the control (represented by embryos deriving from adults of sea urchins fed with *U. rigida*). Fold differences greater than ± 1.5 were considered significant. Data are reported as mean ± SD, N=3.

		C. closterium	SD	*N. shiloi*	SD	*Diploneis* sp.	SD
Stress	*hsp70*	1.7	1.04	2.2	2.40	-4.4	1.13
	hsp60	2.2	0.19	2.3	0.82	-5.3	2.36
	hsp56	2.5	0.16	-3.4	2.83	-2.9	0.58
	MTase	-1.6	1.76	-3.1	1.62	2.0	0.28
	GS	2.7	0.91	1.8	0.95	3.9	0.07
	cytb	3.3	0.72	2.7	0.63	3.0	0.19
	p38 MAPK	0.33	0.89	-0.7	0.88	-0.5	1.84
	14-3-3 ε	3.9	0.90	2.9	1.70	-2.5	0.30
	caspase 3/7	-1.1	0.21	-2.4	1.98	1.4	0.07
	caspase-8	2.7	0.40	1.3	0.68	2.9	0.85
	NF-kB	-1.8	1.41	3.0	0.03	5.3	0.28
	p53	-3.7	1.31	-3.4	2.21	4.4	1.12
	HIF1A	-1.9	1.25	-2.7	1.84	-2.7	0.35
	ERCC3	0.9	0.03	-0.3	0.34	4.7	0.10
Skeletogenesis	*SM30*	-2.1	0.90	-3.7	0.43	2.9	1.25
	SM50	1.9	0.25	0.7	0.94	0.7	0.98
	BMP5-7	-1.7	0.41	-3.8	0.86	2.8	0.21
	Nec	2.7	2.00	3.1	0.67	1.1	0.84
	uni	-3.4	0.31	-2.7	1.36	-3.0	2.59
	p16	1.1	0.33	1.2	0.39	5.0	0.55
	p19	3.6	1.44	4.8	0.64	0.5	0.71
	C-jun	2.8	1.10	2.4	0.41	-0.2	0.42
Dev/Diff	*hat*	-1.3	1.46	1.0	0.03	3.1	0.61
	sox9	2.0	0.60	0.9	1.08	0.3	4.81
	BP10	-1.2	0.65	-1.2	0.38	0.3	0.71
	Blimp	2.2	0.19	1.6	0.43	3.0	1.13
	Alix	0.2	0.41	0.7	0.14	7.3	1.41
	Wnt5	1.0	0.59	0.9	0.97	9.4	0.49
	Wnt6	1.9	0.89	1.5	0.10	-0.5	1.04
	Wnt8	1.8	0.25	-1.2	0.66	-3.1	2.41
	δ-2-catenin	-2.0	0.97	-2.9	1.14	-5.2	0.49
	nodal	2.8	1.56	2.8	1.56	-0.7	2.05
	tcf4	-0.8	0.52	0.46	0.28	-3.7	1.28
	TCF7	2.3	1.51	-2.8	1.59	-0.7	1.27
	FoxG	1.9	1.86	2.2	0.15	-0.4	0.49
	FOXA	-3.2	1.12	-1.7	1.19	0.0	0.40
	Foxo	2.1	0.97	2.5	0.80	-0.3	0.71

	GFI1	-3.1	1.20	-2.9	1.32	-1.9	0.92
	OneCut	4.8	1.70	4.8	1.40	-0.3	0.50
	TAK1	4.2	1.94	-2.3	0.45	1.2	0.75
	VEGF	-3.0	1.30	-2.8	1.70	-1.3	0.78
	JNK	0.8	0.19	0.7	0.35	0.2	1.00
Detoxification	*MT*	4.6	2.08	3.4	2.34	11.1	0.49
	MT4	1.1	1.34	4.1	1.37	-3.9	0.07
	MT5	2.0	0.15	4.4	3.46	2.6	0.00
	MT6	-2.3	2.22	3.6	0.03	-3.9	0.38
	MT7	2.7	1.37	3.5	0.85	-0.1	0.21
	MT8	-2.5	1.21	-2.9	2.17	-11.1	1.28
	MDR1	2.7	1.29	2.9	0.97	6.0	0.14
	CAT	1.7	1.36	1.6	0.03	6.8	0.51

Some of these targeted genes were common to the genes from RNA-seq, confirming transcriptomic results. **Figure 4.24** shows some examples of these common genes: *FoxG*, *catenin*, *cytochrome*, *metallotionein* and *14-3-3ε*.

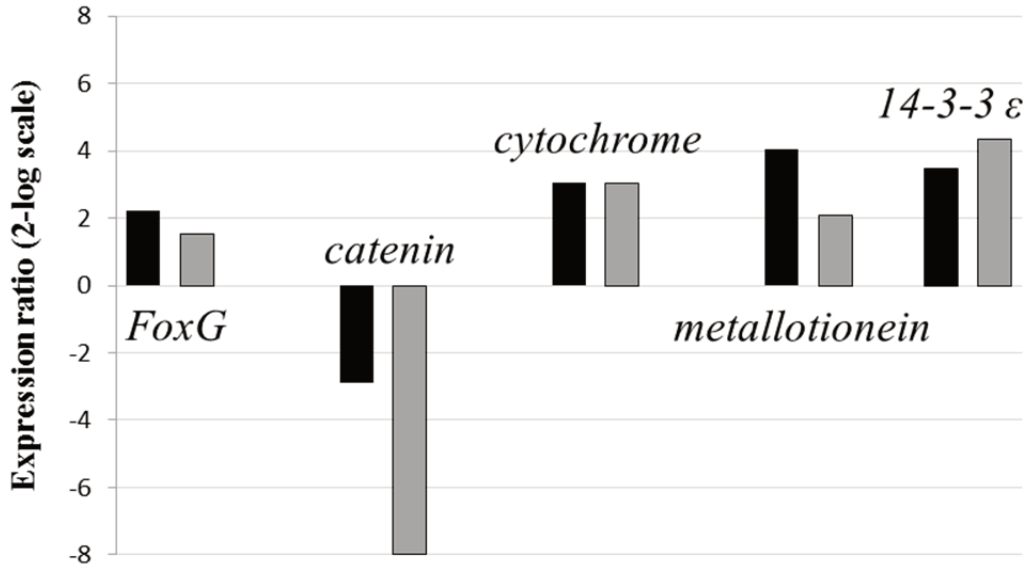


Figure 4.24. Five genes (*FoxG*, *catenin*, *cytochrome*, *metallotionein* and *14-3-3ε*) validated by *Real Time qPCR*. Black bars correspond to data from *Real Time qPCR* experiments; grey bars correspond to data from RNA-seq. Fold differences greater than ± 1.5 were considered significant.

4.4 Discussion

Feeding experiments revealed a noxious effect of three benthic diatom species (*C. closterium*, *N. shiloi* and *Diploneis* sp.) on embryos spawned from adult sea urchins fed for one month on these diets. Embryos showed morphological malformations affecting the arms, spicules and apex. These malformations were the same as those observed when sea urchin embryos were treated with some oxylipins, such as polyunsatured aldehydes (PUAs) and hydroxyacids (HEPEs), produced by planktonic diatoms (Varrella et al., 2014, 2016b). These oxylipins have been shown to affect embryonic development in several marine invertebrates, including copepods and the sea urchin *P. lividus* (Lauritano et al., 2016; Varrella et al., 2014, 2016a,b; Caldwell et al., 2009). Volatile organic compounds (VOCs), including PUAs, have been identified in several freshwater benthic diatoms, such as *Achnanthes biasolettiana* (Fink et al., 2006), *Gomphonema parvulum*, *Amphora veneta*, *Fragilaria* sp. and *Melosira varians* (Wendel and Jüttner, 1996) but not much is known on the effects of these compounds on grazing invertebrates. Very recently, Pezzolesi et al. (2017) highlighted the production of PUAs in three diatoms, *Tabularia affinis*, *Proschkinia complanatoides* and *Navicula* sp, commonly occurring in the microphytobenthic communities in temperate regions. The existence of a large family of PUAs, including some with four unsaturations, such as decatetraenal, undecatetraenal and tridecatetraenal, was observed for the first time.

Feeding on these diatoms also increased the levels of some lipids in the gonads of *P. lividus*. In particular, it has been observed a strong increase in linoleic acid and cholesterol, compared to when sea urchins were fed an *Ulva* diet. Gonads of this sea urchin are a rich source of polyunsatured fatty acids (PUFAs) (Archana and Babu, 2016;

Kabeya et al., 2017) and linoleic (18:2 ω6) and α-linolenic (18:3 ω3) acids are found in higher proportions in the gonads of *P. lividus* (Martínez-Pita et al., 2010). The increased lipids in *P. lividus* gonads in the present experiments could be due to the diet they were fed upon since diatoms are known to be rich in lipids, mainly PUFAs, usually comprising up to 15-25% of dry biomass (Levitan et al., 2014; Mangas-Sanchez et al., 2015; Yi et al., 2017). *C. closterium* is particularly rich in 16:0 and 16:1 (ω7) fatty acids that can comprise up to 64% of the total lipids (Song et al., 2015). Furthermore, a comprehensive study looking at the sterol composition of more than 100 diatom species revealed that cholesterol contributed for >90% of the total sterols in C. *closterium* and *N. shiloi* (Rampen et al., 2010).

Present findings also indicate an increase in the aqueous (polar) phase of acetoacetate and six amino acids tyrosine, proline, valine, isoleucine, leucine and lysine in the gonads of sea urchins fed with *C. closterium* and *N. shiloi*. Lower levels of these amino acids were found in control gonads from adults fed with *U. rigida*.

Essential amino acids such as phenylalanine, threonine, valine, lysine, leucine, isoleucine and histidine (about 32.1% of total amino acids) cannot be synthesized *de novo* by the organisms and thus must be supplied by the diet. The increase in essential amino acids (valine, isoleucine, leucine and lysine) after feeding with these benthic diatoms could be ascribed to the diatom diets. Diatoms, like other photoautotrophs synthesize a wide range of amino acids for building proteins and other compounds (Bromke, 2013). In fact, they are known to produce high levels of leucine and fairly abundant quantities of lysine (Werner, 1977; Bromke, 2013).

On the contrary, gonads from sea urchins fed with *C. scutellum* and *Diploneis* sp. showed a decrease of several essential amino acids (lysine, leucine, isoleucine, phenylalanine and valine) respect to the control diet. This difference could be explained taking into account the different periods in which sea urchins were collected for feeding experiments on fast- and slow-growing species (see paragraph 4.2.4). In fact, a seasonal variation has been observed in the amino acidic content of the sea urchin gonads (Liyana-Pathirana et al., 2002; Chen et al., 2013; Verachia et al., 2012). Among amino acids, both diatoms increased the amount of citrulline, an intermediate of the ornithine-urea cycle, which represents a key pathway for anaplerotic carbon fixation into nitrogenous compounds that are essential for diatom growth and for the contribution of diatoms to marine productivity (Allen et al., 2011).

Finally, *C. scutellum* was the only diatom increasing the levels of proline in sea urchin gonads. This amino acid is normally accumulated in diatom cells in osmotic stress conditions, protecting membranes against a salt-induced alteration in their hydration sphere (Schobert, 1977; Scholz and Liebezeit, 2012b). Moreover, it is also one of the most dominant free amino acid of the sea urchin gonad (Chen et al., 2013). Unfortunately, specific data on the amino acidic and protein compositions of benthic diatoms under analysis is not available, but it is known that benthic diatoms belonging to the genus Cylindrotheca are quite rich in proteins (Brown et al., 1995).

To date, knowledge on proteins and amino acid composition of gonads, eggs and larvae of echinoids are scarce and the possibility to modify their profiles through diet manipulations is still unknown and debated. For example, significant increase in the amino acid profile has been found in the gonads of *Strongylocentrotus droebachiensis*

when sea urchins were fed on the artificial soy-based diet (Liyana-Pathirana et al., 2001). On the contrary, Gago et al. (2016) have recently found very few differences in the protein content of *P. lividus* eggs, prisms and pre-plutei and the amino acid composition of eggs from captive broodstock fed prepared diets or those obtained from wild broodstock. However the present results indicated that diet can significantly affect lipid, amino acid and protein levels of cultured sea urchins.

Another interesting result is the large-scale genomic information on *P. lividus* generated in this study. Analysis of DE genes indicated that metabolic, cellular, reproductive, developmental, immune system, oxidation-reduction process, NADH dehydrogenase activity, cytochrome c oxidase, ATP/RNA/GTP/heme/calcium and metal ion binding, biological regulation and response to stimuli and biological adhesion processes were affected by feeding of adult sea urchins *P. lividus* on the benthic diatoms under analysis (**Figures 4.20-4.22**).

The interpretation of *de novo* transcriptomic results were also improved with the analysis by *Real Time qPCR* of a set of fifty genes previously used to study the response of *P. lividus* embryos to oxylipins produced by planktonic diatoms. These genes have key roles in different functional processes such as stress response, skeletogenesis, embryonic development, cell differentiation, morphogenesis and detoxification processes (see **Figure 4.2;** for further details on gene functions see Appendix A). Firstly, all fifty genes were molecular targets of the benthic diatoms, with the only exception of *p38MAPK*, *BP10* and *JNK* (see **Figure 4.23**, **Table 4.4**). Summarizing the *Real Time qPCR* experiments (**Figure 4.25**): i) twenty-two genes were targeted by all

three benthic diatoms; ii) thirty-five genes were common targets for *C. closterium* and *N. shiloi*; iii) six genes were specifically affected only by *Diploneis* sp.

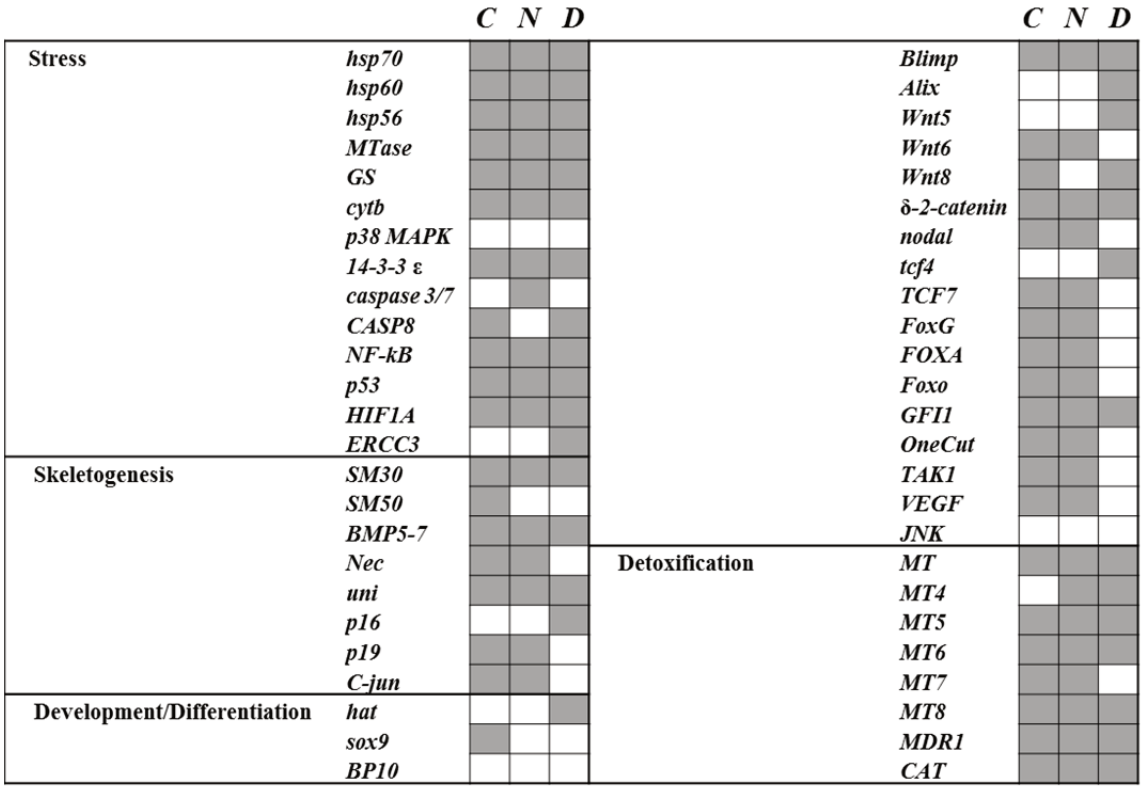


Figure 4.25. Schematic overview of *P. lividus* genes affected by the benthic diatoms under analysis. The grey squares indicate the genes with changing expression levels (up-regulated and down-regulated genes) with respect to the control. *C= C. closterium*, *N= N. shiloi*, *D= Diploneis* sp.

These data suggest that the plutei deriving from adults fed with *C. closterium* and *N. shiloi* were very similar, as also shown in the Heatmap reported in **Figure 4.26**. In fact, the molecular response to *Diploneis* sp. appeared quite different in comparison with the other two diatoms: *C. closterium* mainly up-regulated genes (26 up-regulated genes versus 13 down-regulated genes; see also **Table 4.4**) compared to *N. shiloi* (22 up-regulated genes versus 15 down-regulated genes) and *Diploneis* sp. (18 up-regulated genes versus 13 down-regulated genes). Even if there were no differences observed

between the three diatom diets at the morphological level, the molecular results (and mainly the *de novo* transcriptome) suggest that the toxic effect of feeding of *P. lividus* on the diatom *C. closterium* was higher than that of *N. shiloi*.

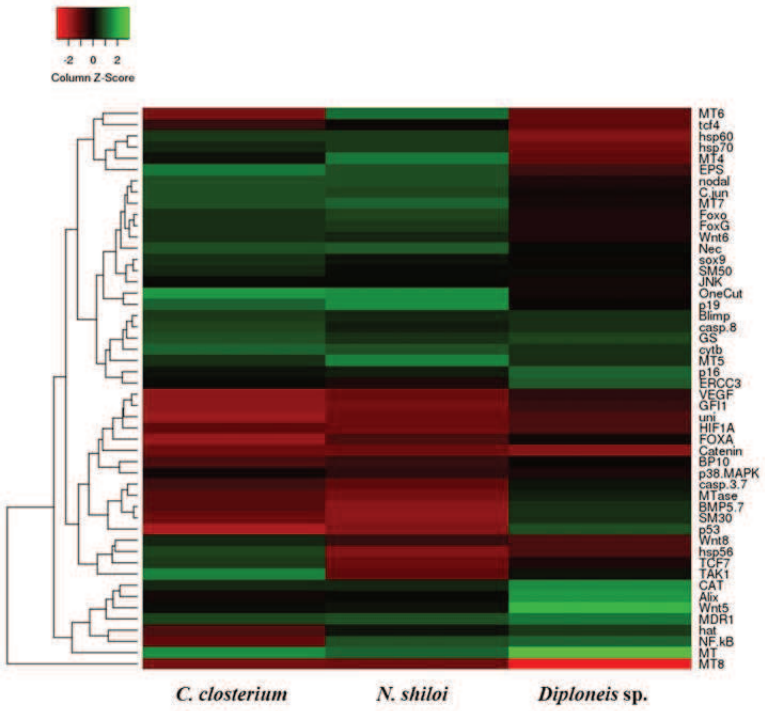


Figure 4.26. Heatmaps showing the expression profiles and hierarchical clustering of fifty genes analyzed through *Real Time qPCR* in embryos deriving from sea urchins fed with *C. closterium*, *N. shiloi* and *Diploneis* sp. Color code: red, down-regulated genes with respect to the control; green, up-regulated genes with respect to the control; black, genes for which there was no variation in expression with respect to the control. Heatmap scale bars indicate normalized expression values.

All together these molecular results, supported by morphological findings, revealed that the majority of malformations affected the skeleton, the developmental plan and differentiation of sea urchin embryos. In fact, several genes belonging to the skeletogenic, developmental and differentiation classes were affected by the ingestion of benthic diatoms. In conclusion, this study is the first demonstration of the toxic effects of benthic epiphytic diatoms on embryos and larvae of the sea urchin *P. lividus* due to the feeding of adults during gonadal maturation.

5. Chemical analysis of benthic diatoms

5.1 Introduction

Chemical analyses were performed on four benthic diatoms, *Cylindrotheca closterium*, *Nanofrustulum shiloi*, *Diploneis* sp. and *Cocconeis scutellum,* previously investigated for their effects on sea urchin reproduction (see Chapter 4).

At the end of 1990s, diatom species were first reported to produce polyunsaturated aldehydes (PUAs), initially identified as decatrienal and decadienal that induced abortions or congenital malformations in planktonic copepods that fed heavily on these diatoms (Ban et al., 1997; Ianora et al., 2003; Miralto et al., 1999). As extensively reported in Chapter 1, these short-chained aldehydes, whose chemistry has been later revised in decatrienal, octadienal, octatrienal and heptadienal (Fontana et al., 2007a,b), were proposed to act as a mechanism of chemical defence against herbivorous zooplankton. In analogy to the synthesis of hexenal and nonenal in terrestrial plants (Blée, 1998), diatom PUAs derive from lipoxygenase (LOX)/hydroperoxide lyase (HPL) pathway acting on membrane-derived PUFAs (Cutignano et al., 2006; d'Ippolito et al., 2003, 2004, 2006). PUAs have been described only in a small number of diatom species (Wichard et al., 2004), whereas almost all diatoms possess LOX pathways for the synthesis of non-volatile oxylipins (NVOs), including fatty acid derivatives with hydroxy-, keto-, oxo- and epoxy functionalities.

Oxylipins and LOX enzymes are classified with respect to the substrate specificity, as well as the position and the stereochemistry of the addition of oxygen along the

polyunsatured fatty acid (PUFA) carbon chains, with the use of stereoisomer nomenclature (*S* and *R*) as appropriate (Brash, 1999).

Very recently, new active compounds, called Sterol Sulfates (StS) have been described in the planktonic diatom *Skeletonema marinoi* (Gallo et al., 2017). StS are rather common in other marine organisms, mostly sponges and echinoderms, even if very little is known about their biosynthesis and function in these invertebrates (Kornprobst et al., 1998). StS derive from sulfation (also found as sulfurylation) of sterols performed by sulfotransferases (SULTs). This activity has been described in cnidarians (Cormier et al., 1970) and sea urchins (Malins and Roubal, 1982), as well as sulfation has been reported in algae, microalgae, and other phototrophic organisms (Hernández-Sebastià et al., 2008). Concerning diatoms, in addition to *S. marinoi*, StS have been previously described in only two species containing 24-methylenecholesterol sulfate (Anderson et al., 1978; Kates et al., 1978) and an epidioxy derivative of 24-methyl-$\Delta^{5,7}$-cholestadienol (Toume and Ishibashi, 2002).

Gallo et al., 2017 demonstrated that StS induce programmed cell death (PCD) in diatom cells. It has been proposed that these molecules mediate reactive oxygen species (ROS) burst and, through undetermined mechanisms that also involve nitric oxide (NO) pathways, ultimately sensitize cells to PCD by apoptotic-like processes. Since StS production increases with cell ageing, this study hypothesized that these molecules could probably be involved in regulatory mechanisms such as growth, acting alone or in conjunction with other intracellular mediators in signal transduction pathways (d'Ippolito et al., 2009; Fontana et al., 2007a,b; Lamari et al., 2013; Vardi et al., 2006, 2008).

Despite the well-documented literature regarding the chemical ecology of planktonic diatoms, there are few studies on benthic species (Zupo, 1994; Zupo and Messina, 2007; Maibam et al., 2014). Since feeding experiments have revealed toxic effects of benthic diatoms on sea urchin embryos (see Chapter 4), GC-MS (Gas Chromatography-Mass Spectrometry) and LC-MS (Liquid Chromatography-Mass Spectrometry) approaches, were applied to detect PUAs and NVOs, respectively. In addition, a complete analysis of total fatty acids content was performed using GC-MS. Furthermore, LOX activity by ferrous oxidation-xylenol orange 2 (FOX2) assay (Orefice et al., 2015) was also used to confirm the enzymatic production of NVOs and PUAs. FOX2 assay is highly specific for the determination of the primary LOX-products, namely fatty acid hydroperoxydes (FAHs), and specifically allows the quantification of low concentrations of these molecules in the presence of high background levels of non-peroxidized fatty acids or other oxygenated chemical species.

Finally, a methanol extraction and LC-MS investigations have been performed for StS detection from the four benthic diatoms under analysis in order to confirm the presence of these mediators in the benthic species.

5.2 Materials and methods

5.2.1 Cell culturing and collection

Diatoms were grown at temperature, photon flux and photoperiod conditions reported in paragraph 4.2.1. Once biomass was collected from intermediate cultures (grown as described in paragraph 4.2.3), massive cultures were inoculated and grown in 17-cm glass Petri dishes (Raniello et al., 2007) until cells entered into stationary phase. Cells

were finally harvested by centrifugation at 3000 g for 10 min at 12° C in a swing-out rotor and immediately processed or frozen in liquid nitrogen and kept at -80° C until further analysis.

5.2.2 Fatty acids analysis

In order to determine the fatty acid composition of each diatom (300 mg, ww), a methanol extraction was performed. Pellets were suspended in methanol (5 mL/gr pellet), sonicated and centrifuged at 3000 g for 6 min at 4° C. The supernatant was recovered and additional methanol was added to the residue. This step was repeated three times. Part of each raw extract (0.5 mg) was subjected to alkaline methanolyses (saponification), adding catalytic amount of Na_2CO_3 in 500 µL MeOH. The reaction mixture was incubated over night at room temperature before neutralization with 1% AcOH and extraction with diethyl ether/water. After removal of the organic solvent by nitrogen flow, dry sample was dissolved in n-hexane (1µg μL^{-1}) and analyzed by GC-MS equipped with an ion-trap, on a 5% diphenyl column, in electron ionization (EI, 70 eV) and negative mode. Elution of free fatty acid methyl esters required an increasing gradient of temperature according to the following program: 200° C for 2.5 min then 15° C/min up to 290° C, followed by 7 min at 290° C. Each sample (2 µL) was directly injected and the analysis was performed with a blink window of 3 min (inlet temperature of 270° C, transfer line set at 280° C and ion source temperature of 250° C).

5.2.3 CHNS

Carbon, hydrogen, nitrogen, and sulfur (CHNS) were analyzed on a Thermo Electron Corporation FlashEA 1112 CHNS elemental analyzer with autosampler. This instrument uses combustion in an oxygen-rich environment to oxidize the sample into predictable forms of the single elements (C/H/N/S) which are then detected through GC (gas chromatography) separation followed by quantification using thermal conductivity detection. Catalysts are also added to the combustion tube in order to aid the conversion process. In the combustion process (furnace at ca. 1000° C), carbon is converted to carbon dioxide, hydrogen to water, nitrogen to nitrogen gas/oxides of nitrogen and sulphur to sulphur dioxide. The resulting products are swept out of the combustion chamber by inert carrier gas such as helium and passed over heated (about 600° C) high purity copper. The function of copper is to remove any oxygen not consumed in the initial combustion and to convert any oxides of nitrogen to nitrogen gas. The gases are finally passed through the absorbent traps in order to leave only carbon dioxide, water, nitrogen and sulphur dioxide.

For the analyses, ~ 300 mg wet biomass of each diatom stored at -80° C was firstly dried overnight using a freeze-drying chamber and then disrupted into small pieces. Each species (~ 3 mg dry weight, dw) was weighed in a tin sample cup with vanadium pentoxide (catalyst). In addition, sulfanilamide (the standard) and vanadium pentoxide for blank have been used. Samples were analyzed in triplicates.

5.2.4 Oxylipins extraction procedure

Oxylipins were extracted according to Cutignano et al., 2011. Cell pellets (1 g wet biomass) were re-suspended in 1 mL of MilliQ water. Then, the internal standards, 4*E*-decenal (60 μg/g of pellet) and 16-hydroxy-hexadecanoic acid (10 μg/g of pellet), were added. Samples were sonicated for 5 min and left for 30 min at room temperature to allow the LOX reaction occurring after cell disruption. Resulting homogenates were diluted with 1 volume of acetone and then centrifuged at 3000 g for 6 min at 4° C. Pellets were re-suspended in H_2O-acetone 1:1 and the extraction process was repeated three times. The supernatants were combined and partitioned by an equal volume of CH_2Cl_2. The combined organic phases were dried over Na_2SO_4 and evaporated under reduced pressure by a rotary evaporator (Buchi, Rotavapor R-200).

5.2.5 PUAs analysis by GC-MS

For PUAs detection, two thirds of the total extract of each sample was treated with carbetoxyethylidene (CET)-triphenylphosphorane (1.2 mg per mg of extract) for 20 h at room temperature (d'Ippolito et al., 2002a). The Wittig reaction (**Figure 5.1**) resulting from addition of CET-triphenylphosphorane converts aldehydes into ethyl esters that gives characteristic fragmentation patterns in the high resolution MS spectrometry. In fact, CET derivatives display intense molecular ions $(M)^+$ and fragmentation peaks arising from loss of $M\text{-}45^+$ and $M\text{-}29^+$ in the saturated and conjugated series, respectively (d'Ippolito et al., 2002a).

For chemical determination, samples were dissolved in CH_2Cl_2 (1 μg μL^{-1}) and analyzed through GC-MS (2 μL) using a temperature gradient of 3° C min^{-1} from 130° C to 220° C. Injector temperature was 240° C, detector temperature was 260° C and nitrogen flow was 1 mL min^{-1}). Electron voltage was set at 70 eV.

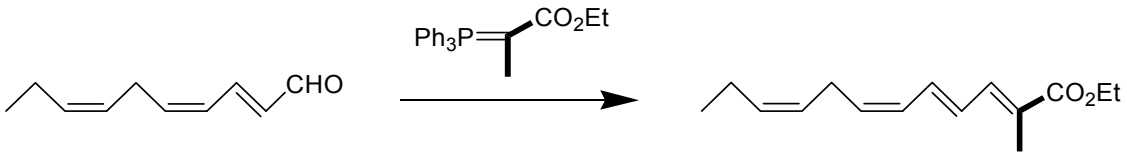


Figure 5.1. Derivatization of 2*E*,4*Z*,7*Z*-decatrienal by carbethoxyethylidene (CET) triphenylphosphorane.

For PUAs quantification, peaks were integrated by Xcalibur™ software and values were normalized by carbon content measured by CHNS Analyzer (Carbon, Hydrogen, Nitrogen, Sulfate). Data were reported as mean ± SD from three biological replicates.

5.2.6 NVOs detection through LC-MS

One third of the extract was methylated with diazomethane (0.4 mL per 10 mg extract) for 30 min at room temperature to convert free fatty acids in methyl esters. After removing the organic solvent under nitrogen flow, the resulting methylated extract was used for analysis of NVOs by LC-MS. Oxylipins were revealed as sodium adducts $(M+Na)^+$ with molecular ions at *m/z* M+23 and were inversely eluted according to number of double bonds and length of the alkyl chain, with epoxy-alcohols preceding keto- and hydroxy-fatty acids (Cutignano et al., 2011).

Samples were analyzed using two different MS platforms: a Micro-qToF mass spectrometer (Waters) with electrospray ionization (ESI) source (positive mode) and

coupled with a Waters Alliance HPLC system equipped with a C-18 Kromasil column (4.6 × 250 mm, 100 Å, Phenomenex) and a Q-Exactive Hybrid Quadrupole-Orbitrap spectrometer (Thermo Scientific) with an ESI source and interfaced to an Infinity 1290 UHPLC System (Agilent Technologies) equipped with a Luna 5 μ C-18 column (2.0 × 150 mm, 100 Å, Phenomenex).

Analytical conditions of MicroMass qTof were set as follows. Solvent gradient: MeOH:H_2O 75/25 (v/v) to MeOH:H_2O 94/6 (v/v) in 28 min, MeOH:H_2O 94/6 (v/v) to 100% MeOH in 2 min, 100% MeOH for 15 min; flow rate: 1 mL min^{-1}; ToF MS function: Start mass 80.0, end mass 800.0, start time (min) 0.0, end time (min) 50.0, scan time (s) 0.8, interscan time (s) 0.1, scans to sum 1,000,000; Gas setting: Collision gas 1.0, inlet gas 1.0, cone (L h^{-1}) 30, desolvation (L h^{-1}) 600; Instrument parameters: Polarity ES^+, capillary (V) 3300.0, sample cone (V) 30.0, extraction cone (V) 2.0, desolvation temp (°C) 220.0, source temp (°C) 100.0, ion energy (V) 1.0, collision energy 7.0 for MS analysis and 22 for tandem mass spectrometry (MS/MS), low mass resolution 5.0, high mass resolution 5.0, MCP detector (V) 2600.0, acceleration (V) 200.0, TOF flight tube (V) 5630.0, resolution 5000.0; Collision gas (Argon) at a pressure of 22 mbar. Samples were dissolved in MeOH (final concentration 1 μg μL^{-1}) and 5 μL were loaded.

Analytical conditions of Q-Exactive have been applied as follows. Solvent gradient: MeOH:H_2O 75/25 (v/v) to MeOH:H_2O 94/6 (v/v) in 28 min, MeOH:H_2O 94/6 (v/v) to 100% MeOH in 2 min, 100% MeOH for 15 min; Flow rate: 0.3 mL min^{-1}; Q-Exactive Orbitrap parameters: spray voltage negative polarity 3.0 kV, capillary temperature 320° C, s-lens RF level 60, auxiliary gas temperature 320° C, sheath gas flow rate 50,

auxiliary gas flow rate 30. Full MS scans were acquired over the range 150-500 with a mass resolution of 70,000. 5 µL of samples (final concentration 0.05 µg μL^{-1}), diluted in MeOH, were injected.

Oxylipins were quantified by Xcalibur™ software on Q-Exactive. All values were normalized by carbon content measured through CHNS Analyzer (Carbon, Hydrogen, Nitrogen, Sulfate). Data are reported as mean ± SD from three biological replicates.

5.2.7 FOX2 assay

LOX activity was evaluated by a colorimetric assay (FOX2) based on levels of FAHs. This assay is based on the oxidation of iron (II) to iron (III) by oxidizing agents present in the sample. The reagent iron (III) then binds to the xylenol orange reagent giving a color complex with an absorbance maximum at 560 nm (**Figure 5.2**).

Using the antioxidant butylated hydroxytoluene (BHT), FOX2 assay is high specific for FAHs originated by LOX activity since it prevents the generation of other hydroperoxides generated by other enzymes or spontaneous radical reactions.

FOX2 solution was preliminarly prepared using $FeSO_4$, xylenol orange and BHT. Pellets (50 mg) were suspended in 500 µL of TRIS HCl 50 mM pH 8, NaCl 0.5M and sonicated two times for 2 min separated by 30 sec. After 10 min of sonication, two replicates of three different quantities, diluted in TRIS HCl 50 mM pH 8, NaCl 0.5M, were prepared (final volume: 200 µL). Then, 800 µL of FOX2 solution was added. Samples were vortexed and left at room temperature for 15 min and prior to centrifugation at 11290 g for 4 min at 16° C. Finally, the absorbance was read at 560 nm.

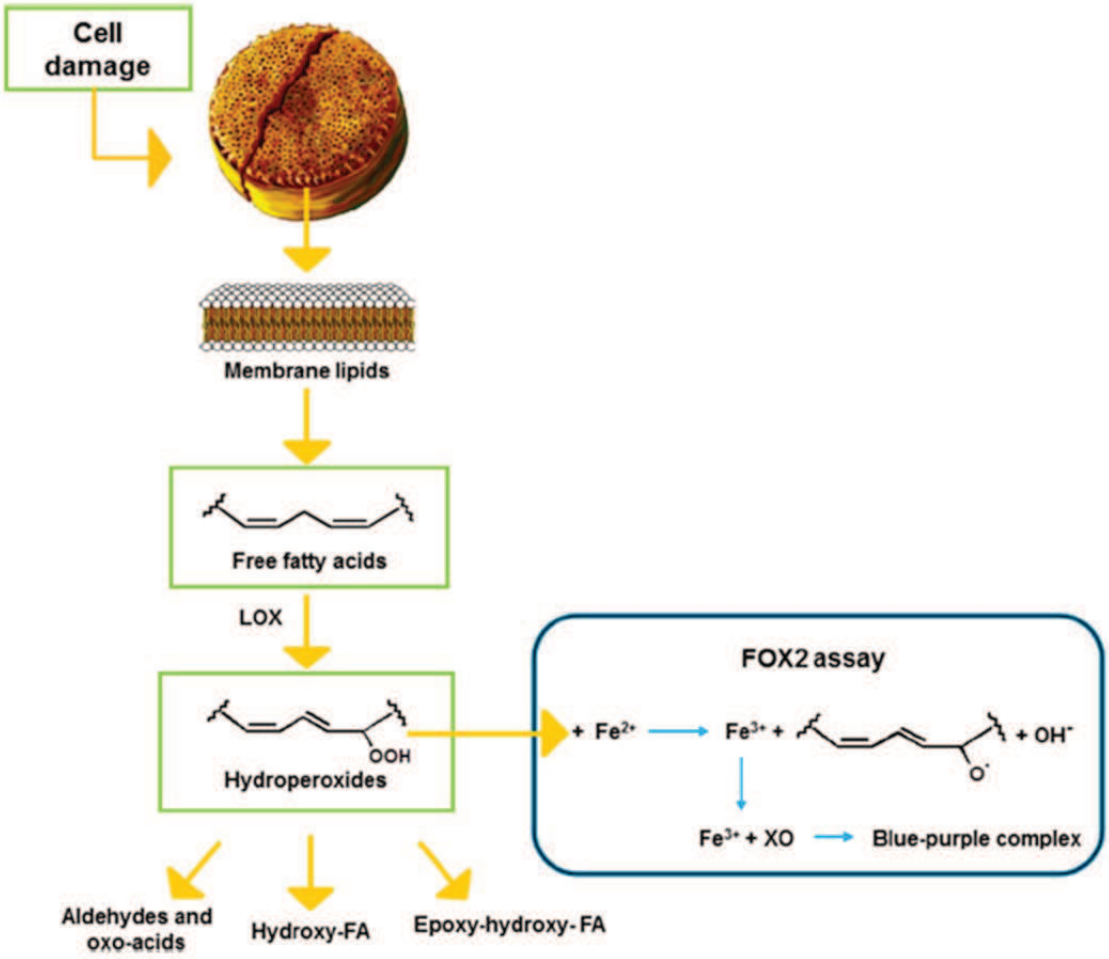


Figure 5.2. Schematic representation of the FOX2 reaction. FA: fatty acid, XO: xylenol orange. (From Orefice et al., 2015).

A control reaction with tris(2-carboxyethyl)phosphine (TCEP) was performed at the same time. TCEP reduces FAHs to the corresponding hydroxyacids with a consequent decrease of absorbance compared to TCEP-untreated samples.

FAHs concentrations were measured using a standard curve (**Figure 5.3**) generated by increasing concentrations of H_2O_2 and values were normalized for protein (Lowry Assay) content. Data are reported as mean ± SD from three biological replicates.

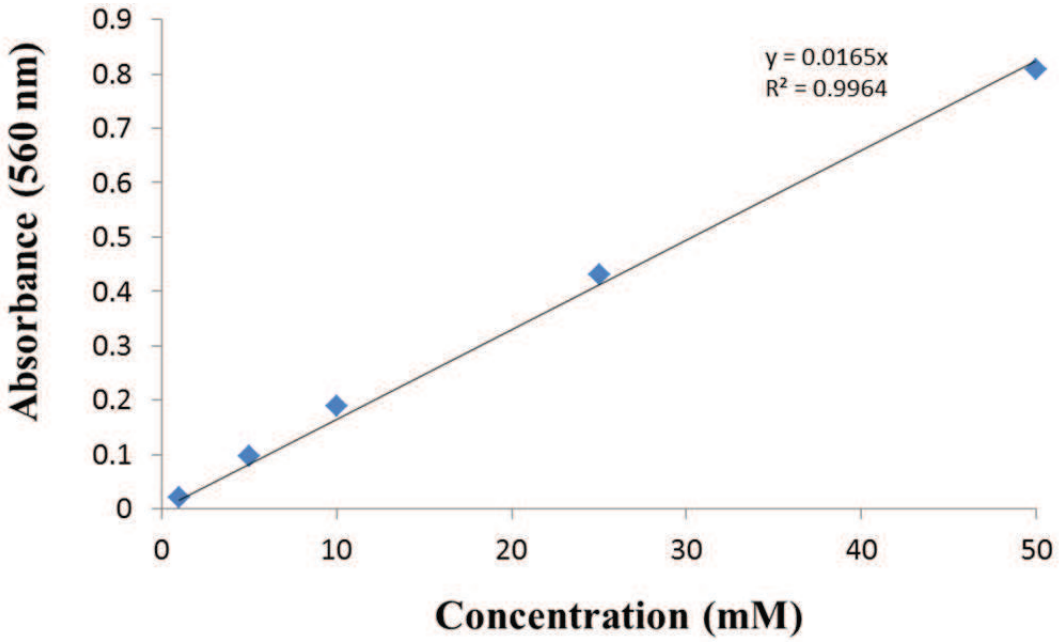


Figure 5.3. Calibration curve with standard samples of H_2O_2 within concentrations ranging from 1 to 50 mM.

5.2.8 StS extraction and LC-MS analysis

Diatoms were grown and collected as reported in paragraph 5.1.1. StS were extracted with MeOH (5 mL g^{-1} wet biomass) from 300 mg wet biomass. [25,26,26,26,27,27,27-D7]-Cholesterol sulfate (C/D/N Isotopes, *m*/*z* 472.3) was added, as internal standard (10 µg STD/100 mg wet biomass). Samples were then vortexed for 10 s and sonicated for 5 min. Suspensions were centrifuged at 3016 for 10 min at 4° C. The extraction was repeated three times. Finally, the hydroalcoholic solutions were combined, dried by a rotary evaporator (Buchi, Rotavapor R-200) and transferred to glass vials. Solvent was removed under nitrogen and dry extracts were stored at -80° C until analysis.

A Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization (ESI) source and Infinity 1290 UHPLC (Agilent

Technologies) Reversed-phase column, Kinetex Biphenyl (Phenomenex) 2.1 × 150 mm, 2.6 μm, was used for StS identification.

Analytical conditions of Q-Exactive have been applied as follows. Solvent gradient: MeOH:H_2O 70:30 (v/v) for 2 min, MeOH:H_2O 70:30 (v/v) to MeOH:H_2O 85:15 (v/v) in 8 min, MeOH:H_2O 85:15 (v/v) to 100% MeOH in 5 min, 100% MeOH for 5 min; Flow rate: 0.3 mL min^{-1}; Q-Exactive parameters: Spray voltage negative polarity 3.0 kV, capillary temperature 320° C, S-lens RF level 60, auxiliary gas temperature 320° C, sheath gas flow rate 50 L min^{-1}, auxiliary gas flow rate 30 L min^{-1}. Full MS scans range: 200-600, mass resolution: 70,000.

Samples (final concentration 0.05 μg μL^{-1}), diluted in MeOH, were injected. StS were evaluated by HR-MS (high resolution - mass spectrometry) and identified according to the diagnostic fragment at *m*/*z* 97 in the MS/MS spectra due to the loss of sulfate. Peaks were integrated by Xcalibur™ software and data were normalized by carbon content (CHNS Analyzer).

5.2.9 Statistical analyses

Data were measured as the average of three biological replicates and reported as 'mean ± standard deviation (SD)'. Statistical significance of differences between groups was evaluated using unpaired *t*-tests (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com). P values < 0.05 were considered statistically significant.

5.3 Results

5.3.1 GC-MS analysis of free fatty acids

Fatty acid composition was identified for each benthic diatom. Fatty acids were assigned comparing the retention time (RT) and the molecular weight with a fatty acids standard mixture (PUFA, Marine Source, Sigma-Aldrich).

C. closterium resulted rich in C20:5 ω3 and C16:1. A lower amount of other fatty acids, including polyunsatured C_{16}, C_{18}, C_{22} chains and C20:4 ω6, were also detected (**Figure 5.4**).

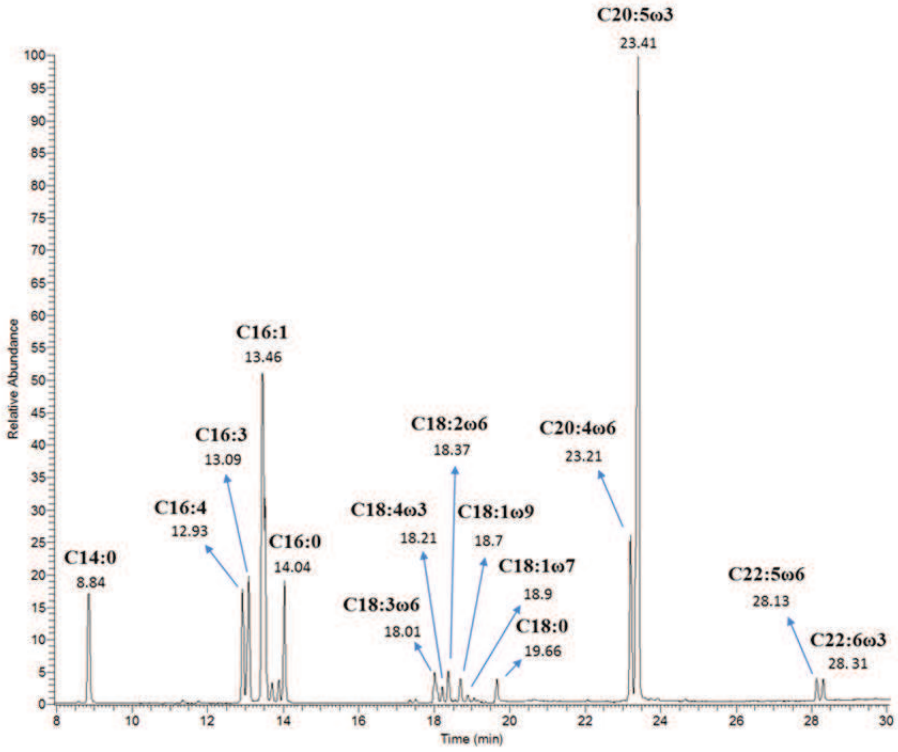


Figure 5.4. GC-MS profile of fatty acids from *C. closterium*.

GC-MS profiles from *N. shiloi* displayed mostly C16:1 and C20:5 ω3 with a minor amount of C20:4 ω6 and C_{18} fatty acids. Interestingly, a total absence of C_{22} chains was found (**Figure 5.5**).

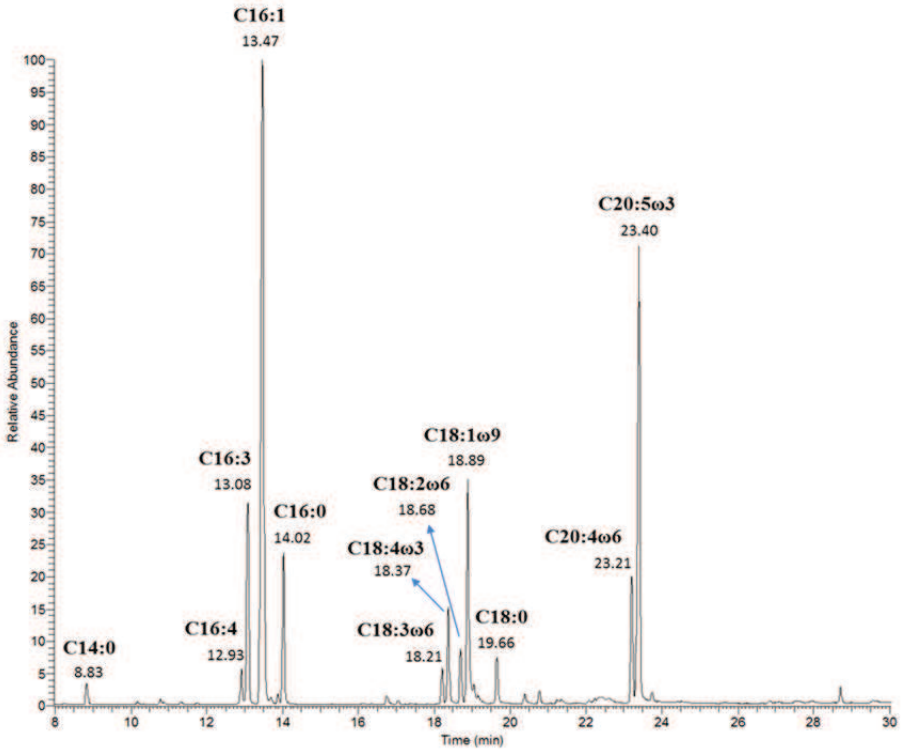


Figure 5.5. GC-MS profile of fatty acids from *N. shiloi*.

On the contrary, *C. scutellum* exhibited a poor content of free fatty acids, with saturated C_{16} and C20:5 ω3 being the most abundant species (**Figure 5.6**).

The fourth species, *Diploneis* sp., appeared to be almost exclusively rich in fatty acids with a high level of unsaturation. In fact, peaks of C16:4 and C20:5 ω3 were the most abundant. A lower amount of C16:0, C16:1 and C18:0 were detected (**Figure 5.7**).

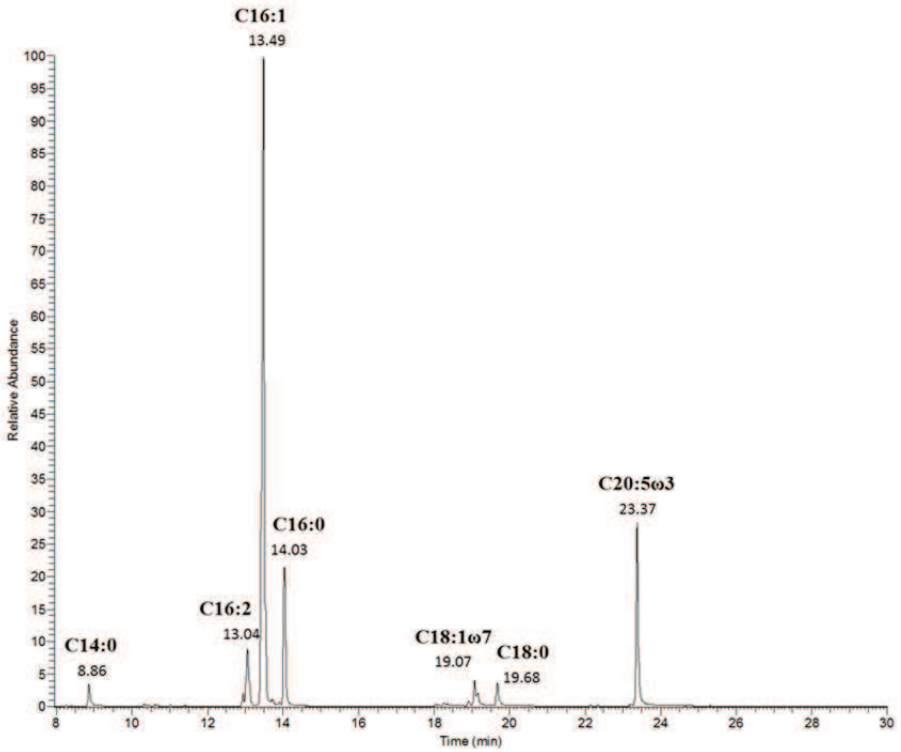


Figure 5.6. GC-MS profile of fatty acids from *C. scutellum*.

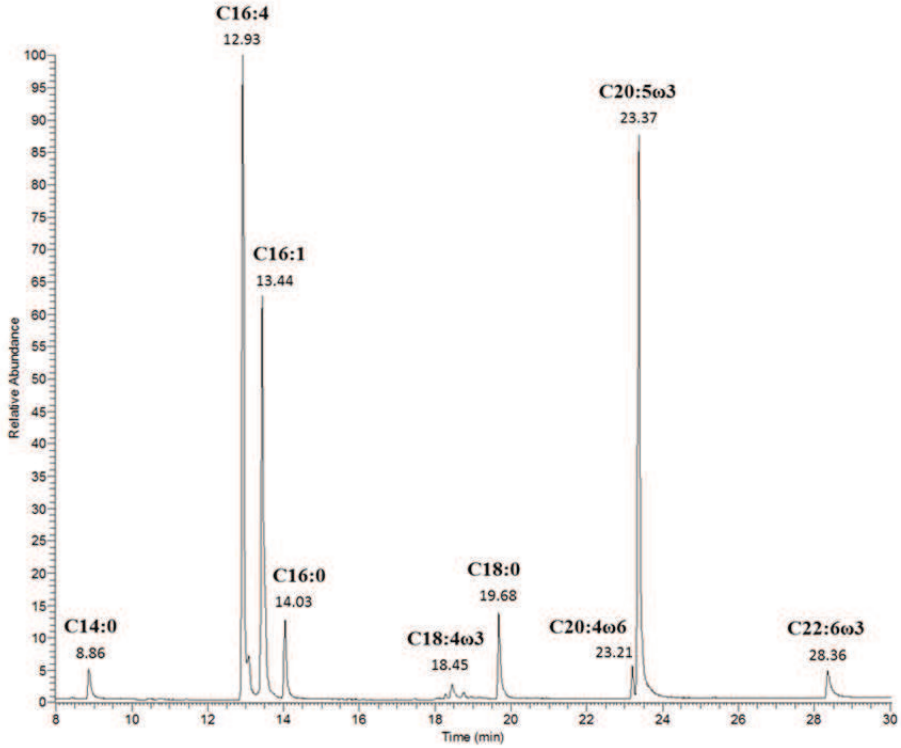


Figure 5.7. GC-MS profile of fatty acids from *Diploneis* sp.

5.3.2 GC-MS analysis of PUAs

GC-MS profiles displayed that *N. shiloi* was the only diatoms producing PUAs among the four benthic diatoms under analysis (**Figure 5.8**). GC-MS analyses showed peaks correlated to 2*E*,4*Z*-octadienal, 2*E*,4*Z*,7*Z*-decatrienal and 2*E*,4*Z*-decadienal, with 2*E*,4*Z*,7*Z*-decatrienal resulting the most abundant. Traces of artifacts, normally originated by CET derivatization, were also detected.

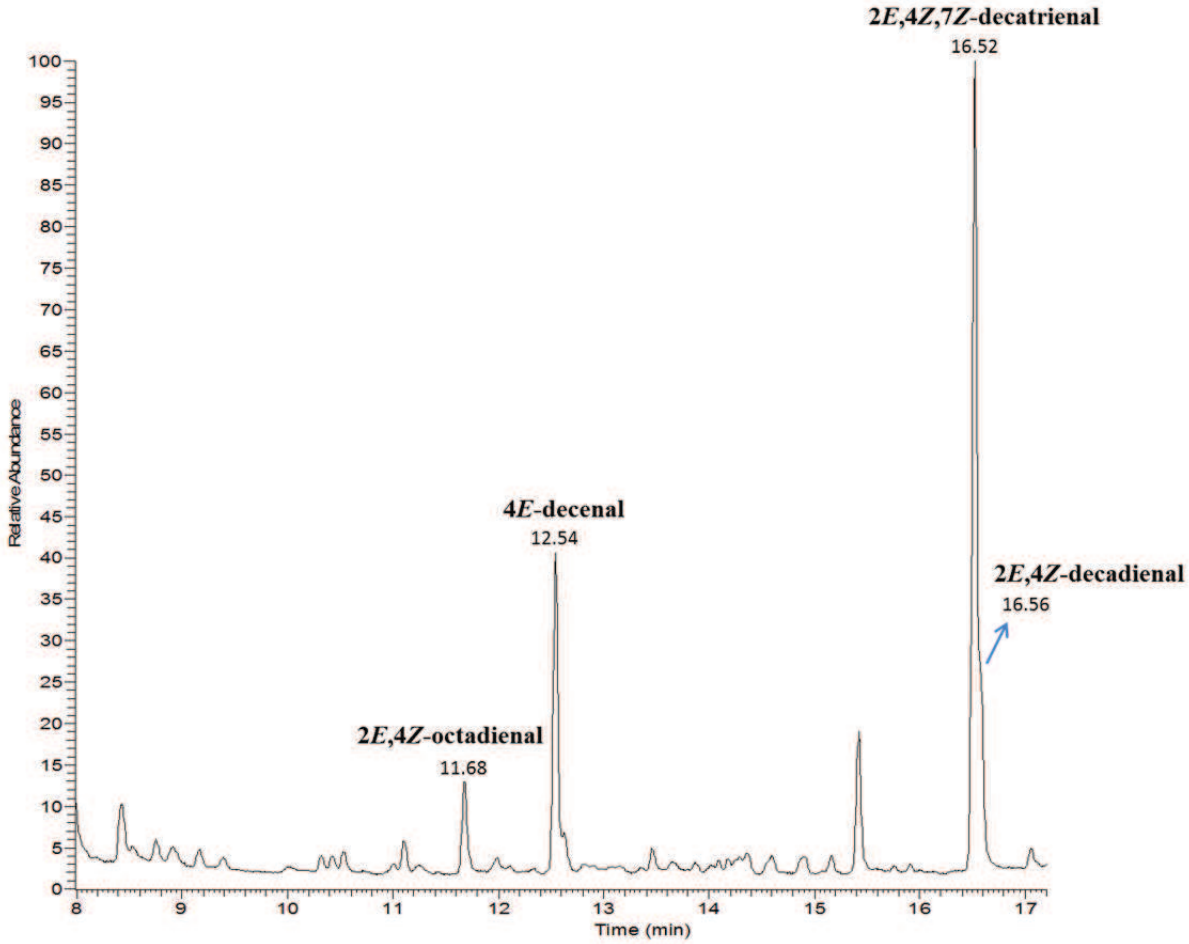


Figure 5.8. GC-MS profile from *N. shiloi* extract. CET derivatives of 2*E*,4*Z*-octadienal, 2*E*,4*Z*,7*Z*-decatrienal, 2*E*,4*Z*-decadienal were found; traces of the artifacts originated by CET reactions were visible. Internal standard: 4*E*-decenal.

2*E*,4*Z*,octadienal showed a retention time (RT) of 11.68. The identification of octadienal was accomplished by MS spectra displaying a molecular ion *m*/*z* 208 and two fragments *m*/*z* 163 $(M-45)^+$ and *m*/*z* 179 $(M-29)^+$. Concerning 2*E*,4*Z*,decadienal, a RT of 16.56, an intense molecular ion at *m*/*z* 236 and two fragments at *m*/*z* 191 $(M-45)^+$ and *m*/*z* 207 $(M-29)^+$ were detected.

Octadienal and decadienal were also identified comparing the RT and the MS spectra to analytical standards injected and analyzed using the same GC-MS conditions (**Figure 5.9-5.10**).

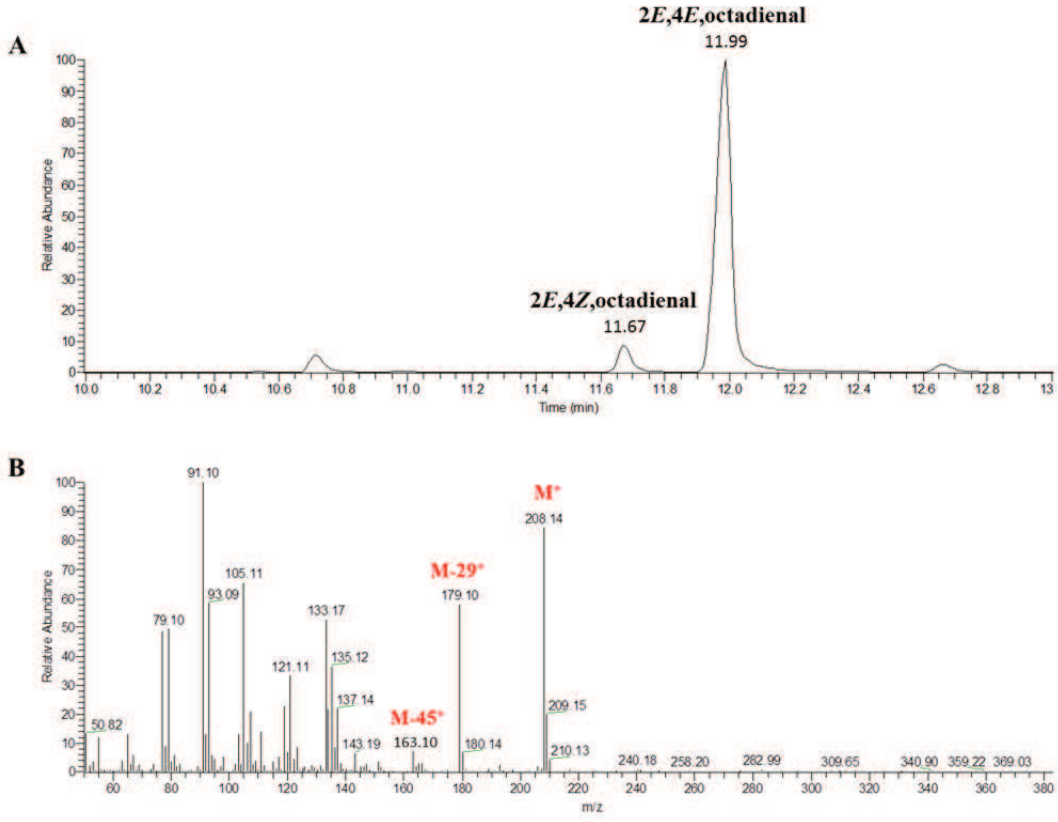


Figure 5.9. GC-MS profile (A) and MS spectrum (B) of octadienal standard mixture. 2*E*,4*Z*-octadienal is the natural isomer deriving from LOX pathway; 2*E*,4*E*-octadienal is the more stable isomer normally found in analytical conditions. Mass spectrum shows in red the molecular ion *m*/*z* 208 and the two fragments *m*/*z* 163 $(M-45)^+$ and *m*/*z* 179 $(M-29)^+$.

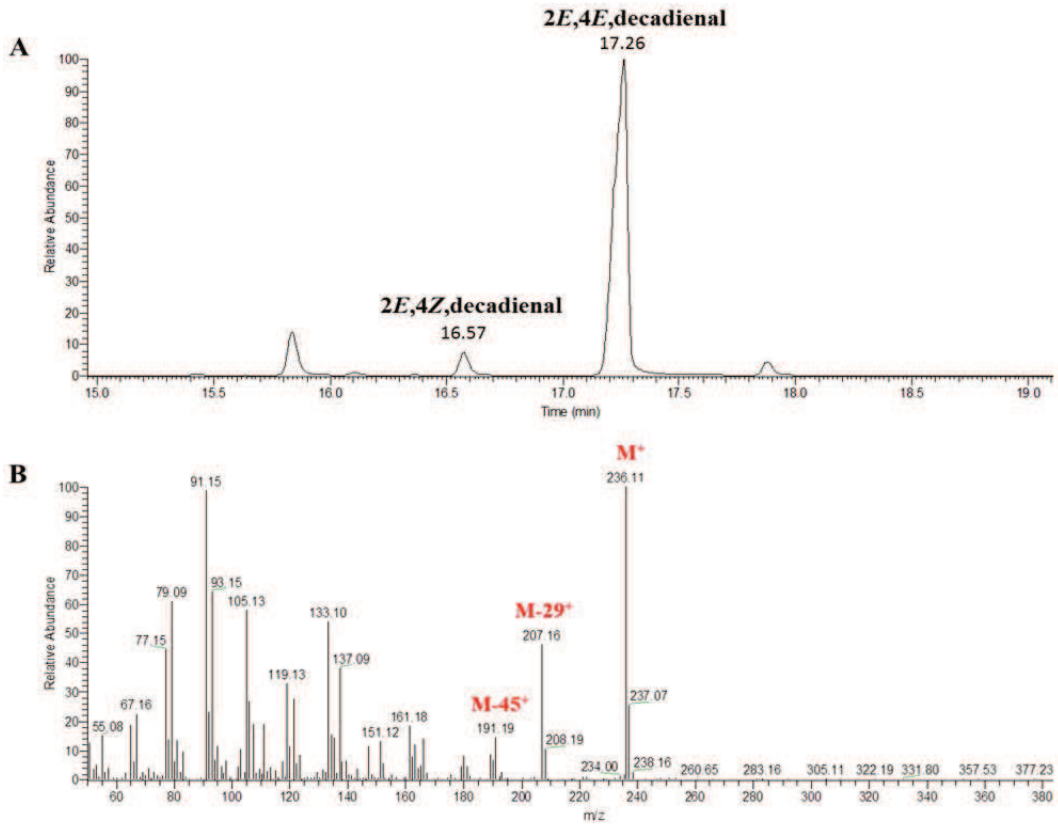


Figure 5.10. GC-MS profile (A) and MS spectrum (B) of decadienal standard mixture. 2*E*,4*Z*-decadienal is the natural isomer deriving from LOX pathway; 2*E*,4*E*-decadienal is the more stable isomer normally found in analytical conditions. Mass spectrum shows in red the molecular ion *m*/*z* 236 and the two fragments *m*/*z* 191 $(M-45)^+$ and *m*/*z* 207 $(M-29)^+$.

Furthermore, 2*E*,4*Z*,7*Z*-decatrienal (RT= 16.52) was identified by the molecular ion at *m*/*z* 234 and two fragments at *m*/*z* 189 $(M-45)^+$ and *m*/*z* 205 $(M-29)^+$. In this gradient condition 2*E*,4*Z*,7*Z*-decatrienal eluted closer to 2*E*,4*Z*-decadienal, therefore a slower temperature gradient (from 130° C to 220° C at 2° C min^{-1}) was applied to better separate these compounds (**Figure 5.11**).

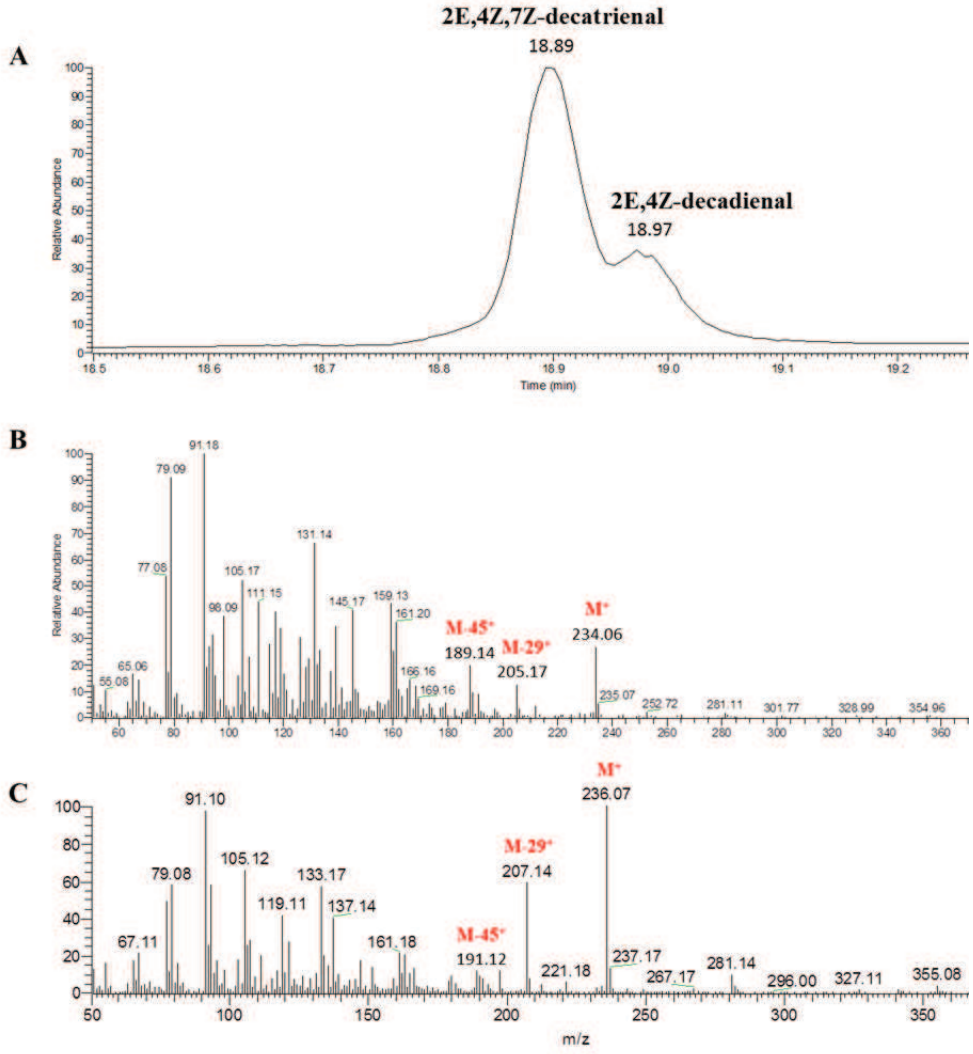


Figure 5.11. GC-MS profile (A) and MS spectrum of 2*E*,4*Z*,7*Z*-decatrienal (RT= 18.89) (B) and 2*E*,4*Z*-decadienal (RT= 18.97) (C) by a slower temperature gradient condition. Mass spectrum of 2*E*,4*Z*,7*Z*-decatrienal (B) shows in red the molecular ion *m*/*z* 234 and the two fragments *m*/*z* 189 $(M-45)^+$ and *m*/*z* 205 $(M-29)^+$. As already reported in **Figure 5.9**, mass spectrum of 2*E*,4*Z*-decadienal (C) shows in red the molecular ion *m*/*z* 236 and the two fragments *m*/*z* 191 $(M-45)^+$ and *m*/*z* 207 $(M-29)^+$.

5.3.3 LC-MS analysis of NVOs

The specific assignment of oxylipins of the benthic diatoms under analysis have been carried out by comparison of the LC-MS results on the microalgal extracts with the data previously published in literature (d'Ippolito et al., 2005, 2006, 2009; Fontana et al., 2007a,b; Nanjappa et al., 2014). In fact, LC-MS investigations displayed that *C.*

closterium, *N. shiloi* and *C. scutellum* produce oxylipins already described in planktonic diatoms. The *C. closterium* profile (**Figure 5.12**) showed oxylipins deriving from both eicosa-5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-pentaenoic acid (EPA, C20:5 ω3) and docosa-4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-esenoic acid (DHA, C22:6 ω3).

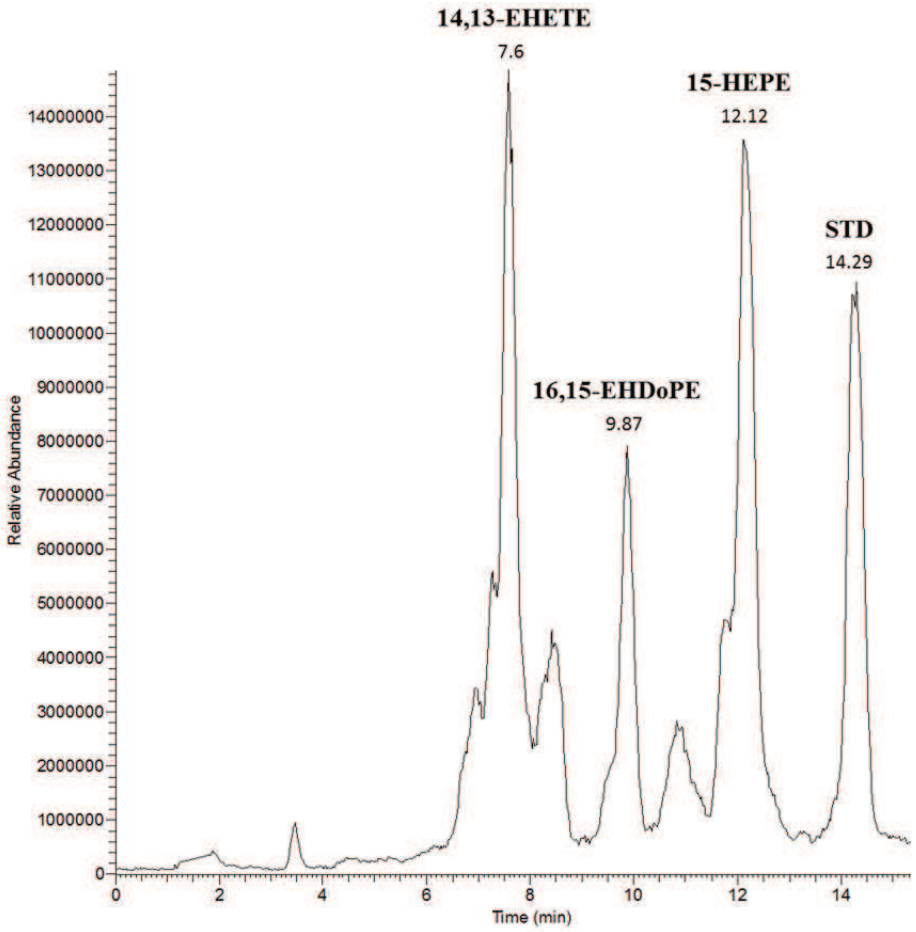


Figure 5.12. LC-MS profile of *C. closterium* showing peaks related to 14,15-epoxy-13-hydroxy-eicosa-5*Z*,8*Z*,11*Z*,17*Z*-tetraenoic acid (14,13-EHETE), 16,17-epoxy-15-hydroxy-docosa-4*Z*,7*Z*,10*Z*,13*Z*,19*Z*-pentaenoic acid (16,15-EHDoPE) and 15-hydroxy-eicosa-5*Z*,8*Z*,11*Z*,13*E*,17*Z*-pentaenoic acid (15*S*-HEPE). Internal standard (STD, RT=14.29): 16-hydroxy-hexadecanoic acid.

These oxylipins were identified as methyl derivatives of hydroxyacid (*m*/*z* 355 $[M+Na]^+$) and epoxyalcohol (*m*/*z* 371 $[M+Na]^+$) of EPA and epoxyalcohol (*m*/*z* 397 $[M+Na]^+$) of DHA.

The compound at *m/z* 371 (RT= 7.6) was identified by the MS/MS fragmentation (*m/z* 259, 273 and 289) as 14,15-epoxy-13-hydroxy-eicosa-5*Z*,8*Z*,11*Z*,17*Z*-tetraenoic acid (14,13-EHETE, 0.0045 ± 0.0006 µmol mg^{-1} C) (**Figure 5.12-5.13A**; d'Ippolito et al., 2009), whereas the corresponding hydroxyacid at *m/z* 355 (RT= 12.12), was characterized as 15-hydroxy-eicosa-5*Z*,8*Z*,11*Z*,13*E*,17*Z*-pentaenoic acid (15-HEPE, 0.0027 ± 0.0001 µmol mg^{-1} C) by the characteristic UV maxima (UV_{max}) at 234 nm of the diene system (d'Ippolito et al., 2009).

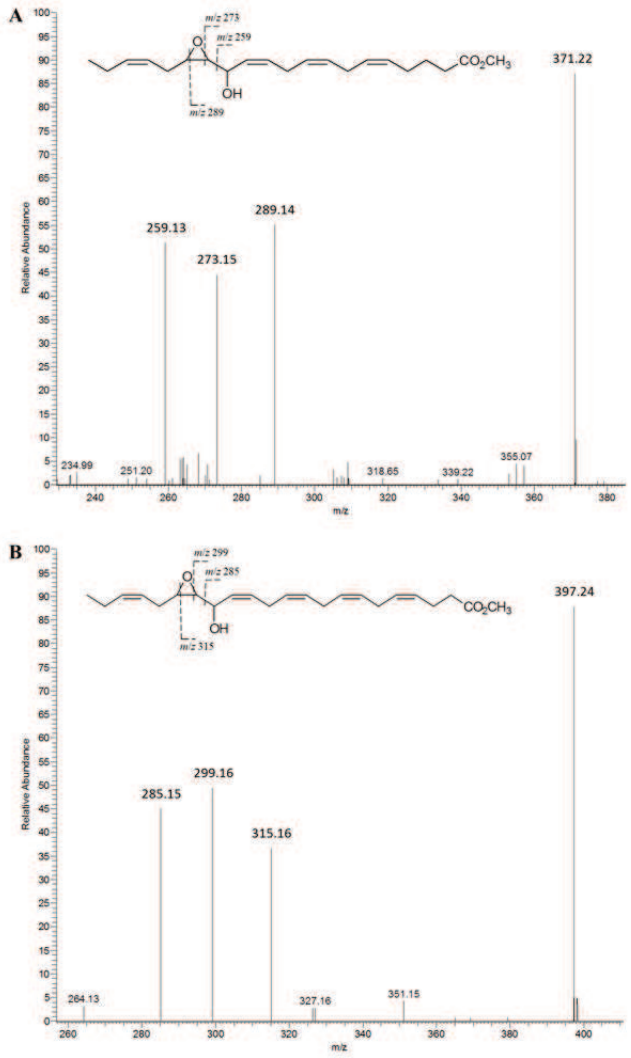


Figure 5.13. MS/MS spectra: 14,13-EHETE (A, *m/z* 371, $[M+Na]^+$) shows fragments at *m/z* 259, 273 and 289 and 16,15-EHDoPE (B, *m/z* 397, $M+Na^+$) shows fragments at *m/z* 285, 299 and 315.

Analogously to 14,13-EHETE, the methyl ester at *m*/*z* 397 (RT= 9.87) was described as 16,17-epoxy-15-hydroxy-docosa-4*Z*,7*Z*,10*Z*,13*Z*,19*Z*-pentaenoic acid (16,15-EHDoPE, 0.0013 ± 0.0001 μmol mg^{-1} C) by the MS cluster at *m*/*z* 285, 299 and 315 (**Figure 5.12-5.13B**) (Nanjappa et al., 2014).

Since a clear correlation has been established between the positions of hydroxy and epoxy groups with the site of peroxygenation, MS/MS characterization of the epoxyalcohols from *C. closterium* extracts was indicative of EPA:dependent 15*S*-LOX and DHA:dependent 17-LOX.

Analysis of *N. shiloi* revealed two major peaks of epoxyalcohols corresponding. The first one was identified as the C_{16} derivative, 9,10-epoxy-11-hydroxy-hexadecadienoic acid (9,11-EHHDE, 0.002 ± 0.0007 μmol mg^{-1} C), with *m*/*z* 319 ($[M+Na]^+$, RT= 4.17) and characteristic fragment at *m*/*z* 207 (**Figure 5.14-5.15A**; Fontana et al., 2007b).

The second oxylipins was the EPA derivative, 11,12-epoxy-10-hydroxy-eicosa-5*Z*,8*Z*,14*Z*,17*Z*-tetraenoic acid (11,10-EHETE, 0.005 ± 0.0005 μmol mg^{-1} C) with *m*/*z* 371 ($[M+Na]^+$, RT= 7.56, fragment at *m*/*z* 233; **Figure 5.14-5.15B**; d'Ippolito et al., 2006). In addition, LC-MS profiles showed a peak at 12.33 min (*m*/*z* 355 $[M+Na]^+$, UV_{max}= 234 nm) of 11-hydroxy-eicosa-5*Z*,8*Z*,12*E*,14*Z*,17*Z*-pentaenoic acid (11-HEPE, 0.0017 ± 0.0001 μmol mg^{-1} C) (**Figure 5.14**; d'Ippolito et al., 2006). The occurrence of these products well supported the presence of 9*S*-LOX and 11-LOX activities.

Moreover, the extracts of *N. shiloi* contained 9-ketohexadec-7*E*-enoic acid (9-KHME, *m*/*z* 305 $[M+Na]^+$, RT= 10.20, 0.0014 ± 0.0001 μmol mg^{-1} C) and 9-hydroxyhexadec-7*E*-enoic acid (9-HHME, *m*/*z* 307 $[M+Na]^+$, RT= 11.27, UV_{max}= 214 nm, 0.012 ± 0.0009 μmol mg^{-1} C), respectively (see below **Table 5.1**).

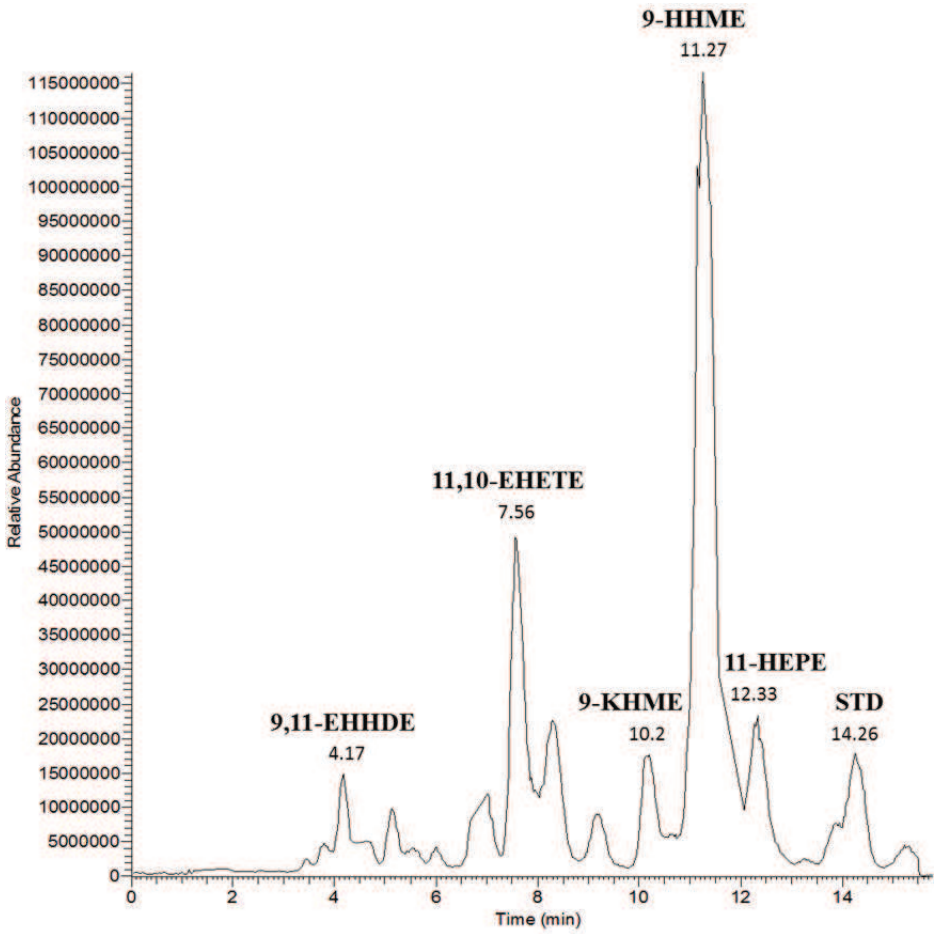


Figure 5.14. LC-MS profile of *N. shiloi* showing peaks related to 9,10-epoxy-11-hydroxy-hexadecadienoic acid (9,11-EHHDE), 11,12-epoxy-10-hydroxy-eicosa-5*Z*,8*Z*,14*Z*,17*Z*-tetraenoic acid (11,10-EHETE), 11-hydroxy-eicosa-5*Z*,8*Z*,12*E*,14*Z*,17*Z*-pentaenoic acid (11-HEPE), 9-ketohexadec-7*E*-enoic acid (9-KHME) and 9-hydroxyhexadec-7*E*-enoic acid (9-HHME). Internal standard (STD, RT= 14.26): 16-hydroxy-hexadecanoic acid.

In agreement with d'Ippolito et al., 2005, these metabolites are oxygenated derivatives of palmitoleic acid (C16:1) but their synthesis involves oxygenase activity not attributable to lipoxygenases.

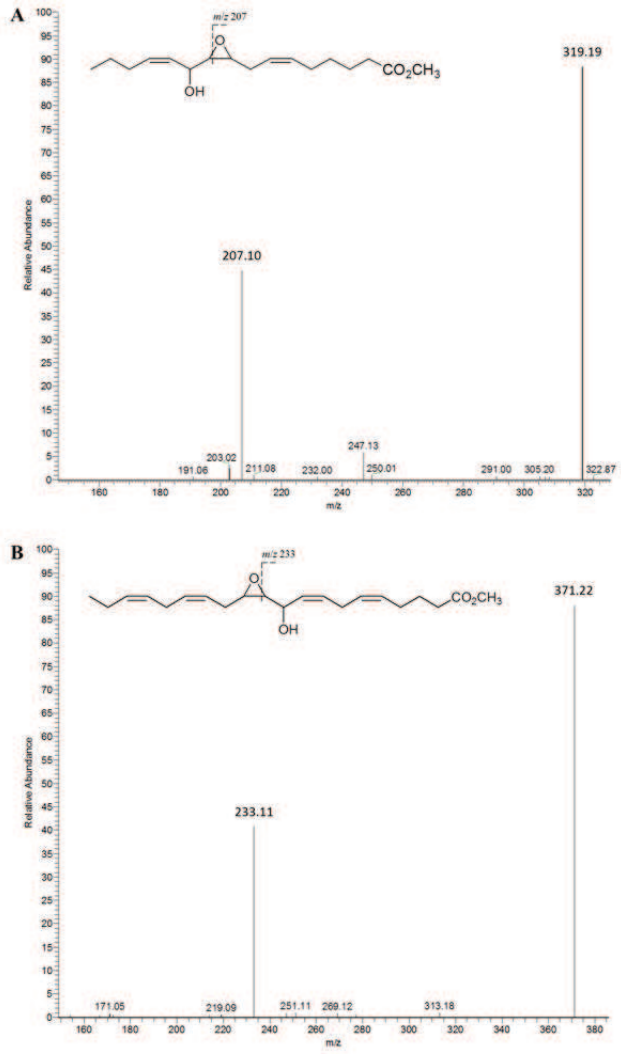


Figure 5.15. MS/MS spectrum of 9,11-EHHDE (A, *m/z* 319, $M+Na^+$) shows a fragment at *m/z* 207 and 11,10-EHETE (B, *m/z* 371, $[M+Na]^+$) shows a fragment at *m/z* 233.

Concerning the slow growing species *C. scutellum*, LC-MS profiling (**Figure 5.16**) showed two peaks related to known oxylipins deriving from 15-LOX activity, as also found in *C. closterium*. In fact, LC-MS analysis displayed the epoxyalcohol 14,13-EHETE (*m/z* 371, RT= 8.48, MS cluster *m/z* 259, 273 and 289, 0.0012 ± 0.0001 µmol mg^{-1} C) and the corresponding hydroxyacid 15-HEPE (*m/z* 355, RT= 12.22, UV_{max}= 234 nm, 0.0011 ± 0.0007 µmol mg^{-1} C) (**Figure 5.12-5.13A**; d'Ippolito et al., 2009).

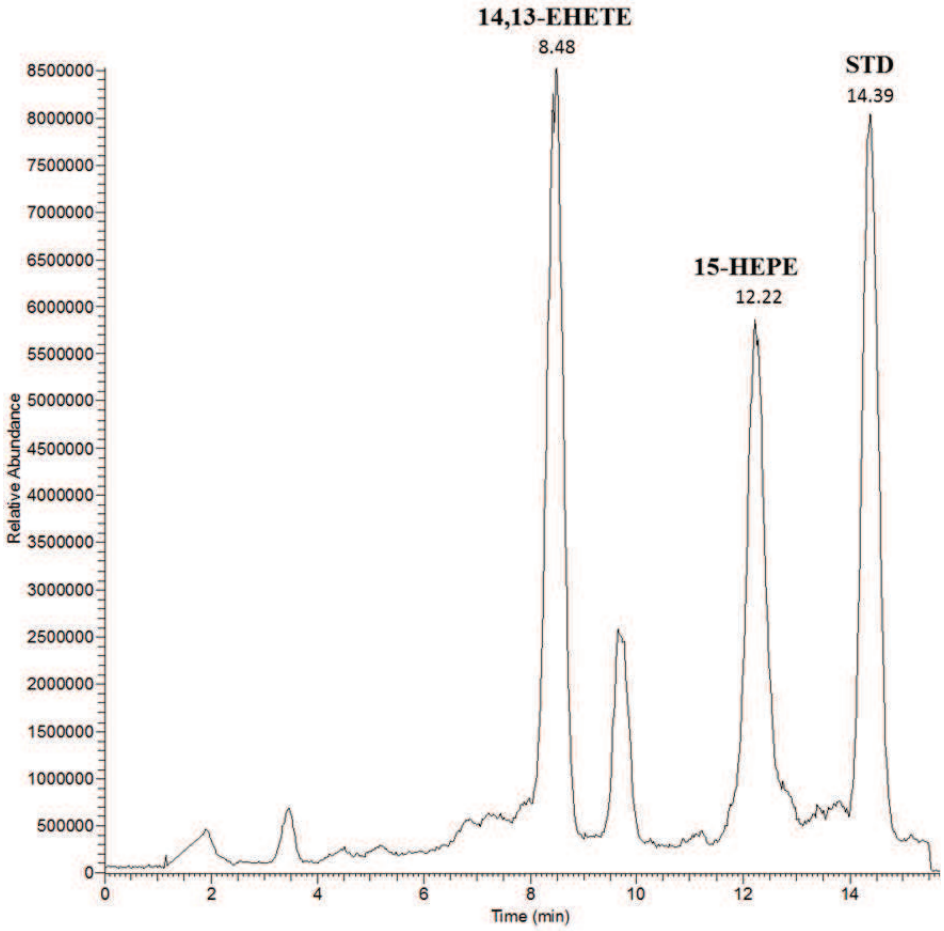


Figure 5.16. LC-MS profile of *C. scutellum* showing peaks related to 14,15-epoxy-13-hydroxy-eicosa-5*Z*,8*Z*,11*Z*,17*Z*-tetraenoic acid (14,13-EHETE) and 15-hydroxy-eicosa-5*Z*,8*Z*,11*Z*,13*E*,17*Z*-pentaenoic acid (15-HEPE). Internal standard (STD, RT= 14.39): 16-hydroxy-hexadecanoic acid.

The fourth species, *Diploneis* sp., exhibited a diverse metabolic profile in LC-MS (**Figure 5.17**), with two sets of abundant peaks in the chromatographic region of oxylipins not correlated to known compounds. In detail, the two peaks displayed a molecular weight at *m*/*z* 381 ($[M+Na]^+$, RT= 5.02 and 5.67, 0.005 ± 0.0004 µmol mg^{-1} C) associated to a specific fragmentation cluster in MS/MS (*m*/*z* 281, 303 and 321, **Figure 5.18**) and UV_{max}= 370 nm; whereas, other two peaks at *m*/*z* 345 ($[M+Na]^+$, RT= 7.76 and 8.46, 0.004 ± 0.0003 µmol mg^{-1} C) did not display any characteristic fragmentation.

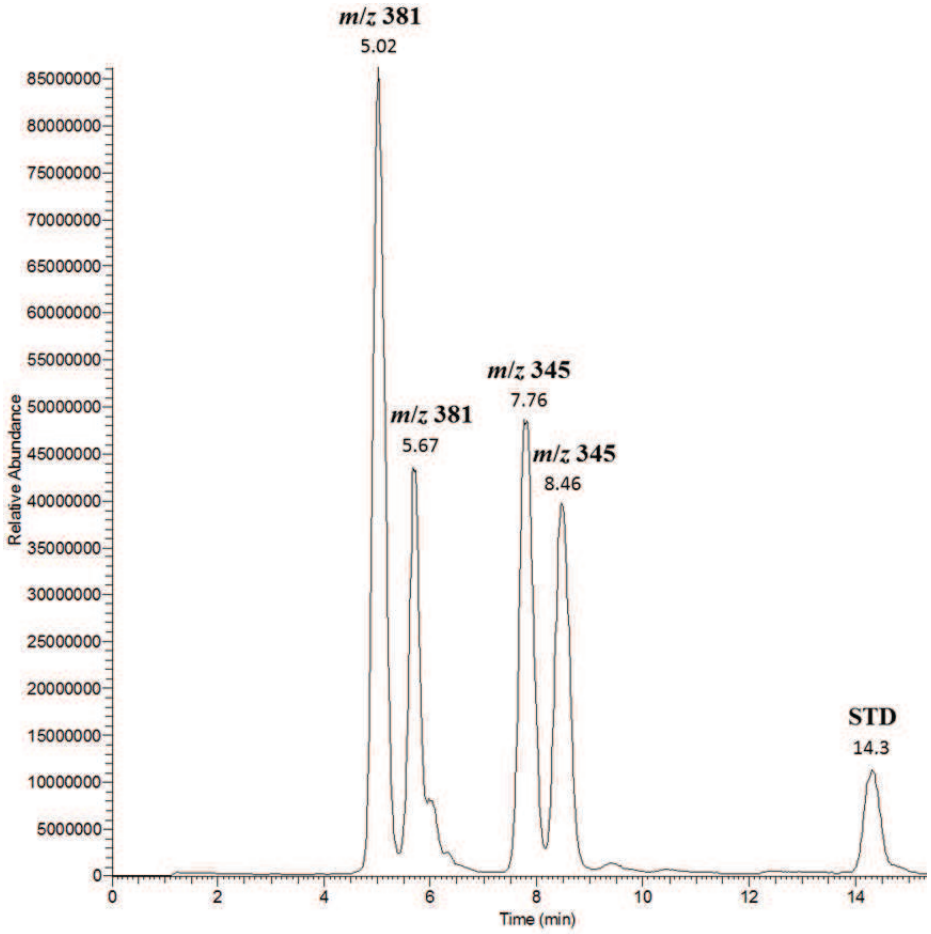


Figure 5.17. LC-MS profile of *Diploneis* sp. showing two peaks at *m*/*z* 381 and two peaks at *m*/*z* 345. Internal standard (STD, RT= 14.30): 16-hydroxy-hexadecanoic acid.

Interestingly, these compounds were very abundant as proved by the comparison of the area of their peaks with that of the internal standard (16-hydroxy-hexadecanoic acid). Since these compounds at *m*/*z* 381 and *m*/*z* 345 have been found for the first time in diatoms, further purifications and NMR analyses will be necessary in order to clarify the whole structure and a possible correlation with the oxylipin pathway.

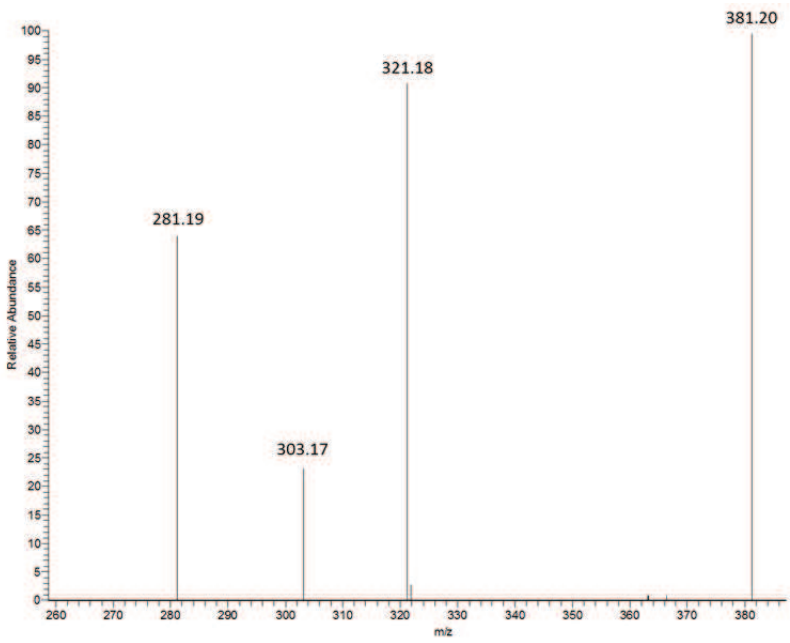


Figure 5.18. MS/MS spectrum of the peak at *m*/*z* 381displaying fragments at *m*/*z* 281, 303 and 321.

5.3.4 FOX2 Assay

According to oxylipins analysis, *N. shiloi* displayed the highest LOX activity (1.4 ± 0.05 µmol of FAHs per mg C) with significant differences with respect to *C. closterium*, *C. scutellum* and *Diploneis* sp. (p value < 0.001).

Moreover, *C. closterium* exhibited higher LOX activity (FAHs= 0.4 ± 0.05 µmol mg^{-1} C) than *C. scutellum* (0.28 ± 0.01 µmol mg^{-1} C; p value = 0.0012), in good agreement with the level of oxylipins detected by LC-MS in these species (**Figure 5.19**).

Only little evidence of LOX activity was revealed by FOX2 assay in *Diploneis* sp. (0.016 ± 0.001 µmol FAHs per mg C; p value < 0.001). This result was again in agreement with the LC-MS analysis and supported hypothesis of the biosynthesis of the uncharacterized compounds found in this species by other biochemical pathways (**Figure 5.17**).

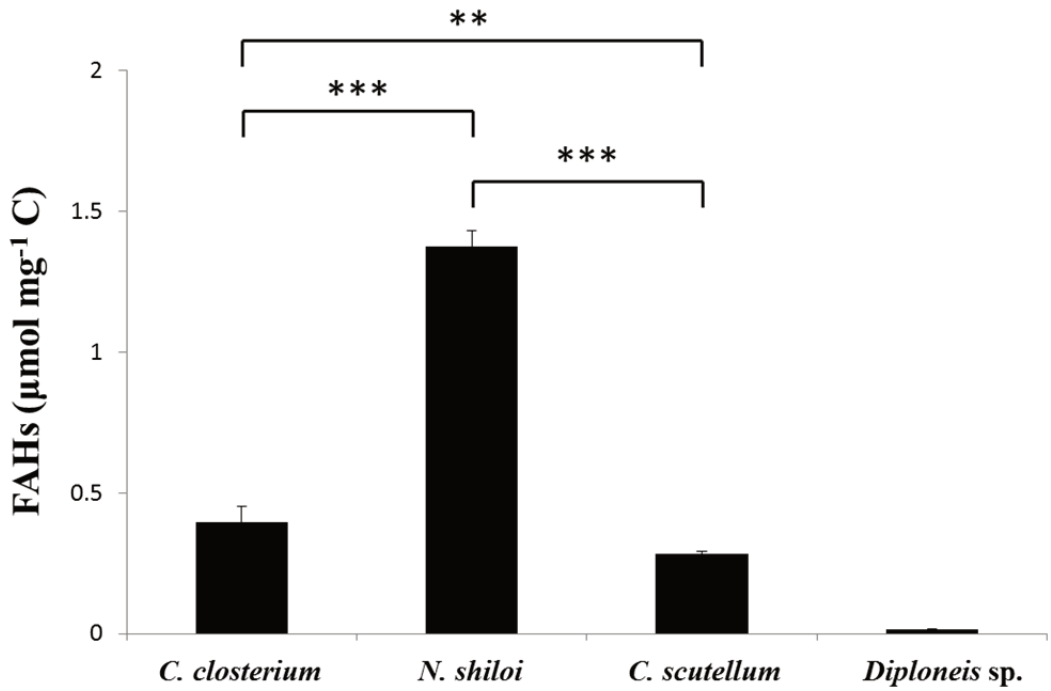


Figure 5.19. FAHs production expressed as µmol per carbon mg measured as the difference between TCEP treated and untreated samples. Data are reported as means ± SD (N=3). Statistical differences were evaluated by unpaired *t*-tests (*p<0.05, **p<0.01, ***p<0.001).

5.3.5 StS analysis by LC-MS

LC-MS analysis of the MeOH extracts of the four benthic diatoms showed presence of StS in every sample even if the compounds differed qualitatively and quantitatively (**Figure 5.20-5.21-5.25**). In particular, *N. shiloi* produced the highest amount of StS, reaching 0.13 µmol mg^{-1} C (p values < 0.01). In turn, *C. closterium* displayed higher StS levels (0.05 µmol mg^{-1} C) than *C. scutellum* and *Diploneis* sp. (0.029 and 0.023 µmol mg^{-1} C, respectively; p values < 0.05) (**Figure 5.25**).

After deep analysis corroborated by literature data and comparison with standards (Gallo et al., 2017, 2018), it was possible to conclude that *N. shiloi* biosynthesizes fucosterol sulfate (RT= 5.74; **Figure 5.20-5.21**), featured by molecular weight at *m*/*z*

491, and brassicasterol sulfate (RT= 5.57; **Figure 5.21**) with *m/z* 477. This latter compound was also present in the extracts of *C. scutellum* (RT= 5.59; **Figure 5.20-5.21**) and *Diploneis* sp. (RT= 5.64; **Figure 5.20-5.21**).

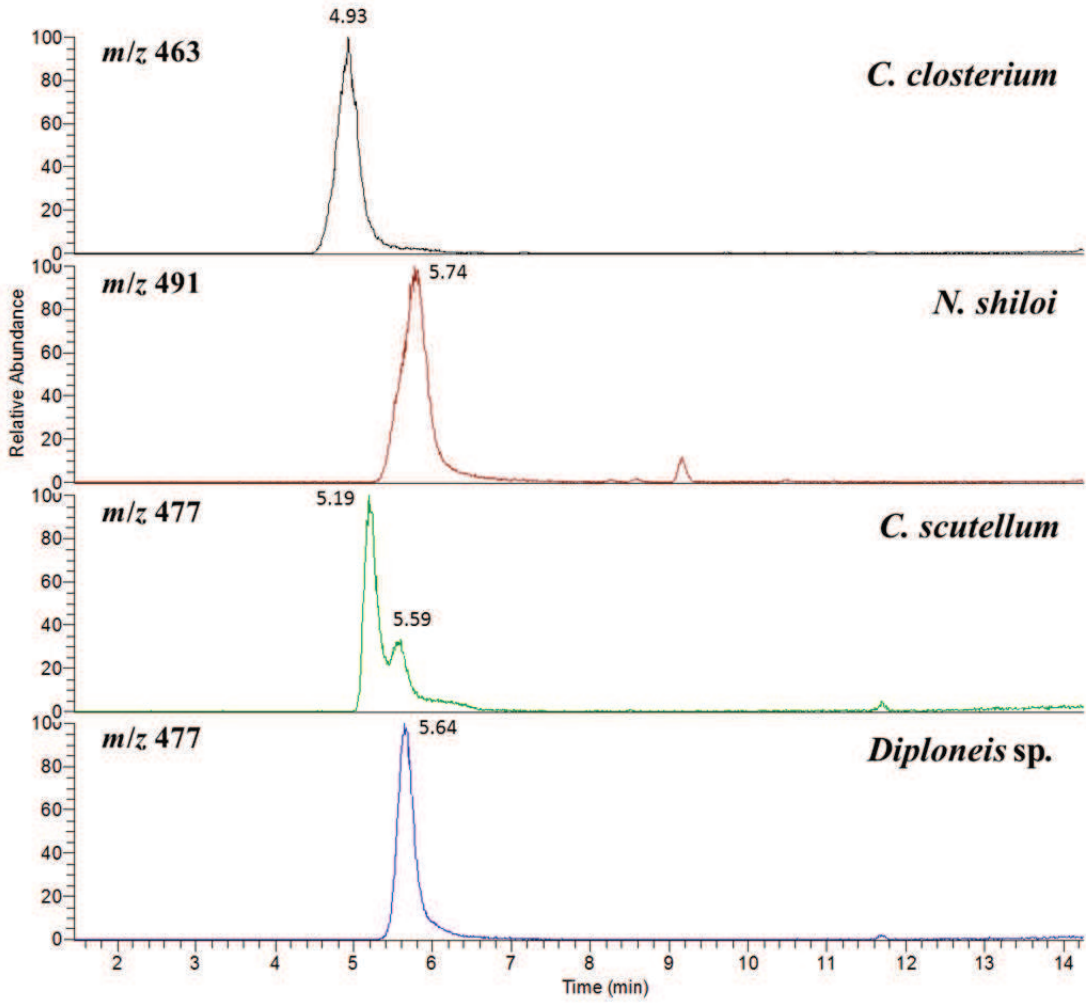


Figure 5.20. LC-MS peaks assigned as StS reported for each diatom under analysis.

In addition, *C. scutellum* showed the presence of a second product with the same molecular weight (*m/z* 477) but with a different retention time (RT= 5.19). This compound was described as 24-methylene cholesterol sulfate (**Figure 5.20-5.21**) when it was compared with the *Cyclotella cryptica* profile, used as a natural standard.

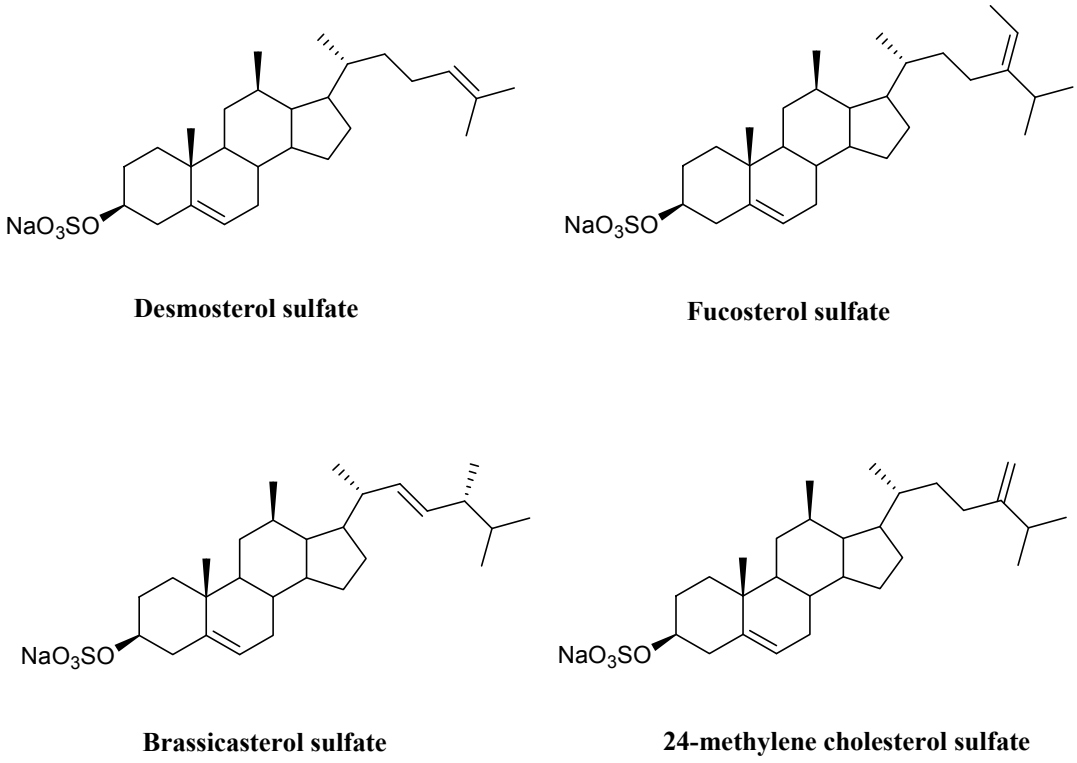


Figure 5.21. Chemical structures of StS found in the four benthic diatoms under analysis.

C. closterium profile (**Figure 5.20**) revealed a single StS, displaying *m*/*z* 463, which was assigned to an unsatured cholesterol derivative (RT= 4.93; **Figure 5.21**). This peak has been tentatively assigned to desmosterol sulfate but the chemical analysis is still in progress to corroborate the identification.

5.4 Discussion

Chemical and biochemical results reported in the present Chapter have revealed that the four benthic diatoms under analysis produce interesting compounds with cytotoxic properties. As summarized in **Table 5.1**, three of the four species under analysis, namely *C. closterium*, *N. shiloi* and *C. scutellum*, showed LOX activity in analogy with what

has been described in the planktonic counterparts (d'Ippolito et al., 2002a,b, 2005, 2009; Nanjappa et al., 2014).

C. closterium and *C. scutellum* displayed the same enzymatic pathway based on a 15-LOX, likely 15*S*-LOX (d'Ippolito et al., 2009), which is the most common among diatoms. *C. closterium* also showed an additional epoxyalcohol from DHA precursor produced by a putative LOX enzyme able to oxidize this fatty acid at C_{17}. The simultaneous presence of products deriving by EPA:dependent 15-LOX and DHA:dependent 17-LOX has been previously reported in diatoms of the *Leptocynlindrus* species (Nanjappa et al., 2014). As previously suggested in this species, it is possible that both series of compounds synthesized by a single enzyme whose low catalytic specificity is determined by the identity of the terminal end of DHA and EPA (**Figure 5.22**).

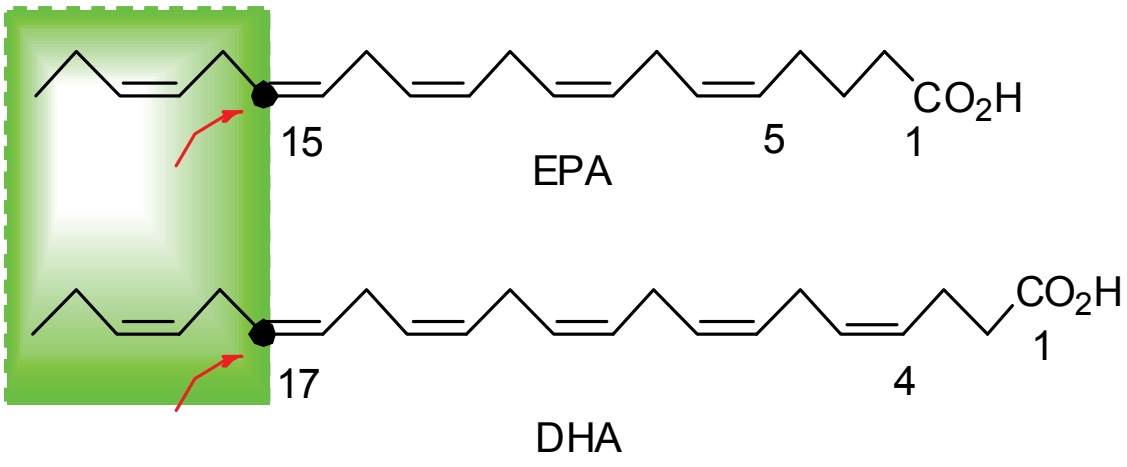
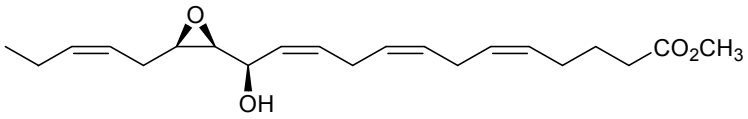
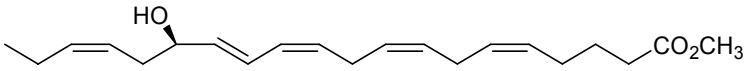
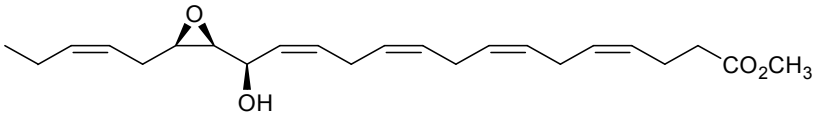
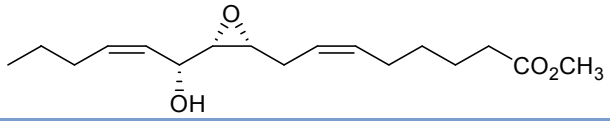
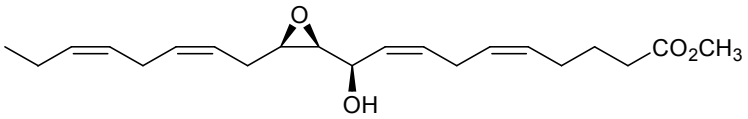
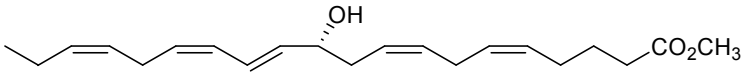
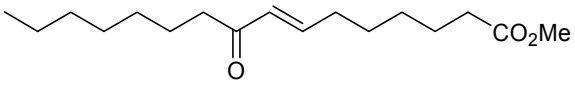
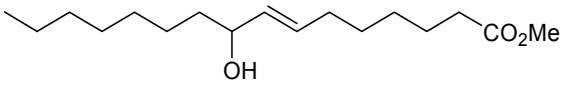
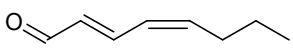
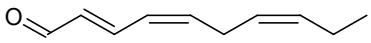
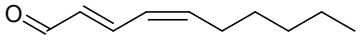
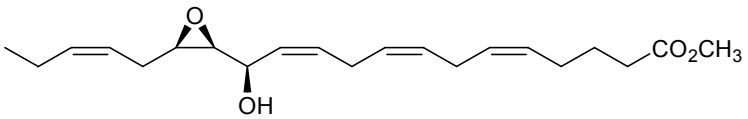
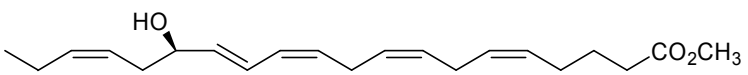


Figure 5.22. EPA and DHA chemical structures highlighting the identity of the terminal part of EPA and DHA (green area). The positions 15 and 17 are at the same distance from the methyl group, thus suggesting that a single LOX can be responsible for both oxidation. Red arrows indicate the insertion of O_2 catalyzed by LOX.

Table 5.1. Summary of the compounds found in the four diatoms under analysis, specific enzymatic activity and their chemical structures.

SPECIES	ENZYME	CHEMICAL STRUCTURE
C. closterium		
14,13-EHETE	EPA:15-LOX	
15-HEPE	EPA:15-LOX	
16,15-EHDoPE	DHA:17-LOX	
N. shiloi		
9,11-EHHDE	HTE:9-LOX	
11,10-EHETE	EPA:11-LOX	
11-HEPE	EPA:11-LOX	
9-KHME	HDE:oxygenase	
9-HHME	HDE:oxygenase	
2E,4Z-octadienal	HTE:9-LOX	
2E,4Z,7Z-decatrienal	EPA:11-LOX	
2E,4Z-decadienal	AA:11-LOX	
C. scutellum		
14,13-EHETE	EPA:15-LOX	
15-HEPE	EPA:15-LOX	

On the contrary, *N. shiloi* showed the most diverse oxylipins pattern with compounds deriving from a C_{16}-dependent 9-LOX, likely 9*S*-LOX (d'Ippolito et al., 2003, 2005) and a C_{20}-dependent 11-LOX activity (**Table 5.1**). This diatom was the only species to produce PUAs (2*E*,4*Z*-octadienal, 2*E*,4*Z*,7*Z*-decatrienal and 2*E*,4*Z*-decadienal) among the four benthic diatoms that are the subject of this thesis. Using labeled precursors and cell preparations of *T. rotula*, it has been demonstrated that PUAs are the end products of a LOX/HPL pathway. In particular, octadienal originates by a 9*S*-LOX enzymatic cascade starting from 6-hydroxy-hexadeca-7*E*,9*Z*,12*Z*-trienoic acid (6-HHTrE, C16:3 ω4), while decatrienal biosynthesis depends on the enzymatic modification of EPA (C20:5 ω3) by 11-LOX (d'Ippolito et al., 2006; see Chapter 1). An identical pathway is also possible for decadienal from arachidonic acid (AA, C20:4 ω6). Despite the original report from Miralto et al. 1999, this aldehyde is not common in diatoms as AA is not frequent in this group of microalgae. However, the fatty acid analysis of *N. shiloi* revealed presence of AA that justifies the finding of decadienal in this species. Furthermore, a considerable amount of oxygenated derivatives of palmitoleic acid, namely 9-KHME and 9-HHME, were also detected in *N. shiloi*. Interestingly these compounds have been so far reported only in *T. rotula* (d'Ippolito et al., 2005; **Table 5.1**).

On the whole, oxygenase metabolism of *N. shiloi* is very similar to that described in the planktonic diatoms *S. marinoi* and *T. rotula*, already associated to negative effects on copepod reproduction (Ianora and Poulet, 1993; Carotenuto et al., 2002).

This consideration is quite interesting since the biological tests (see Chapter 4) evinced that, among the four benthic diatoms under analysis, *N. shiloi* was the most potent in

impairing sea urchin embryo development (**Figure 5.23A**). In the case of *C. closterium* a considerable percentage of abnormal plutei has been detected, whereas *C. scutellum* did not affect embryo development (see Chapter 4).

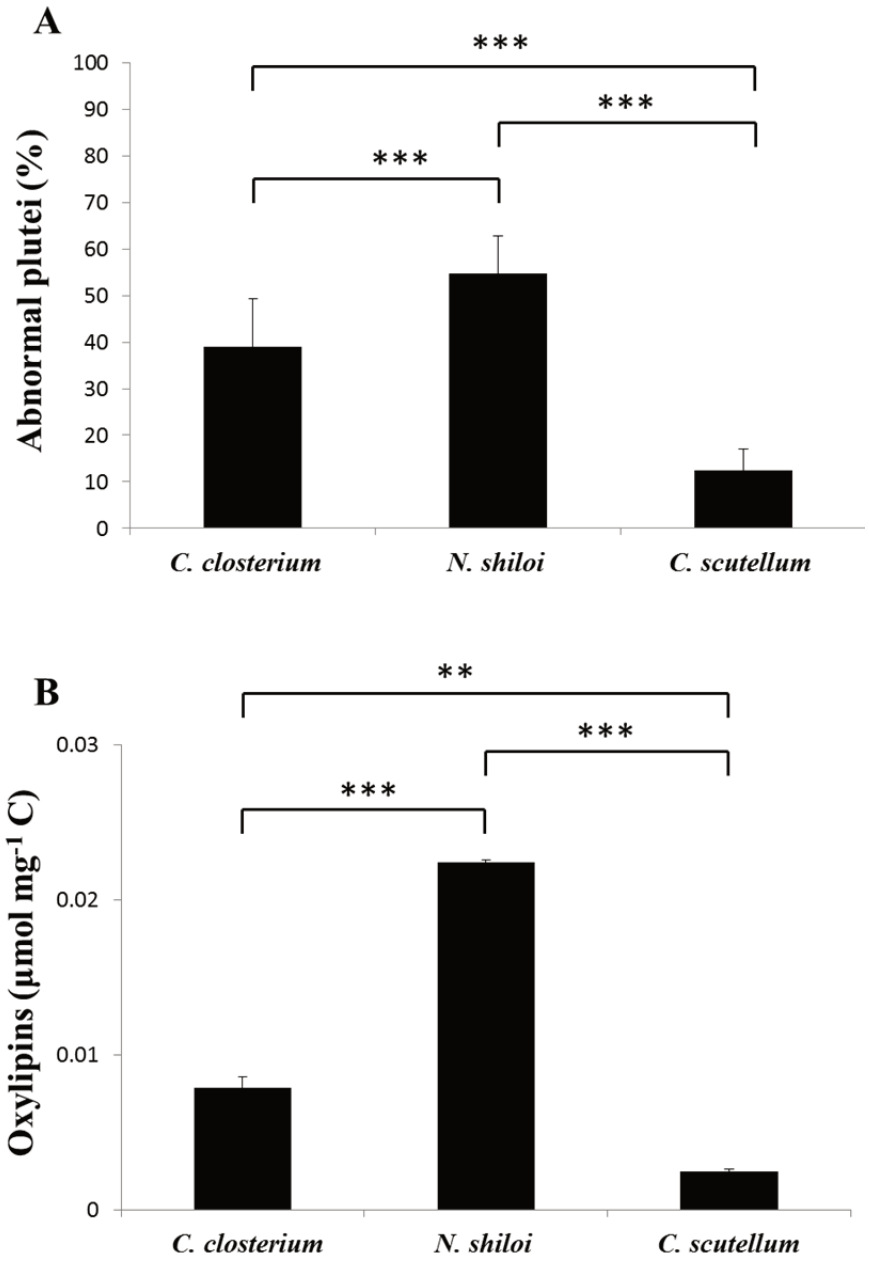


Figure 5.23. Percentage of abnormal plutei (embryos at 48 hpf) deriving from sea urchins fed with *C. closterium*, *N. shiloi* and *C. scutellum* (A; see Chapter 4, Figure 4.11). Oxylipins content measured by LC-MS expressed as µmol mg^{-1} C. Data are reported as means ± SD (N=3). Statistical differences were evaluated by unpaired *t*-tests (B) (*p<0.05, **p<0.01, ***p<0.001).

Chemical analyses have revealed that *C. closterium* produce a level of oxylipins higher than *C. scutellum* (**Figure 5.23B**). This could explain the different biological activity, probably due to the different dose given to sea urchins in one month feeding (**Figure 5.23A**). LOX differences in *C. closterium*, *N. shiloi* and *C. scutellum* were also confirmed by FOX assay that detected FAHs (**Figure 5.19**) with the same trend of concentrations found in oxylipins by LC-MS quantifications.

GC-MS profiles from *C. closterium*, *N. shiloi* and *C. scutellum* showed that C_{16} and C_{20} fatty acids were highly represented. This finding is in agreement with the occurrence of C_{16} and C_{20} oxylipin in these species. Only *C. closterium* showed DHA and, consistently, was the only species to produce oxylipins deriving from this fatty acid.

As discussed above, LOX products of DHA have been reported in other species of planktonic diatoms, including three species of the Leptocylindraceae family (Nanjappa et al., 2014) that likely synthesize these molecules by a LOX pathway parallel to EPA-dependent 15-LOX (see **Figure 5.22**).

Among the four species studied, *Diploneis* sp. was the only one without recognizable oxylipins (**Table 5.1**). The LC-MS profile of this species was characterized by a family of undetermined products that cannot be ascribed to LOX activities although they show typical chemical features of oxygenated derivatives of fatty acids. In particular, the compound at *m*/*z* 381 showed a high UV_{max} (370 nm) and a specific fragmentation pattern (*m*/*z* 281, 303 and 321) that are reminiscent of polyoxygenated fatty acid derivatives. The absence of LOX activity (**Figure 5.19**), as established by FOX2 assay, is consistent with the lack of correlation with LOX products in GC- and LC-MS (**Figure 5.17**), thus corroborating the hypothesis of the origin from other oxygenase enzymes.

The structure characterization of these compounds was outside the aim of this thesis. However, similar molecules have been previously observed in other diatoms and their identification may shed light on the presence of an additional class of chemical mediators in this lineage of microalgae. Despite the absence of LOX pathways, *Diploneis* sp. induced significant aberrations on sea urchin embryo development with respect *C. scutellum* (p value < 0.001) with level of activity similar to *C. closterium* (p value > 0.05) and not far from *N. shiloi* (p value < 0.001; **Figure 5.24**).

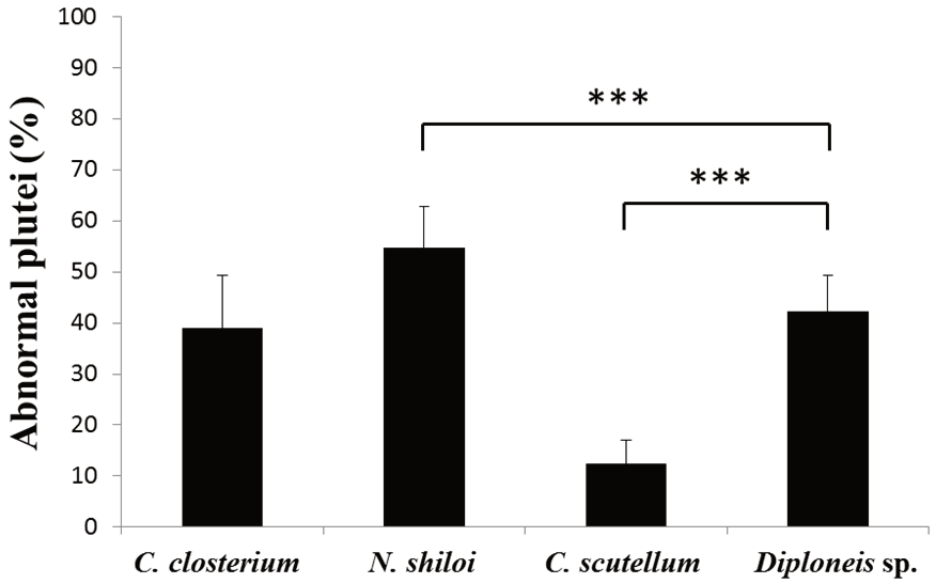


Figure 5.24. Percentage of abnormal plutei deriving from sea urchins fed with *C. closterium*, *N. shiloi*, *C. scutellum* and *Diploneis* sp. (see Chapter 4).

Since the teratogenic activity in copepods has been associated to oxidative burst induced by high levels of LOX-derived hydroperoxides (e.g. fatty acid hydroperoxides) (Fontana et al., 2007b) it is possible that the uncharacterized oxygenase pathway can lead to a similar accumulation of free radicals. This would explain the inhibitory activity of *Diploneis* sp. on sea urchins and the toxicological analogies with the other three diatoms

that, instead, produce oxylipins. In such a case, it would be possible to have two different pathways committed to the same biological activity. Obviously, it is not possible to exclude that the toxicity of *Diploneis* sp. on sea urchins can be due to other compounds that were not considered in the present study.

In relation to the role of StS as intracellular mediators of PCD (Gallo et al., 2017) in diatoms, chemical analyses also focused on StS in the four benthic diatoms. LC-MS/MS investigation revealed significant level of StS in these samples. This is the first report of this family of compounds in benthic diatoms. In detail, three of the four species (*N. shiloi*, *C. scutellum* and *Diploneis* sp.) contained sterol sulfates with carbon skeleton typical of plant sterols, namely brassicasterol and fucosterol. On the other hand, the extracts of *C. closterium* contained only an uncharacterized product whose structure is probably related to that of cholesterol sulfate, the typical animal sterol. This diversity of structures and the biosynthetic implications are intriguing since the occurrence of sterol skeletons is usually Phyla dependent. In nature, there are very few examples of organisms (e.g. sponges) that are able to biosynthesize both plant and animal sterols. Thus, the finding of brassicasterol and unsaturated cholesterol in the benthic diatoms is atypical and deserves further investigations.

The quantity of StS measured in each species (**Figure 5.25**) reveals a quite similar trend displayed by oxylipins. *N. shiloi* revealed the highest amount of StS (0.13 ± 0.01 μmol mg^{-1} C, p values < 0.01). As in the case of oxylipins, *C. closterium* (0.06 ± 0.005 μmol mg^{-1} C) showed a significant difference (p value < 0.05) with respect *C. scutellum* (0.03 ± 0.01 μmol mg^{-1} C). Only *Diploneis* sp. diverged by the correlation between StS and oxylipins.

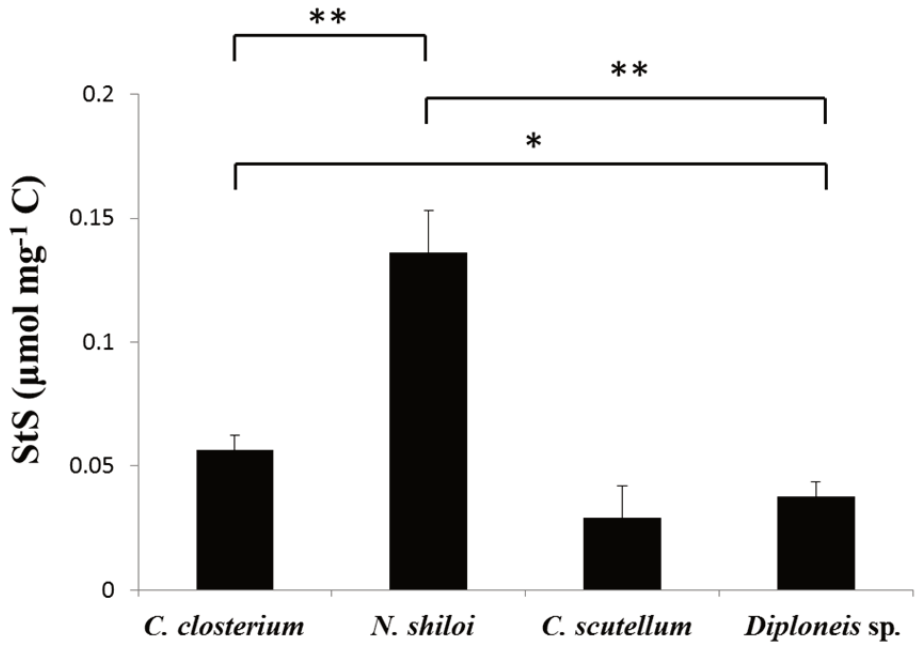


Figure 5.25. StS concentrations measured as µmol per carbon mg, in the four benthic diatoms. Data are reported as means ± SD (N=3). Statistical differences evaluated by unpaired *t*-tests (*p<0.05, **p<0.01, ***p<0.001).

However, if we consider the products of the uncharacterized oxygenase pathway, there is a good agreement between StS and the level of these products. The physiological or ecological meaning of this parallelism has to be investigated. Oxylipins and StS share a few effects (apoptosis, oxidative burst, NO release) and, probably, common effectors. Thus it is not unlikely that there might be a cross-talk between the two classes of mediators.

6. Conclusions

6.1 Contribution of the Ph. D. work

Diatoms are a highly diverse and abundant group of marine photosynthetic microorganisms responsible for almost 50 % of primary productivity, thus sustaining the entire life food chain in oceans, seas and lakes. These microalgae are regarded as "nourishment" of marine grazers even if, as summarized by the *paradox of diatom-copepod interactions* (Ban et al., 1997), several studies have emphasized the negative effect that many diatom species induce in natural zooplankton grazers (Miralto et al., 1999; Ianora et al., 2003). To date, most of these toxic outcomes have been related to production of cytotoxic compounds belonging to the chemical families of oxylipins and sterol sulfates (StS). Oxylipins arise from enzymatic oxidation of the polyunsatured fatty acids stored in cell and chloroplast membranes. They have been found in several planktonic species (Pohnert, 2000, 2002; Pohnert et al., 2002; d'Ippolito et al., 2002a,b, 2003, 2005, 2009; Fontana et al., 2007a,b; Lamari et al., 2013; Nanjappa et al., 2014) but there are very few data about their occurrence and function in benthic species (Jüttner et al., 2010; Pezzolesi et al., 2017). Concerning StS, despite their wide distribution in several marine invertebrates (sea stars, sponges), they have only been described in a recent paper in a planktonic diatom, *Skeletonema marinoi* (Gallo et al., 2017), and there was no report of their presence in benthic species until now.

In this work, the impact of benthic diatoms was examined for the first time on the sea urchin *P. lividus*, comparing their biological activities with species of planktonic diatoms. A multidisciplinary approach has been utilized, integrating developmental

biology, metabolomics and molecular biology to address the differences induced by four benthic diatoms (*Cylindrotheca closterium*, *Nanofrustulum shiloi*, *Cocconeis scutellum* and *Diploneis* sp.) from the morphological and molecular viewpoints. More specifically, the effects of these benthic diatoms have been studied on the grazing activity of *P. lividus*.

The major findings achieved in this Ph. D. research are:

(i) morphological observations revealed a noxious effects of *C. closterium*, *N. shiloi* and *Diploneis* sp. on embryos deriving from adult sea urchin *P. lividus* fed for one month on these diets, with *N. shiloi* showing the strongest effects (this observation has been supported by chemical analysis; see below); on the contrary, *C. scutellum* showed no effects, producing embryos as those deriving from the control diet (see Figure 4.11). Malformations were very similar to those observed after treatment with planktonic diatom-derived oxylipins.

(ii) metabolomic analysis showed that feeding on these diatoms was able to induce variations in the levels of lipids and/or amino acids in the gonads of *P. lividus* (see Figures 4.14-4.16).

(iii) molecular results through *Real Time qPCR* and *Next Generation Sequencing* (NGS) approaches showed that benthic diatoms were able to affect several biological processes (see Figures 4.20, 4.22, 4.23).

(iv) chemical analyses (focused on two classes of diatom-derived secondary metabolites, the oxylipins and StS) revealed that all benthic diatoms produced oxylipins with the only exception of *Diploneis* sp., which in

turn produced some unknown compounds deriving from polyunsaturated fatty acids metabolism. In addition, all four benthic diatoms showed the presence of StS.

(v) biochemical and biological data showed a clear correlation between diatoms toxicity in sea urchin embryos and levels of oxylipins.

Overall, the results indicate that benthic diatoms are able to induce morphological and molecular toxigenic effects in the sea urchin *P. lividus*. Interestingly, the only exception is represented by *C. scutellum*, the only diatom already studied for its apoptogenic activity in the marine shrimp *Hippolyte inermis* (Zupo, 2000; Zupo and Messina, 2007). These findings suggest that this diatom has a species-specific apoptotic mechanism in the androgenic gland of *H. inermis*.

Molecular findings from *de novo* transcriptomic data and *Real Time qPCR* highlighted that *P. lividus* activates a great number of genes, involved in different biological and cellular processes, in order to react against diatom-derived toxic metabolites.

In addition, despite the importance of sea urchins as a model organism for the marine environment, to date there are few molecular tools available for *P. lividus* since the genome has not been annotated. For this reason, *de novo* transcriptome obtained in the present Ph. D. work, represents a promising molecular tool to identify new *P. lividus* genes involved in stress response. Taken together, these genes could represent general biomarkers to detect exposure to other stress-inducing molecules in the sea urchin *P. lividus*, including pollutants such as copper, zinc and hydrocarbons. Furthermore, these findings support the idea that marine organisms possess a series of cellular strategies to counteract the negative effects of toxic compounds, including the massive

reorganization of gene expression networks. A novel tool has been proposed for understanding how changes in gene expression levels may be used as an early indicator of stressful conditions in the marine environment. As observed in most adaptive responses, the control of gene expression is tightly regulated and displays fast response kinetics, which enables the cell to change its transcriptional capacity within minutes in the presence of stress and to return to its basal state after the stress is removed (de Nadal et al., 2011). Combining standard embryological assays with gene expression is useful to tease out interesting data in terms of how benthic diatoms interact with the sea urchin embryo transcriptome. The present study highlights substantial gene plasticity in embryos of the sea urchin *P. lividus* in response to feeding on benthic diatoms. These data are in accordance with some experiments performed on the larvae from the red sea urchin *Strongylocentrotus franciscanus*, showing a variation in *heat shock proteins* transcript level when exposed to elevated CO_2 conditions and then subjected to a temperature shift (O'Donnell et al., 2010). In that case, the Authors suggested that marine organisms could have the capability to protect themselves against ocean acidification, but this could come at a significant cost to an organism's ability to tolerate increased stress intensity.

The chemical analyses described herewith have revealed that, as already described for planktonic species, benthic diatoms are able to produce oxylipins and StS. In particular, three species, *C. closterium*, *N. shiloi* and *C. scutellum* displayed a common lipoxygenase (LOX) activity, with a clear correlation between oxylipin levels and the consequent teratogenic effects on sea urchin embryos. Considering *Diploneis* sp., feeding tests revealed a comparable result to *C. closterium*, suggesting that its unknown

compounds could negatively affect embryo development. Moreover, all diatoms displayed a good production of StS, even if with a high chemical variability among the four species. All chemical findings well supported the negative effect observed at the morphological level: for example *N. shiloi* was the only benthic diatoms producing PUAs and the highest amount of NVOs and StS; this could be linked to the strongest morphological effect induced by this species. The most interesting hypothesis is that the two classes of diatom-derived secondary metabolites analyzed in this work, the oxylipins and StS, both involved in cell death programs, could act in a synergic or inter-related way in the cells. In fact, marine organisms are constantly subjected to a mixture of environmental stressors and natural and/or dissolved anthropogenic compounds, including both physical (e.g. cold, heat and osmotic conditions) and chemical (e.g. endocrine disruptor chemicals and hydrocarbons) stressors (Kozlowsky-Suzuki et al., 2009). In a situation of exposure to multiple xenobiotics or inter- and intraspecific signals, single compounds may act independently as in a single exposure, or they may interact to modulate the effects of total multiple exposure in synergistic, antagonistic or additive toxicity (Koppe et al., 2006; Xie et al., 2006).

6.2 General conclusions and future perspectives

The work, presented in this Thesis, extends the information on the effects of planktonic diatoms on marine organisms, opening interesting future perspectives. As extensively reported in Chapter 1, in spite of extensive studies in the literature on planktonic diatoms, secondary metabolites from benthic diatoms and their interactions with grazers remain an unexplored topic. These data are good as a starting point to

improve knowledge on benthic diatoms. In fact, they extend the information of the negative effects of diatoms on the reproductive success of marine organisms by showing that benthic species induce similar effects as those described for planktonic species.

In addition to the well-known role as predator deterrents, diatom oxylipins have been also discovered to be potent allelochemicals able to suppress the growth of other phytoplankton. For instance, the PUA decadienal was showed to inhibit the growth of *Thalassiosira weissflogii* in a dose- and time-dependent manner (Casotti et al., 2005). Furthermore, among phytoplankton, smaller species such as the prymnesiophyte *Isochrysis galbana* were found more sensitive to PUAs compared to larger species like the chlorophyte *Tetraselmis suecica* and the diatom *Skeletonema marinoi* (Ribalet et al., 2007a). Since decadienal has been demonstrated to induce programmed cell death (PCD) by the generation of nitric oxide (NO) and further treatments with sublethal doses of decadienal triggered resistance to subsequent lethal doses, a sophisticated stress surveillance system has been proposed (Vardi et al., 2006). In fact, according to these authors, PUA concentrations could exceed a certain threshold during algal bloom and act as a diffusible bloom-termination signal regulating population dynamics.

In agreement with the numerous reports on oxylipins (Vardi et al., 2006), it has been recently demonstrated that StS act as intracellular mediators in signal transduction pathways leading to PCD through the activation of NO pathways and oxidative burst. The intracellular concentrations of StS linearly correlate with culture ageing and reach auto inhibitory levels in the declining phase (Gallo et al., 2017), suggesting a crucial role of these small molecules in the regulation of cell growth.

Furthermore, phytoplankton-bacteria interactions effectively transport carbon dioxide from the atmosphere deep into the ocean. Phytoplankton usually lives in the sunlit surface waters of the ocean and converts atmospheric-derived carbon dioxide into their biomass through photosynthesis. Bacteria colonized this biomass, which for a part sinks into the darker depths, turning it back into carbon dioxide through respiration. The effects of oxylipins on the activity and community structure of microbial assemblages associated with sinking particles were also investigated. PUA doses ranging from 1 to 10 μM stimulated respiration, organic matter hydrolysis, and cell growth, while PUA dosages near 100 μM appeared to be toxic, resulting in decreased bacterial cell abundance and metabolism, as well as pronounced shifts in bacterial community composition (Edwards et al., 2015). Overall, these studies have opened the hypothesis that PUAs induce an increase in remineralization of sinking particles with a reduction of organic carbon export from surface waters and in turn to the retention of phosphorus and other nutrients in shallower waters, increasing primary productivity on interannual timescales (Edwards et al., 2015).

The present thesis suggests that the four benthic diatoms under analysis produce oxylipins and sterol sulfates with a high variability in terms of chemical structure and concentration. In agreement with previous papers in planktonic species, these results indicate that mechanism of cell signaling during bloom dynamics and regulation of carbon export efficiency could be also applied to benthic species.

Taking into account these interesting findings, future experiments will be devoted to the isolation and chemical characterization of new benthic species with the aim to expand the current knowledge of the dynamics of chemical interactions in benthic

diatoms. In addition, since toxigenic effects of diatoms have been tested only on sea urchins and copepods (Miralto et al., 1999; Romano et al., 2003; Poulet et al., 2003; Ianora et al., 2004; Fontana et al., 2007b), new feeding experiments will be performed using other marine invertebrates to reveal the ecological impact of diatoms on the marine fauna, considering the huge abundance of them during bloom events.

Furthermore, the chemical analyses of the benthic diatom *Diploneis* sp. opened interesting new perspectives on the identification of uncharacterized compounds. In fact, as extensively described in the Chapter 5, the LC-MS profile of this species showed still uncharacterized products that cannot be ascribed to LOX activities and that are reminiscent of polyoxygenated fatty acid derivatives. Similar molecules have been previously observed in other planktonic diatoms (unpublished results) and their identification may shed light on the presence of an additional class of chemical mediators in this lineage of microalgae.

The data provided in this Ph. D. thesis will possibly provide new hints for the bioprospecting of secondary metabolites from benthic diatom species. In the case of planktonic species, it has been recently demonstrated that diatom-derived PUAs activated cell death in human cancer cell lines (Sansone et al., 2014). Considering the renewed interest for new bioactive compounds from the marine habitat (Ruocco et al., 2017c) and given the importance of diatoms as a rich source of such compounds (Ruocco et al., 2016c; Romano et al., 2017; Giordano et al., 2018), screening of benthic diatom-derived organic extracts on cell lines would be helpful to explore these natural compounds for their possible antitumor, antioxidant, antinflammatory, antiaging, antidiabetes, antiobesity, antineurodegenerative, depigmenting and exfoliating activities

for biotechnological applications in the pharmacological, cosmaceutical and nutraceutical fields.

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Appendix A

NAME	ACRONYMS	FUNCTIONS	REFERENCES
STRESS RESPONSE			
Heat Shock Protein 70 *Heat Shock Protein 60* *Heat Shock Protein 56*	*hsp70* *hsp60* *hsp56*	A family of proteins that are produced by cells in response to exposure to stressful conditions. They were first described in relation to heat shock, but are now known to also be expressed during other stresses including exposure to cold, UV light and during wound healing or tissue remodeling; many members of this group perform chaperone function by stabilizing new proteins to ensure correct folding or by helping to refold proteins that were damaged by the cell stress.	Marrone et al., 2012
DNA-methyltransferase 1	*MTase*	A large group of enzymes that all methylate their substrates but can be split into several subclasses based on their structural features; these enzymatic reactions are found in many pathways and are implicated in genetic diseases, cancer, and metabolic diseases.	Marrone et al., 2012
Glutamine synthetase	*GS*	An enzyme that plays an essential role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine; the glutamine produced is an essential precursor for purine and pyrimidine synthesis, a modulator of protein turnover or an intermediate for gluconeogenesis and acid-base balance	Marrone et al., 2012

Cytochrome b	*cyt b*	A protein found in the mitochondria of eukaryotic cells; it works as part of the electron transport chain and is the main subunit of trans membrane cytochrome bc1 and b6f complexes.	Marrone et al., 2012
p38 mitogen-activated protein kinase	*p38 MAPK*	A class of protein kinases that are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in cell differentiation, apoptosis and autophagy.	Marrone et al., 2012
14-3-3 epsilon protein	*14-3-3ε*	A family of conserved regulatory molecules that are expressed in all eukaryotic cells; they bind a multitude of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors.	Marrone et al., 2012
Sp-Cspe3/7L caspase-8	*caspase 3/7 CASP8*	Protease enzymes playing essential roles in programmed cell death and inflammation; they are named caspases due to their specific cysteine protease activity.	Ruocco et al., 2016; Romano et al., 2011
Nuclear factor kappa-light-chain-enhancer of activated B cells	*NF-κB*	A protein complex that controls transcription of DNA, cytokine production and cell survival; it is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, heavy metals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens.	Russo et al., 2014

Tumor protein p53	*p53*	Tumor suppressors; they bind to DNA and regulate gene expression to prevent mutations of the genome.	Varrella et al., 2016a
Hypoxia inducible factor 1-alpha	*HIF1A*	A subunit of a heterodimeric transcription factor hypoxia-inducible factor 1 (HIF-1) that is encoded by the HIF1A gene; it is considered as the master transcriptional regulator of cellular and developmental response to hypoxia.	Varrella et al., 2016a
ERCC excision repair 3	*ERCC3*	Together with ERCC4, ERCC1 forms the ERCC1-XPF enzyme complex that participates in DNA repair and DNA recombination.	Still unpublished
SKELETOGENESIS			
Spicule matrix protein 30 *Spicule matrix protein 50*	*SM30* *SM50*	These proteins direct spicules growth in certain orientations and inhibit growth in others.	Zito et al., 2003; Marrone et al., 2012
Bone morphogenetic protein 5-7	*BMP5-7*	Promote the oral-aboral ectoderm specification in the sea urchin embryo.	Zito et al., 2003; Marrone et al., 2012
Nectin	*Nec*	Families of cellular adhesion molecules involved in Ca^{2+}-independent cellular adhesion.	Marrone et al., 2012
Univin	*Uni*	Encodes for the Transforming growth factor beta (TGF-β) promoting the interaction of ectodermal cells and the growth of skeleton in sea urchin embryos.	Zito et al., 2003; Marrone et al., 2012

Pl-p16 *Pl-p19*	*p16* *p19*	Two small acidic proteins involved in the formation of the biomineralized skeleton of sea urchin embryos and adults.	Costa et al., 2012
Jun	*Jun*	Transcription factor required for the progression through the G1 phase of the cell cycle.	Russo et al., 2014
DEVELOPMENT AND DIFFERENTIATION			
Hatching enzyme	*Hat*	Secreted by the early blastula to proteolyze the fertilization envelope allowing the embryo to hatch.	Marrone et al., 2012
SRY (sex determining region Y)-box9	*sox9*	Trascription factor involved in the left-right asymmetry process.	Marrone et al., 2012
Blastula protease 10	*BP10*	Astacin metalloprotease, role in the early differentiation of blastomers along the primordial axis of the embryo.	Marrone et al., 2012
Blimp	*Blimp*	Zinc finger transcription factor, which plays a central role in both early and late endomesoderm specification.	Varrella et al., 2014
ALG-2 interacting protein X/1	*Alix*	Protein involved in endocytic membrane trafficking, filamentous (F)-actin remodeling and cytokinesis.	Varrella et al., 2014
Wnt 5	*Wnt5*	Initiates the specification of the sea urchin posterior ectoderm.	Varrella et al., 2014
Wnt 6	*Wnt6*	Activates endoderm in the sea urchin gene regulatory network.	Varrella et al., 2014

Wnt 8	*Wnt8*	Regulates endomesodermal specification, embryo patterning, and early primary mesenchyme cells-gene regulatory network.	Varrella et al., 2014
Cadherin-associated protein (catenin) delta 2	*δ-2-catenin*	Protein involved in the coordination of cell-cell adhesion and gene transcription; this protein promotes cell movement.	Varrella et al., 2016a
Nodal	*nodal*	Regulates left-right asymmetry during cleavage and early blastula stages, acting on the right side of the embryo.	Ruocco et al., 2017b
Transcription factor 4 *Transcription factor 7*	*tcf4* *TCF7*	Members of the Tcf/Lef family responsible for the specification of cell fates along the sea urchin animal-vegetal axis, by interacting with β-catenin.	Ruocco et al., 2017b
Forkhead box protein A *Forkhead box protein G* *Forkhead box protein O*	*FOXA* *FoxG* *Foxo*	Members of the Forkhead transcription factors involved in the regulation of embryonic development, cell fate specification, cell differentiation, and morphogenesis.	Ruocco et al., 2017b
Growth factor indipendent 1	*GFI-1*	Zinc finger transcription factor expressed in the presumptive ciliary band at the mesenchyme blastula stage.	Ruocco et al., 2017b
One Cut Homeobox 1	*OneCut*	Transcription factor expressed in the early gastrula stage giving rise to the future ciliary band regions and later in the definitive ciliary band of the sea urchin pluteus, including the apical organ.	Ruocco et al., 2017b

TGF beta-activated kinase	*TAK1*	The major intracellular mediator of the highly conserved TGF beta/BMP signaling pathway implicated in many other different signaling pathways including TNF and interleukin as well as JNK and p38 activities.	Ruocco et al., 2017b
Vascular endothelial growth factor	*VEGF*	VEGF/VEGFR signaling between ectoderm and the primary mesenchyme cells (PMCs) plays a key role in the positioning and differentiation of these migrating cells during gastrulation and in the morphogenesis of the sea urchin embryonic skeleton.	Ruocco et al., 2017b
c-Jun N-terminal kinase	*JNK*	Required for cell movements during embryonic development, especially for invagination of the archenteron.	Ruocco et al., 2017b
DETOXIFICATION			
Metallothionein *Metallothionein 4* *Metallothionein 5* *Metallothionein 6* *Metallothionein 7* *Metallothionein 8*	*MT* *MT4* *MT5* *MT6* *MT7* *MT8*	Proteins capable of binding to heavy metals, involved in the transport of heavy metals and cellular detoxification.	Marrone et al., 2012; Ragusa et al., 2013
Multi drug resistance protein 1	*MDR1*	ATP-binding cassette protein.	Varrella et al., 2014
Catalase	*CAT*	Catalyzes the decomposition of hydrogen peroxide to water and oxygen; it is important in protecting the cell from oxidative damage by reactive oxygen species.	Varrella et al., 2014

Appendix B

Invertebrate Reproduction & Development

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Experimental evaluation of the feeding rate, growth and fertility of the sea urchins *Paracentrotus lividus*

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Experimental evaluation of the feeding rate, growth and fertility of the sea urchins *Paracentrotus lividus*

Nadia Ruocco[a,b,c], Valerio Zupo[d], Davide Caramiello[e], Francesca Glaviano[e], Gianluca Polese[b], Luisa Albarano[a,b] and Maria Costantini[a]

[a]Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Napoli, Italy; [b]Department of Biology, University of Naples Federico II, Complesso Universitario di Monte Sant'Angelo, Napoli, Italy; [c]Bio-Organic Chemistry Unit, Institute of Biomolecular Chemistry-CNR, Pozzuoli, Naples, Italy; [d]Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Napoli, Italy; [e]Unit Marine Resources for Research, Stazione Zoologica Anton Dohrn, Naples, Italy

ABSTRACT

The trophic ecology of the sea urchin *Paracentrotus lividus*, a key species in several shallow benthic communities, has been intensively studied, but the role of various foods in the processes of growth and gonadal maturation is still scarcely understood. This research assessed the effects of two fundamental food items for wild specimens of the sea urchin *Paracentrotus lividus*, the tissues of the seagrass *Posidonia oceanic*a and of the green alga *Ulva rigida*, compared to the effect of a commercial compound feed on the somatic growth, gonad development, fertilization success and post-embryonic development. Consumption rates along with the C/N ratios were measured in the feeds and in the faecal pellets. We demonstrated that feeding for three months on *U. rigida* and *P. oceanica* did not affect growth and gonadal index of adults, fertilization processes and first cleavage and development, as well as field-collected animals. In contrast, a diet based on formulated pellets triggered a significant increase of gonadal index, but lack of gamete production, due to a follicular hyperthrophy. Our work will be useful for the definition of optimal diets for the production of mature broodstocks of an ecologically important marine model organism.

Impact statement

– We aim at defining the daily feeding rate of the sea urchin P. lividus
– P. lividus represents a key species in various benthic communities.
– Feeds are important in the processes of growth and gonadal maturation of sea urchins.
– Several factors influence sea urchin feeding rates.

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Introduction

Paracentrotus lividus is a species of sea urchin belonging to the family *Parechinidae* within the large phylum *Echinodermata* (Pawson 2007). This species widely occurs in different marine environment, such as the Mediterranean Sea and the Eastern Atlantic Ocean from western Scotland and Ireland to the Azores, Canary Islands and Morocco (Boudouresque and Verlaque 2001).

In the Mediterranean sea, this sea urchin is considered to be a key species for several coastal communities associated to vegetated ecosystems, thanks to its role in their food webs (Zupo and Fresi 1984). It is an important consumer of plant tissues (Boudouresque et al. 2007), and it is also a well-established model organism for eco-toxicological and physiological studies. In addition, the gonads of *P. lividus* are considered a gastronomic delicacy and consequently its market demand has significantly increased since the early 1970s, causing a depletion of this species in different site of the Mediterranean (Guidetti et al. 2004; Lawrence 2001). In fact, harvesting of *P. lividus* is reflected in population structures from fished and control locations: since humans selectively collect the largest sea urchins (>4 cm), large-sized *P. lividus* were rare at the exploited locations (Guidetti et al. 2004).

Several attempts have been applied to identify effective formulated diets with the aim to promote body growth and gonadal maturation of adults in land based systems (Caltagirone et al. 1992; Fabbrocini et al. 2012). An extensive literature investigates the effect of diets on growth of *P. lividus*, as well as dietary effects on reproductive success (Bayed et al. 2005; Frantzis and Gremare 1993; Carboni et al. 2012; Lawrence 2013). However, some key issues still limit its industrial exploitation.

CONTACT Valerio Zupo vzupo@szn.it; Maria Costantini maria.costantini@szn.it

Yet the wide development of a major commercial urchin-farming industry has been restrained by the lack of a fully developed technology for the cost-effective production of sea urchins with the desired gonad quality (Carboni et al. 2012). Furthermore, it is important to consider that the feeding rates measured in the field may differ from those taken in aquaculture, also according to the composition and quality of its feeds (Bayed et al. 2005). Feeds appear to play a pivotal role in the regulation of the reproductive cycle and it has been proven that gonadic growth is strongly correlated with the availability, quantity and quality of food (Boudouresque and Verlaque, 2001; Boudouresque and Verlaque 2013). Therefore, the measurement of feeding rates according to the quality and quantity of foods can be important to plan correct feeding procedures to finalize the culture of this species and a rapid maturation of gonads (Spirlet et al. 1998).

In this study, we aimed to experimentally define the feeding rates of *P. lividus* reared in test tanks, according to the feeds used, and to measure the effects of feeds on its body growth and the maturation of gonads, as compared to the well-known patterns observed in the environment (Vadas et al. 2000). Adults of the sea urchins *P. lividus* were fed on the green alga *Ulva rigida*, the seagrass *Posidonia oceanica* and a commercial compound feed used in aquaculture for three months. More in details, fresh *U. rigida* and *P. oceanica* represent natural dietetic items, also because both these plants characterized environments usually populated by *P. lividus*. In fact, this sea urchin is one of the major macro-herbivores in the Mediterranean Sea eating a range of red, green and brown algae in addition to seagrass (Boudouresque and Verlaque 2001). *U. rigida* is considered a control food in several feeding experiments on fish and invertebrates (Valente et al. 2006) and it is included in various diets for sea urchins (Frantzis and Gremare 1993), demonstrating that it affected the growth rate of *P. lividus* (Frantzis et al. 1992). Moreover, *P. lividus* represents the dominant grazer for *P. oceanica*, choosing this seagrass because of the greater availability of shelter and food in the seagrass (Pinna et al. 2012). It prefers leaves covered with epibiota and adult, thicker leaves (Vergés et al. 2011), consuming all parts of the seagrass as a 'preferred' species for feeding during spring and summer (Boudouresque and Verlaque 2001). In fact, the sea urchin is one of the main consumers of *P. oceanica* (Verlaque 1987), avoiding other species that synthesize toxic or repellent secondary metabolites (Guerriero et al. 1992; Lemée et al. 1996; Tejada et al. 2013). Moreover, *P. lividus* is a key species that controls the dynamics of seaweeds and seagrasses, by eliminating, when at high densities, the erect stratum of algae and seagrasses (Sala and Zabala 1996). Sea urchins were also fed with a pre-hydrated pelletized formulated feed (Classic K®; Hendrix SpA, Mozzecane-VR, Italy). Previous data demonstrated that this commercial pellets provided rapid fattening of gonads (Fabbrocini and D'Adamo 2010, 2011; Fabbrocini et al. 2012), representing a positive control on the size and quality of gonads. It is also inexpensive, available on the market and producing negligible amounts of wastes (Fabbrocini et al. 2012).

Materials and methods

Ethics statement

Wild individuals of *P. lividus* (Lamarck) were collected from a site in the Bay of Naples that is not privately owned or protected in any way, according to the Italian legislation (DPR 1639/68, 09/19/1980 confirmed on 01/10/2000). Field studies did not include endangered or protected species. All experimental procedures on animals were in compliance with the welfare guidelines of the European Union (Directive 609/86).

Sea urchin collection

Adult sea urchins *Paracentrotus lividus* were collected during in January (corresponding to the start of reproductive cycle; Byrne 1990) by scuba-divers in the site 'Rocce Verdi' in the Gulf of Naples, Italy. Sixty individuals of an average weight of 40 g were collected and immediately transported to the laboratory, using a thermally insulated box containing seawater. Further, they were transferred to plastic tanks with recirculating seawater, prior to start the feeding experiments. Sea urchins were individually measured using a calliper, to record the maximum horizontal diameter of thecae; adult specimens with a diameter between 4 and 5 cm (excluding the spines), that is a typical diameters for mature adults, were selected for the experiments.

Experimental rearing apparatus

A continuous open flow-through (35 l per hour) system was set, consisting of nine rectangular glass tanks (three tanks for each diet used; chamber size 30 × 35 × 40 with 35 l of sea water): seawater was pumped from the sea, collected in an outdoor basin, then filtered twice on gauze filters (200 µm) and moved to an indoor basin, filtered again by means of a protein skimmer, a UV sterilizer, a refrigerator and a mechanical filter, then moved to the experimental tanks. The aeration in the

tanks was provided by airstones. Used water was released through outflow tubes from each tank at a rate of 1 full change per hour. The main abiotic parameters were recorded three times a week using a multiparametric probe (YSI 85, YSI, Incorporated) and kept constant using water chillers, circulation pumps and filters (temperature 18 ± 1°C; salinity 38 ± 1;, dissolved O_2 7 mg/l; pH 8.1). The internal surfaces of tanks were manually cleaned of their epiphytes and fouling organisms three times a week, using synthetic sponges and scrapers.

Feeding experiments

Preliminary experiments were performed to define the daily feeding rates of adult sea urchins. Ten adults of *P. lividus* were reared in each tank of the continuous open flow-through system, and then fed with 10, 20 and 30 g wet weight (WW) per day of the green macroalgae *U. rigida* (often used as a control to feed sea urchins in laboratory experiments) to define their daily feeding rate. *U rigida* was chosen cause it is considered among the most palatable and nutritionally suitable feeds for *P. lividus* (Hiratsuka and Uehara 2007; Cyrus et al. 2015), and therefore, its consumption is close to the maximum feeding rates for this species. The other feeds were provided at the same rate and the presence of small residuals of all the feeds was checked prior to start the experiments. Food consumption was measured after 24, 48 and 72 h to check that residuals were still present, for all the items, after 1 day and that they could be still consumed in further days. Doses were set in order to assure that the residual food was maintained in low abundance, to avoid water pollution and lixiviation (Sartori and Gaion 2016). Once determined this initial dose, guaranteeing an *ad libitum* consumption in 24 h, on all items considered, we started the feeding experiments.

Twenty adult *P. lividus* (10 females and 10 males for each diet treatment, identified according to the morphology of the apical system of females and the different genital plates between females and males under the dissecting microscope; Philip and Foster 1971; Jeffery and Emlet 2003), collected in the field in January, were reared in each experimental tank and fed, alternatively, with 20 g per day of (a) fresh *U. rigida*, (b) fresh *P. oceanica* leaves (including both brown and green tissues) and (c) pelletized (2.5 × 2.5 × 5 mm) pre-hydrated formulated feed (Classic K; HENDRIX SpA, Mozzecane, Verona, Italy pre-hydrated formulated feed (HENDRIX,Verona, Italy; a commercial food characterized by a high protein content 465 g kg^{-1}, proteins of animal origin accounting for <50 g kg^{-1}) as suggested by Fabbrocini and D'Adamo (2010). Before starting our experiments, we considered a three-day time frame of starvation, in according with other previous feeding experiments (Ruocco et al. 2018). The short acclimation and starvation time was chosen because our aim was to study the effects of three different feeds on the maturation gonads and increase of gonadal index (GI), starting from the gonadic state characterizing the experimental sea urchins at the moment of the collection. A longer starvation time would produce auto-digestion of gonadic tissues to sustain the metabolism of starved animals (Sartori et al. 2015) and this was in contrast with our experimental aims.

The residual amount of food in each tank was weighed (fresh weight) every day to calculate the individual daily food consumption. Macroalgae and *Posidonia* tissues were still alive when the residuals were removed; therefore, we should exclude any significant process of degradation and weight reduction within the experimental time considered. However, controls of the feeds were placed into own containers, to exclude the influence of feeding sea urchins, to determine possible change in their fresh weight. Since the feeds were daily replaced and they were mainly ingested by sea urchins in the first hours after the administration we can exclude the influence of lixiviation processes in the formulated feeds. Excess moisture was removed from *U. rigida* by blotting the leaves on paper towels before weighing. The total food daily ingested was calculated as the difference between the feed introduced and that removed in each tank. Food intake was calculated in milligrams of feeds (dry weight) consumed per animal, per day. Since adult sea urchins were grouped in a tank for each diet, an average consumption was also evaluated.

Carbon and nitrogen measurements in feeds and faecal pellets

Additional samples of algae and *Posidonia oceanica* tissues were collected and stored for chemical analyses, in order to define the quality of the fresh feeds provided, as described above. To this end, additional thalli of *U. rigida* and leaves of *P. oceanica* were collected in the Gulf of Naples by scuba-divers, transferred to the laboratory and stored at −20°C. Two gram of three independent samples of algae and three of the seagrass were subsequently dried at 65°C for three days up to constant weight. In parallel, *P. lividus* faecal pellets were collected in the experimental tanks after continuous feeding on fresh tissues of *U. rigida* and *P. oceanica*, as well as on formulated diet pellets. As we used a short starvation period (as described above) and considering

that faecal material is still produced for a large number of days after food intake has ceased, we collected faecal pellets 15 days after the beginning of feeding experiments, in order to be sure that gut retention time could not affect this analysis. The faecal pellets were similarly dried up to constant weight, as described above. Dry samples were homogenized in a grinder in order to obtain a thin dust. Samples were weighed and loaded into a Carbon/Nitrogen (CN) Analyzer (FlashEA 1112 Automatic Elemental Analyzer, Thermo Scientific Waltham, MA, USA), following the procedure described by Hedges and Stern (1984). Acetanilide was used as standard. C/N analyses were conducted in duplicate. The data obtained allowed for an interpretation of results obtained according to the three experimental diets.

Gonadal index and histological preparation

Evaluations of the GI% were performed on 20 field-collected adult specimens of the sea urchin *P. lividus* (t0) as compared with 20 specimens fed one month and three months on each of the three feeds, i.e. *U. rigida, P. oceanica* and formulated pellets. These sea urchins were weighed, sacrificed and dissected; their gonads were extracted and weighed (fw) for the evaluation of the GI as (Sánchez-España et al. 2004; Fabbrocini and D'Adamo 2011; Keshavarz et al. 2017):

1) GI = gonadal wet weight (g)/sea urchin wet weight (g) × 100

The gonads of three males and three females for each treatment were dissected, fixed in Bouin, included in paraffin blocks, sliced and stained by hematoxylin, to evaluate the histological structure of tissues and interpret the results of other data sets (Byrne 1990). The slices, after staining, were enclosed into permanent mountings and observed under the optical microscope.

Gamete collection, embryo culture and morphological analysis

After three months of feeding, as described, adults of *P. lividus* reared in the experimental tanks were injected 2 ml of 2M KCl through the peribuccal membrane to trigger the release of gametes. Eggs were immediately washed with filtered seawater (FSW) and kept in FSW until use. Concentrated sperm was collected and kept at 4°C until use. Eggs were fertilized in FSW, utilizing sperm-to-egg ratios of 100:1 (Romano et al. 2011).

Fertilized eggs were incubated at 20°C in a controlled temperature chamber on a 12h/12h light/dark cycle. These experiments were conducted in triplicates, fertilizing 400 eggs in 3 ml of sea water.

Percentages of fertilization of first cleavage at about 1 h post fertilization (hpf) and normal and malformed embryos (48 hpf) were evaluated for at least 200 plutei from each female (fixed in formaldehyde 4% in FSW) using a light microscope (Zeiss Axiovert 135TV; Carl Zeiss, Jena, Germany).

Statistical analyses

The statistical significance of differences among daily feeding rates recorded according to the three feeds, and C and N concentrations in feeds and faecal pellets were evaluated by the average and variation of the data reported as 'mean ± standard deviation (SD)'. SD bars were plotted in order to allow an immediate perception of the intervals of superimposition of our replicates. Statistical significance of differences between individual treatments was evaluated using *t*-tests (Prism 3.0, GraphPad Prism 4.00 for Windows, GraphPad Software, San Diego California USA). $p < 0.05$ was considered as statistically significant.

Results

Daily feeding rate

Preliminary experiments were performed to define the daily feeding rates of adult sea urchins. Ten adults of sea urchins *P. lividus* were reared in each tank of the continuous open flow-through system and then fed with 10, 20 and 30 g WW per day of the green macroalga *U. rigida* (usually used as control to fed sea urchins in SZN Animal Facility) to approximately determine the daily sea urchin feeding rate. Food consumption was measured after 24, 48 and 72 h. Food consumption was higher in the first 24 h on fasted animals with significant differences among the three quantities (Figure 1; 10g versus 20g p value = 0.047, indicated with a; 20 g vs. 30 g, p value = 0.06, indicated with b; 10 g vs. 30 g, p value = 0.0012, indicated with c). At 24 h the food consumption decreased, but differences were not significant among the three quantities; at 72 h, the daily feeding rates stabilized on an average of about 1 g WW per sea urchin, independently of the initial quantity of *U. rigida* administered.

According to these preliminary experiments, sea urchins were fed 20 g (WW) per day of *U. rigida* and *P. oceanica*. In the case of formulated pellets, *ad libitum* feeding was 1.8 g/day per 20 sea urchins, i.e. about 0.10 g/animal.

After three months of treatment, the daily feeding rate (as dry weight, DW) for a sea urchin corresponded

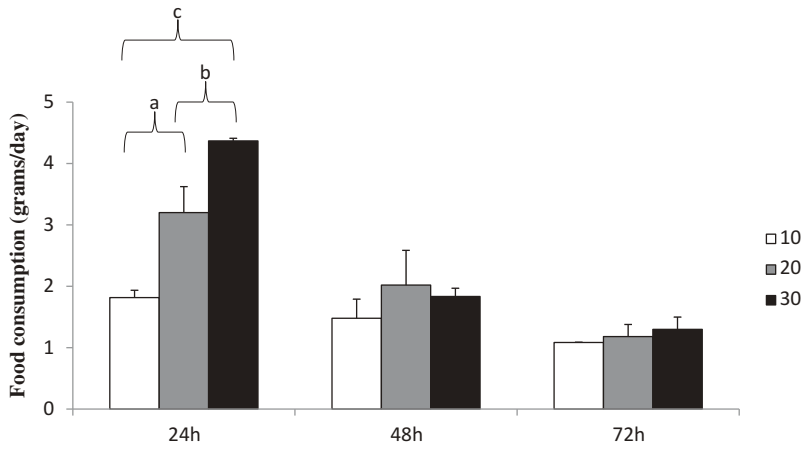

Figure 1. Ten adults of sea urchins *Paracentrotus lividus* were put in a tank of the continuous open flow-through system, fasted for three days and then fed with 10 (white bars), 20 (grey bars) and 30 (black bars) grams wet weight (WW) per day of the green macroalgae *Ulva rigida* (usually used as control to fed sea urchins in SZN Animal Facility). Statistically significant differences have been detected only after 24 h of feeding using 10 and 20 g of *Ulva rigida*: 10 versus 20 g, p value = 0.0471 (a); 10 versus 30 g = 0.0012 (c); 20 versus 30 g, p value = 0.0606 (b). After 48 and 72 h of feeding no significant differences have been detected ($p > 0.05$).

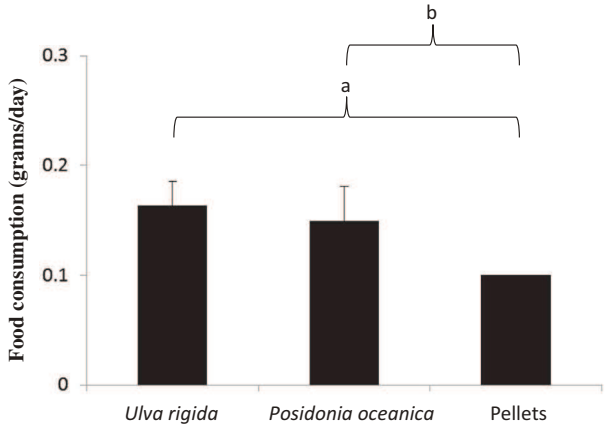

Figure 2. Daily feeding rate. The histogram shows the daily feeding rate per animal after feeding for one month with *Ulva rigida, Posidonia oceanica* and the pre-hydrated formulated feed. *Ulva rigida* versus *Posidonia oceanica, p* value = 0.06 (no significant value $p > 0.05$); formulated pellets versus *Ulva rigida p* value < 0.0001 (a); *P. oceanica* and pellets *p* value < 0.0001 (b).

to 0.16, 0.15 and 0.10 g for *U. rigida, P. oceanica* and formulated pellets, respectively (Figure 2).

Differences in individual daily feeding rates were not significant (*t*-test) between treatments *U. rigida* and *P. oceanica* (p = 0.06). In contrast, the daily feeding rates obtained for formulated pellets were significantly lower both in comparison of *U. rigida* versus pellets p value <0.0001 (indicated with a); *P. oceanica* and pellets p value <0.0001 (indicated with b).

Table 1. Composition in percentage (%) of nitrogen (N) and carbon (C) of the three feeds, *Ulva rigida, Posidonia oceanica* and formulated pellets, and of the faecal pellets collected from sea urchins fed with the three feeds. C/N ratio has also been reported both in the three feeds and in the correspondent faecal pellets.

	Composition (%)					
	Feed		Faecal pellets		C/N ratio	
	N	C	N	C	Feed	Faecal pellets
Ulva rigida	2.19	26.00	1.55	15.88	11.85	10.27
Posidonia Oceanica	2.41	36.64	1.03	30.95	15.23	30.09
Pellets	4.86	42.18	1.83	22.39	8.67	12.25

Carbon and nitrogen contents of feeds and faecal pellets

The amounts of carbon (C) and nitrogen (N) measured by CN Analyzer in the three feeds (namely, *U. rigida, P. oceanica* and formulated pellets) were compared with those measured in the sea urchin faecal pellets (Table 1).

The C/N ratio was highest in *P. oceanica* (15.2) and it decreased in *U. rigida* (11.9) reaching the lowest value (8.7) in the formulated pellets (Figure 3(a)). Statistically significant differences (*t*-test) in the C/N ratios were found between *U. rigida* and *P. oceanica* (p = 0.03, indicated with a), as well as between *U. rigida* and formulated pellets (p = 0.0274, indicated as c). The difference is highly significant between *P. oceanica* and pellets (p = 0.005, indicated with b). Concerning faecal pellets, *P. oceanica* exhibited a higher C/N ratio (30.1) in respect to *U. rigida*. This value decreased in the

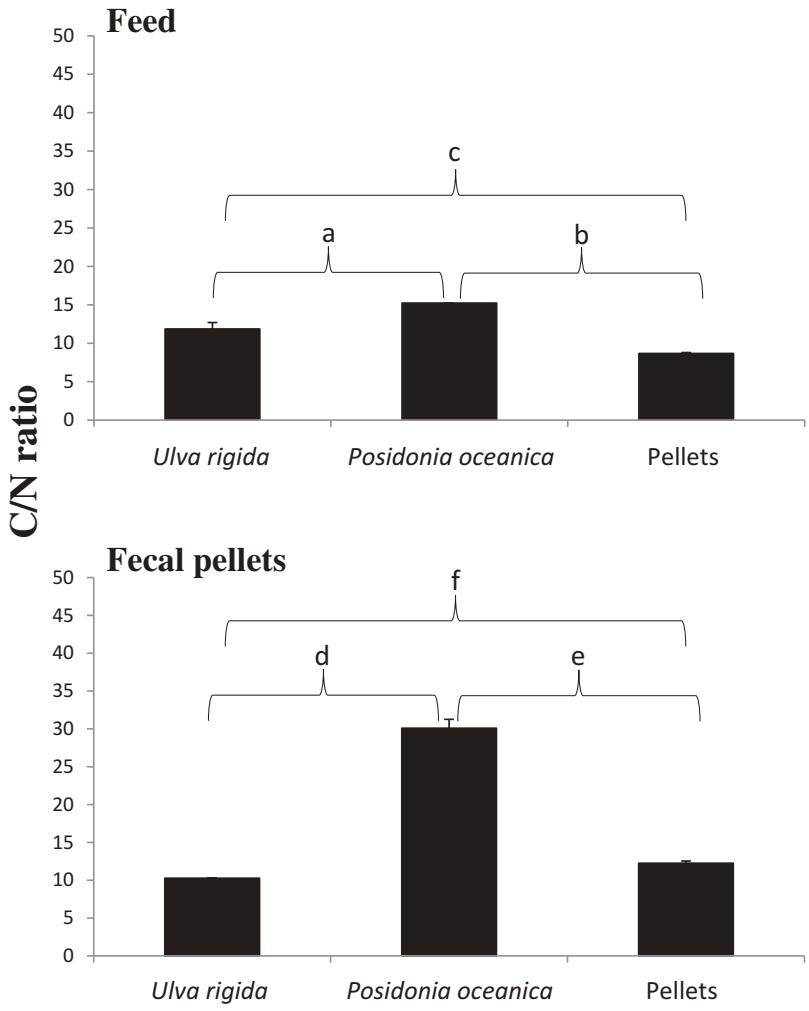

Figure 3. Carbon and nitrogen measurements in feeds and faecal pellets. Carbon (C) and nitrogen (N) ratio (C/N ratio) measured by CN Analyzer (A) in the three feeds, *Ulva rigida, Posidonia oceanica* and formulated pellets, and (B) in the faecal pellets collected from sea urchins fed with these three diets. C/N ratio in feeds: *Ulva rigida* versus *Posidonia oceanica, p* value = 0.03 (a); *Ulva rigida* versus formulated pellets, *p* value = 0.0274 (b); *Posidonia oceanica* and formulated pellets, *p* value = 0.005 (c). C/N ratio in faecal pellets: *Posidonia oceanica* versus both *Ulva rigida, p* value = 0.002 (d) and formulated pellets, *p* value = 0.0024 (e); *Ulva rigida* versus formulated pellets, *p* value = 0.0186 (f).

pellets (12.2) reaching the lowest value in *U. rigida* (10.3) (Figure 3(b)).

The differences in C/N ratios were significant between *P. oceanica* and both *U. rigida* (p = 0.0017, indicated with d) and pellets (p = 0.0024, indicated with e); significant difference were found also between *U. rigida* and pellets (p = 0.0186, indicated with f).

Adult growth and gonadal index

No significant differences in growth rates were found among adult sea urchins fed for one month with *U. rigida, P. oceanica* and pellets in comparison with adults collected in the field at the beginning (t0; Table 2).

Moreover, no significant differences were found among the GI values of sea urchins fed one month on *U. rigida, P. oceanica* and pellets in comparison with those collected in the field at the start of the experiments ($p > 0.05$). Different results have been reached after three months of feeding. In fact, we observed a high significant increase in the GI values in sea urchins fed with formulated pellets, in comparison with those fed with *U. rigida* (p = 0.005, indicated with a) and *P. oceanica* (p = 0.005, indicated with b). In Figure 4, we reported the gonads from sea urchins fed with formulated pellets in comparison with those from adults fed with *U. rigida* and *P. oceanica* after three months of feeding experiments.

Fertility of sea urchins

Gametes were collected from the sea urchins at two distinct periods, i.e. after one and three months of

Table 2. Adult sizes (in millimetres) and gonadal index (GI ± SD, n = 20/group) of adults of sea urchin *Paracentrotus lividus* collected in the field at the beginning (t0) and after one month and three months of the feeding experiments with *Ulva rigida, Posidonia oceanica* and formulated pellets (p value > 0.05 for adult sizes after one and three months of feeding, and GI after one month of feeding; p = 0.005 for GI after three months of feeding in sea urchins fed with formulated pellets, in comparison with those fed with *Ulva rigida* (a) and *Posidonia oceanica* (b).

		Ulva rigida	*Posidonia oceanica*	Formulated pellets
Adult size				
t0	40.2 ± 2.26			
1 month		41.3 ± 2.17	39.9 ± 2.39	40.5 ± 2.65
3 months		41.9 ± 2.45	40.6 ± 1.90	40.9 ± 2.93
GI				
t0	4.0 ± 0.51			
1 month		3.9 ± 0.39	4.2 ± 0.49	4.0 ± 0.60
3 months		4.2 ± 0.44	4.4 ± 0.47	14.7 ± 1.13
			a	
			b	

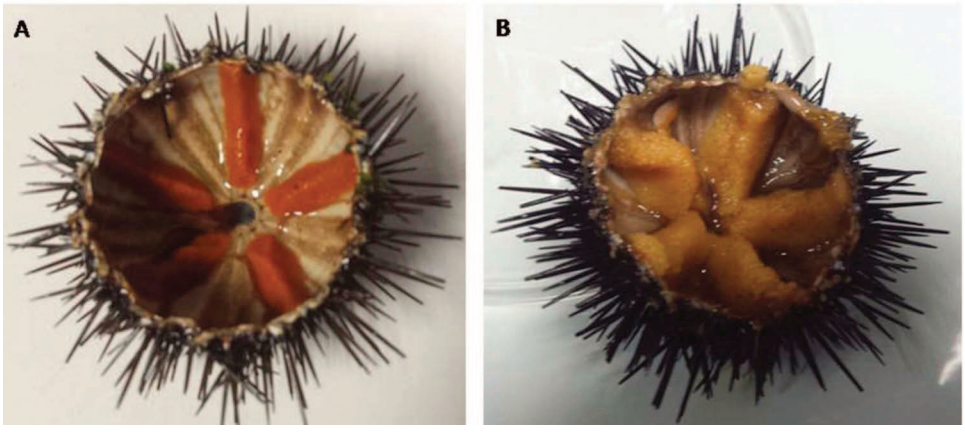

Figure 4. Gonads from (A) adult female fed on *Ulva rigida* and *Posidonia oceanica* and (B) adult female fed on formulated pellets after three months of feeding experiments.

feeding trials (see Materials and Methods Section). Gametes were collected only from those sea urchins in the replicates fed on *U. rigida* and *P. oceanica*. In the case of sea urchins fed on formulated pellets, after one month of feeding gametes were present only in five individuals (three females and two males); after three months gametes were absent in all the individuals under this treatment. As soon as the fertilization occurred, we measured the fertilization success and the first mitotic cleavage (1 hpf) to obtain two blastomeres (Table 3). Then the embryonic development has been followed until the pluteus stage. Fertilization and first cleavage were obtained in 100% of gametes produced by individuals under treatments with *U. rigida* and *P. oceanica*. Morphological observations of the only three females producing gametes revealed that feeding one month on formulated pellets induced significant (p = 0.04) increase in the percentage of abnormal embryos in respect to *U. rigida* and *P. oceanica*.

Histological observations of the gonads for both the treatments with *U. rigida* and formulated pellets demonstrated a stage quite close to a maturity. In fact, *U. rigida*, at the end of the treatment, produced ovaries in pre-mature recovering stage (Figure 5(a)), with oocytes still filling the centre of follicular masses.

The commercial pellet treatment exhibited as well pre-mature ovaries with a lower abundance of oocytes in the centre of follicules and a few mature eggs in theor periferical areas (Figure 5(b)). However, in this case the gonadic tissues appeared hypertrophic and vacuolated in the cortex.

Discussion

The effect of feeding on physiological and reproductive conditions of sea urchins is essential to understand their biology and ecology and to develop novel feeds for echinoculture. However, it is interesting to observe that the feeds ingested vary according to their quality

Table 3. Percentage of fertilization, first cleavage (two blastomeres), normal plutei and malformed plutei in the embryos from sea urchins *Paracentrotus lividus* collected in the field at the beginning (t0) and after three months of feeding with *Ulva rigida, Posidonia oceanica* and formulated pellets. In the case of formulated pellets after one month of feeding only five adults (three females and two males) produced gametes (data reported in the table), whereas after three months of feeding no adults produced gametes.

	t0	*Ulva rigida*	*Posidonia oceanica*	Formulated pellets
Fertilization	100	100	100	100
First cleavage	100	100	100	100
Normal plutei	90	91	90	80
Malformed plutei	10	9	10	20

and this study represents a confirmation of this regulation of their feeding activity. Although considered as an herbivorous species, *P. lividus* has often been classified as an opportunistic omnivore taking advantage of various food sources (Zupo and Fresi 1984; Boudouresque and Verlaque 2001). However, they do have preferences when presented with choice, determined by the chemical and physical characteristic of feeds. Information about nutrition, digestion and digestibility is still limited. Production of sea urchins is the results of ingestion, digestion and absorption, which have important implications for their nutrition (Boudouresque and Verlaque 2013). Even if food is abundantly and continuously available in their own environment, sea urchins do not necessarily feed continuously. In fact, the consumption is high when food is supplied after starvation, in our experimental conditions, and it decreases in conditions of feeding *al libitum* (Bonsdorff 1983). Moreover, food consumption is related to the reproductive stages. Concerning the digestion system, the part corresponding to stomach is the primary site of production of digestive enzymes and that corresponding to intestine is the primary site of uptake of nutrients, although their gut is not structurally differentiated into a stomach and an intestine, but a long digestive tube (Lawrence et al. 2013). Regional differences in digestive enzymatic activity are consistent with regional differences along the intestine tracts. The 'stomach' has much higher amylase activity than the 'intestine'. Almost all studies on digestive enzymes in sea urchins concern carbohydrates. Many studies have shown cellulase activity on the linear, soluble carboxymethylcellulose with minimal cellulase activity on native cellulose. Amylase also occurs in sea urchins, as well as glycogenase and agarose.

Sea urchins play an important role in shaping some coastal shallow benthic communities thanks to their grazing activity in rocky bottoms, also recognized to be able to transform communities dominated by macroalgae into barren areas so reducing biodiversity, altering ecosystem functions and regulating sea urchin population dynamics (Palacín et al. 1998; Sala et al. 1998; Barnes and Crook 2001; Prado et al. 2007; Hereu et al. 2012). Moreover, *P. lividus* plays a central role by directly removing plant biomass (both green and brown tissues), improving nutrient export, and modifying plant production in ecosystems dominated by the seagrass *P. oceanica* (Tomas et al. 2005; Prado et al. 2007; Ruiz et al. 2009; Planes et al. 2011). At the same time, *P. lividus* is intensively exploited in many Mediterranean areas because male and female gonads are considered a delicacy (Guidetti et al. 2004; Furesi et al. 2014).

Our data showed that *P. lividus* daily ingests about the same quantities of *U. rigida* and *P. oceanica* and significantly lower amounts of artificial pellets. This difference in daily feeding rates could be due to a strong preference for *U. rigida* and *P. oceanica* by *P. lividus* or to a different nutritional value of the considered artificial food. In fact, this feed better corresponds to the sea

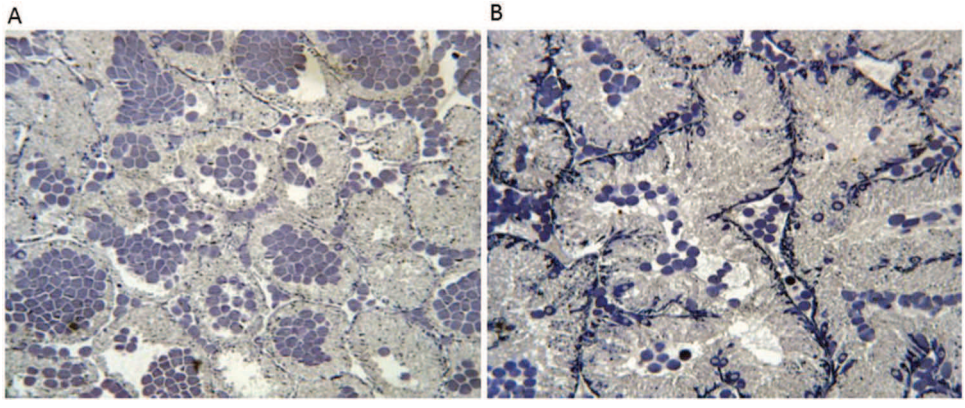

Figure 5. Representative histological sections observed under the optical microscopy of *Paracentrotus lividus* ovaries reared with *Ulva rigida* (left, A) and with formulated pellet (Right, B). The scale bar corresponds to 10 µm.

urchin nutritional needs, probably due to its high protein and lipid contents (Fabbrocini and D'Adamo 2010). In particular, protein content of *U. rigida* is 6.64%, whereas carbohydrate is 22% and total lipid contents 12%, phenol 23% and moisture content 76%, total free amino acid 8.9%, chlorophyll a 13%, chlorophyll b 7.5% and carotenoids 4.5% (Satpati and Pal 2011). In *P. oceanica* the total carbohydrates, proteins and lipids are, respectively, 28.98 mg/g, 607.50 mg/g and 40.50 mg/g (El Din and El-Sherif 2013). The composition of the formulated pellets consists of crude protein 46.5%, crude fat 10.5%, crude fiber 2.4% and ashes 9.5%, proteins of animal origin accounting for less than 5% (Mihranyan 2010).

C/N data revealed that *P. oceanica* had the highest C/N ratio and the faecal pellets of individuals fed on its tissues are as well characterized by high C content. We may hypothesize that these relationships are due to a higher abundance of structural carbohydrates (cellulose) characterizing the seagrass tissues. Cellulose is a complex carbohydrate, representing the structural component of cell walls in both green plants and algae (Baldan et al. 2001). In fact, several green algae have walls containing a cellulosic content up to 70% of their dry weight (Ott and Maurer 1977). According to our hypothesis, sea urchins discharge the excess of carbon due to cellulose through the faecal pellets, as well as it was demonstrated in various studies on invertebrate consumers of *P. oceanica* (Kawamata 1997).

To date, several studies defined the daily feeding rates of different sea urchins in the field. In the case of *Strongylocentrotus nudus* trough mathematical models have been estimated the amount of kelp eaten, using the brown algae *Laminaria* spp., *Eisenia bicyclis* and *Undaria pinnatifida* present in the habitats of this sea urchin (Hiratsuka and Uehara 2007). The predicted feeding rate was 0.5 g wet mass $\times$ d^{-1} $\times$ $animal^{-1}$, considering adults of about 40–50 mm. Feeding rates of four sea urchin species, *Echinometra* sp. A, *E. mathaei, E.* sp. C and *E. oblonga* (belonging to the genus *Echinometra*), were investigated after feeding on a diet prepared from turf algae and agar for a 7-day period (Scheibling and Anthony 2001). In that case, the feeding rates differed significantly among the four species of sea urchins, being between 0.14 and 0.29 g for sea urchins of 30–35 mm. Furthermore, adult *Strongylocentrotus droebachiensis* were fed on two diets, the invasive green alga *Codium fragile* and the brown alga *Laminaria* sp. (Cyrus et al. 2015). The feeding rates declined from June to July and remained low (about 0.1 g DW per urchin of 38–52 mm per day) through September. That these data are comparable with our results, showing that the daily feeding rates (DW) for a sea urchin correspond to 0.16, 0.15 and 0.10 g for *U. rigida, P. oceanica* and formulated pellets, respectively.

Interestingly, the three different diets did not produce size increments of adult sea urchins, but affected their gonad growth and reproduction performance. In fact, we demonstrated that, at the end of feeding experiments (after three months), *U. rigida* and *P. oceanica* didn't produce effects on the gonad growth. Differently, after three months the formulated pellets affected the GI, resulting in a significant growth of the gonads probably due to the high content of crude proteins of this food (as reported above). Our data confirmed its contribution in the production of large gonads (Sartori and Gaion 2016), which represents the major aim of echinoculture practices (Fabbrocini and D'Adamo 2010). Despite the large increase in the volume of their gonads, surprisingly they were not capable of producing gametes. This could be probably correlated to the colour and texture of their gonads, which were very different from those normally observed in sea urchins collected from the field and/or fed with the other two feed used in this work (see Figure 4). Both treatments demonstrated to be sufficient for a rapid increase of the ovary tissues and a maturation of gonads, starting from a spent stage. However, the effects were quite different both from a histological point of view and according to the results of fertilization tests. In fact, *U. rigida* produced a slower maturation and enlargement of ovaries, with the production of several oocytes, while the commercial pellets produced a hypertrophy of the follicular tissues, a diffused vacuolization, and the maturation of a few eggs, which conducted finally to a low fertilization success with compromised post-fertilization embryonic and larval development to pluteus. It is well-known in literature that the nutrients' composition of a diet has a significant effect on the growth (Marsh and Watts 2007). For this reason, optimizing a feed for the best production of gonads in a sea urchin requires a consideration to balance the energy demands with the availability of various protein and non protein dietary principles, including vitamins, carotenoids and fatty acids (Castell et al. 2004; González-Durán et al. 2008). On this line, in the last 20 years, technology for sea urchin culture, including reproduction and diet formulation, has been improved for the supply of sea urchin (Watts et al. 2013). On this line, our data demonstrated that there is strong relationship between GI and dietary contents of proteins, as in the case of commercialized pellets. These data were in agreement with other investigation reported by Pearce et al. (2002c), demonstrating a positive effects of proteins on gonadal increase of *S. droebachiensis* fed with

artificial diets at increasing protein levels. Comparable results have been reported by De Jong-Westman et al. (1995) for adults of *S. droebachiensis* fed with prepared diets at high level of proteins.

In conclusion, this study represents an additional attempt to correlate the daily feeding rate of adult *P. lividus* with the composition of feeds and their effects of growth and reproductive success. Results will be useful for the definition of optimal diets for the production of mature bloodstocks with high quality of eggs and for larval production also in industrial culture of this ecologically important and well-established marine model organism.

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Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Valerio Zupo http://orcid.org/0000-0001-9766-8784

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ORIGINAL ARTICLE

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Roe enhancement of *Paracentrotus lividus*: Nutritional effects of fresh and formulated diets

Valerio Zupo[1,2] | Francesca Glaviano[2] | Marina Paolucci[3] | Nadia Ruocco[4,5,6] | Gianluca Polese[5] | Anna Di Cosmo[5] | Maria Costantini[4] | Mirko Mutalipassi[1]

[1]EMI Department, Benthic Ecology Centre, Stazione Zoologica Anton Dohrn, Ischia, Italy

[2]RIMAR Department, Unit MaRe, Stazione Zoologica Anton Dohrn, Napoli, Italy

[3]Department of Science and Technologies, University of Sannio, Benevento, Italy

[4]Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Napoli, Italy

[5]Department of Biology, University of Napoli Federico II, Naples, Italy

[6]CNR: Bio-Organic Chemistry Unit, Institute of Biomolecular Chemistry-CNR, Naples, Italy

Correspondence
Valerio Zupo, EMI Department, Benthic Ecology Centre, Stazione Zoologica Anton Dohrn, Villa Dohrn, Punta San Pietro, Ischia 80077, Italy.
Email: valerio.zupo@szn.it

Funding information
SZN

Abstract

Sea urchin gonads are exploited both for gastronomic and scientific purposes; thus, the production of large and mature gonads is essential. Natural populations of the Mediterranean Sea urchin *Paracentrotus lividus* are subject to increasing fishing efforts, due to continuous intensification of consumptions. Aquaculture practices may represent an answer, but the availability of artificial feeds accelerating the production of high-quality gonads in terms of size, taste, colour, firmness, maturity and viability of gametes is critical to boost the productions. The accessibility of cheap and effective feeds promoting the fattening of gonads and the quality of gametes is still a bottleneck slowing down the expansion of echinoculture practices. This investigation is aimed at enabling the development of this strategic sector, by comparing the dietetic effects of fresh foods and a commercial feed for aquaculture, to four newly formulated feeds. The protein contents of diets were strongly related to the GSIs. The abundance of fatty acids appeared inversely related to the viability of embryos and abnormalities of larvae. The features of an ideal diet for this sea urchin were defined, based on the results of experimental trials, and the need for increasing levels of plant-derived proteins during the grow-out period was demonstrated.

KEYWORDS
artificial diets, fats, gametes, gonadic index, proteins, sea urchin

1 | INTRODUCTION

Most sea urchins are typical plant feeders. They are the major consumers of huge standing stocks produced by macroalgae and seagrasses in various coastal environments (Lawrence, 1975). Their natural populations are important to control and keep constant the crops of many seagrasses (Boudouresque & Verlaque, 2007; Zupo, 1994), and their trophic role is usually played as large and abundant macroherbivores (Zupo, Alexander, & Edgar, 2017). Their natural diets are quite complex and contain, in addition to the main plant tissues, several items (Zupo, 1993) including small animal prey and various epiphytes, indispensable to complete the assortment of feeding principles ingested (Mazzella et al., 1992). Therefore, they could be considered as opportunistic herbivores (Zupi & Fresi, 1984). In addition, each species of sea urchin is adapted to local ecological conditions and evolved specific dietetic patterns (Lawrence, 2007).

The sea urchin *Paracentrotus lividus* (Lamark, 1816) is quite common throughout the Mediterranean Sea (Boudouresque & Verlaque, 2007), from the North Atlantic coasts of Ireland to southern Morocco (Bayed, Quiniou, Benrha, & Guillou, 2005; Symonds, Kelly, Caris-Veyrat, & Young, 2007). It is an important resource since the last century (Koehler, 1883), both as a marketable good (Devin, 2002; Williams, 2002) and an animal model for research in the life sciences (Buitrago et al., 2005; Yamabe, 1962). Its importance for

scientific investigations is also related to remarkable characters of embryos; in fact, it exhibits transparent eggs and embryos, allowing for a continuous monitoring of the division progressions, up to the development of a larva (Santella & Chun, 2011).

The progressive depletion of its natural stocks, due to overfishing (Barnes & Crook, 2001; Byrne, 1990; Le Direach, 1987), habitat destruction and natural diseases, reinforces the need for effective aquaculture practices (Mercurio & Sugni, 2016). In addition, its market has largely increased in the last three decades due to the intensification of requests (Pais et al., 2007; Williams, 2002). This evidence boosted investigations aiming at the definition of economically productive practices (Fernandez, 1996; Olave, Bustos, Lawrence, & Carcamo, 2001; Pearce, Daggett, & Robinson, 2002a; Sartori, Pellegrini, Macchia, & Gaion, 2016). Intensive aquaculture efforts produced a tremendous amount of information regarding the in vitro fertilization of eggs and the production of juveniles (Yokota, 2002), the best feeding practices for the grow out of adults (Mortensen, Siikavuopio, & Raa, 2004), the practices of caging (James, 2006a,b), as well as the stimulation of gonadal growth (Cook & Kelly, 2007; Pantazis, 2009; Spirlet, Grosjean, & Jangoux, 2000).

However, the economic effectiveness and therefore the feasibility of industrial plants able to produce marketable sized *P. lividus* are still hindered by the availability of excellent feeds, able to guarantee fast growth and gonadal maturation (Devin, 2002; Le Gall, 1990; Spirlet, Grosjean, & Jangoux, 1998). Researches in this field led to interesting and concrete, even if not conclusive, wet formulations (Lawrence, 2001) and dry feeds (Sartori et al., 2016; Woods, James, Moss, Wright, & Siikavuopio, 2008). While the interest in sea urchin aquaculture increased in the last decades, most research attention was devoted to the development of effective feeds for the grow out of adults (Agatsuma, 1998; Pearce, Daggett, & Robinson, 2003) promoting and accelerating the gonadal production (Fabbrocini, Volpe, Coccia, D'Adamo, & Paolucci, 2015; James & Heath, 2008; Lawrence & Lawrence, 2004; Pearce, Daggett, & Robinson, 2002b, 2002c; Pearce et al., 2002a).

Diets proposed for *P. lividus* (Bendich, 1994; Kawakami et al., 1998; Matsuno, 1991; Tsushlma, Kawakami, Mine, & Matsuno, 1997) contained plant carotenoid to improve the colour, maize for rapid fattening (Dado, 1999), spinach as antioxidant and other components useful to retard the chemical and physical degradation of feeds (Cabrita, Robles, & Herráez, 2008; Sartori et al., 2016). Notwithstanding such a prolonged and intense scientific production, the definition of the "perfect" diet for *P. lividus* is still to be attained and any detail adding information to this puzzling theme can be useful to reach well-devised aquaculture procedures (Fernandez, 1990).

Both ovaries and testes, commonly called "roe," are targets of economic interest. Thus, the quality of a feasible feed for adult sea urchins may be judged based on the size and quality of their gonads (Sartori, Scuderi, Sansone, & Gaion, 2015). Both the storage of nutrients in nutritive phagocytes involved in early maturation stages (Byrne, 1990) and the abundance of mature gametes in late maturation stages positively influence the size of sea urchin gonads (Woods et al., 2008). Diets for various species of echinoids have been devised allowing for their transfer to the aquaculture industry, in accordance with quality criteria in terms of taste, smell, firmness, and colour of gonads (de Jong-Westman, March, & Carefoot, 1995; de Jong-Westman, Qian, March, & Carefoot, 1995). Artificial diets were set for several species of echinoids (Cook, Bell, Black, & Kelly, 2000; Fernandez & Boudouresque, 2000; Lawrence, Plank, & Lawrence, 2003; McBride, Price, Tom, Lawrence, & Lawrence, 2004) and they promoted significant increases in gonadic yields.

Most of the mentioned studies rely on sea ranching, to respond to the reduction in productions from capture fisheries and the sharp decline in wild stocks of sea urchins. These techniques, however, can accelerate the harvest of natural populations. Environmental effects of sea ranching are far from being fully understood, and their actual sustainability should be carefully evaluated case by case (Mustafa, 2003). Nevertheless, the results of this study will be applicable to any culture condition, both for experimental or production purposes. Here, we aimed at investigating the gonadosomatic indices, the histological features of gonads and the quality of gametes and larvae produced by individuals reared under a range of dietetic regimes. To this end, we compared two different methods of feed preparation (using synthetic binders or flours), various levels of proteins and fatty acids, and three categories of diets represented by (a) newly developed dried formulations, (b) wet natural diets and (c) commercial pellets already tested for similar purposes (Fabbrocini et al., 2012, 2015). In particular, we compared the dietetic effects of common plant items that characterize the seabeds hosting the target sea urchins (Ortiz et al., 2006; Valente et al., 2006), that is the seagrass *Posidonia oceanica* and the macroalga *Ulva rigida*, to the effect of five formulated diets.

2 | MATERIALS AND METHODS

2.1 | Sampling of urchins

Sea urchins were collected by divers in fall (September) 2016 in the bay of Naples (40°77′N; 14°08′E) from algal mats, wrapped into humid paper sheets to avoid emission of gametes and kept in refrigerated containers up to the transfer into 1-m^2 flow-through seawater-aerated tanks kept at a temperature of 18°C, using an aquarium chiller. Each collected individual was measured (main diameter excluding spines) and weighed (wet weight). They were also inspected for their general health status, to avoid the introduction of individuals showing loss of spines, evident patches, slow movements, etc.; individuals showing such symptoms of stress or malformations were not used for further tests. A 12:12-hr light:dark photoperiod was imposed. The experimental conditions in the tanks hosting the animals were daily checked, by monitoring temperature, light intensity, oxygen concentration, redox potential and concentrations of total nitrogen, ammonia and nitrites.

TABLE 1 Feeding treatments investigated. Each treatment consisted of two replicates of 20 individuals

Treatment	Diet	Form	Ingredients
1	*Posidonia oceanica*	Fresh	Brown and green leaf tissues
2	*Ulva rigida*	Fresh	Freshly collected thalli
3	Hendrix Classic K	Dry pellet	Dehulled toasted soybean meal, wheat meal, fishmeal, sunflower meal, fish oil, soybean oil, rapeseed oil, dicalcium phosphate, vitamins A, D3, E and BHT
4	Test a	Dry pieces	Krill meal, pea flour, corn flour, *Fucus* powder, fish oils, *agar–agar*, vitamins, minerals
5	Test b	Dry pieces	Krill meal, pea flour, corn flour, *Fucus* powder, *agar–agar*, pomegranate oil, vitamins, minerals
6	Test c	Dry pieces	Carrots, *Brassica oleracea*, *Lactuca sativa*, *Ulva rigida*, boiled corn, *Mytilus* pulp, sunflower oil, fishmeal, wheat flour, Brewer's yeast
7	Test d	Dry pieces	*Brassica oleracea*, *Lactuca sativa*, *Ulva rigida*, corn oil, sunflower oil, fishmeal, soybeans, wheat flour, Brewer's yeast

2.2 | Outlining diets

A pelletized formulated feed (Classic K®; Hendrix SpA, Mozzecane—VR, Italy) was chosen as a positive control, because its use for feeding of sea urchins was previously tested and it demonstrated to provide rapid fattening of gonads, even faster with respect to individuals collected in the field (Fabbrocini & D'Adamo, 2010; Fabbrocini et al., 2012). This formulated feed contains vitamins and a considerable amount of proteins (Table 1), and this could be an important factor for gonad growth and maturation (Cook & Kelly, 2007; Jacquin et al., 2006; Schlosser, Lupatsch, Lawrence, Lawrence, & Shpigel, 2005). Therefore, it represented a positive control on the size and quality of gonads, as it is inexpensive, is available on the market and produces negligible amounts of wastes (Fabbrocini et al., 2012).

Fresh *U. rigida* and *P. oceanica* were chosen in their turn as natural dietetic items. These plants both characterize environments often populated by *P. lividus*. *Ulva rigida* is a tough green alga containing up to 250 g of proteins per kg of dry weight (Ortiz et al., 2006; Valente et al., 2006). It is considered a control feed in several feeding experiments on fish and invertebrates (Valente et al., 2006), and it is included in various diets for sea urchins (Frantzis & Grémare, 1993). Benthic macrophytes (both macroalgae and *Posidonia* leaves) were collected in shallow waters in the Bay of Napoli, reared in open cycle tanks and administered daily (Frantzis, Berthon, & Maggiore, 1988). Their supplies were renewed weekly to provide daily fresh feeds. The data obtained using these seaweed diets, whose effects on gonadal maturation are known (Frantzis, Grémare, & Vétion, 1992), have been compared to those obtained with our four experimental diets.

In addition, four newly formulated diets were tested and compared to the results yielded by the pelletized diet (available on the market) and the two fresh plant diets mentioned above. All formulated diets differed for the basic ingredients (Table 1), the proportions of proteins and fats (Table 2), as well as the preparation methods. In particular, Diets a and b contained krill meal as the main source of animal proteins and special oils to provide essential fatty acids (Table 1): They were prepared using synthetic binders to increase their stability in the water and then dried at low temperature. The first two formulated diets also differed for the source of fatty acids: The first (diet a) included fish oil, and the second (diet b) included pomegranate seed oil. Pomegranate oil is rich in PUFA (Siano et al., 2015). These compound diets were represented by moist rectangles (2 × 3 × 2 cm) of 100 g/Kg dry nutrients dissolved into 20 g/kg agar.

Biocomposite preparation was carried out according to Paolucci, Fasulo, and Volpe (2015), with some modifications. To obtain the biocomposites, two grams of agar–agar (Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved into 90 ml of distilled water on a stirring plate until boiling, then left cool down to a temperature of about 50°C. Nutrients (10 g) were ground down to a particle size lower than 250 μm and mixed with 10 ml of distilled water at room temperature to obtain a creamy mixture. The mixture was slowly added to the agar solution and then poured into rectangular containers

TABLE 2 Composition (g/kg) of feeds considered in this study

Feeding principle	Abundances (g/kg)						
Typology	Pellet	*Fresh items*		Biocomposites		Baked	
	Hendrix K[a]	*Posidonia oceanica*	*Ulva rigida*	Diet a	Diet b	Diet c	Diet d
Proteins	465	300–400	100–250	230.6	220.2	354.0	290.2
Crude fats	105	20–40	10–30	69.5	70.4	63.0	90.2
Fibres	24	280–320	50–60	20.2	30.2	60.3	42.0
Ashes	95	250–300	400–450	69.5	70.6	102.2	82.0

Notes. Data from Mathers & Montgomery (1997), Mensi et al. (2005), Shams El Din & El-Sherif (2013) and Peña-Rodriguez et al. (2011). Diets have been allotted according to the typology of their preparation and sources.
[a]Proteins of animal origin account for <5%.

(30 × 50 cm) and allowed to cool. At last, the biocomposites were collected, cut into small rectangles of 2 × 3 cm and stored into vacuum-sealed plastic bags at −20°C.

In a different way, Diets c and d were prepared using various flours to make them tougher and compact, then dried into an oven (150°C for 35 min) and finally freeze-dried to increase their stability. Diets c and d also contained brewer's yeast (to increase the toughness of the foils and the quantity of natural vitamins), as well as various sources of proteins (including soybeans, fish meals, mussel pulp) with the aim of sustaining the gonadal growth and maturation (Frantzis & Grémare, 1993). All formulated diets, after the preparation, were cut into 2 × 3 cm small pieces and kept at −20°C in dry containers, up to their use.

2.3 | Experimental procedures

Sea urchins were starved for 2 weeks prior to the start of the experiment and randomly allotted into groups of 20 individuals per 60-L tanks. Each feeding treatment was composed of two replicates of 20 individuals. Thus, seven feeding treatments were performed (Tables 1 and 2) into 14 tanks, each containing a group of 20 individuals. Feeds were daily administered ad libitum (Pearce et al., 2002a). A preliminary check of the actual consumption was permitted to evaluate the feeding rates in our experimental conditions, in order to avoid an accumulation of organic wastes that could generate water pollution. Faecal pellets produced and feed remains were carefully removed every morning, from each tank, and new feed portions were added.

The experiment lasted 90 days, and the results were recorded at 0, 45 and 90 days. At the end of the experiment (90th day), individuals were tested for their general health status by performing a "righting response test" (Bayed et al., 2005; Hyman, 1955). The temperature was slowly lowered to 16°C during the last 20 days, to facilitate the gonad maturation (Russell, 1998). Pools of five individuals were collected in each tank at 0, 45 and 90 days, and their gonads were dissected to evaluate the gonadosomatic indices (Keshavarz, Kamrani, Biuki, & Zamani, 2017). In addition, their gametes were collected and used for a fertilization test, followed by a morphological analysis of the larvae produced. The gonadosomatic index (GSI %) was calculated as:

$$\text{GSI \%} = \text{wet weight of gonads/wet weight of the sea urchin} \times 100.$$

The gonads collected at the 90th day, after the evaluation of GSIs, were fixed in Bouin's solution, included in paraffin blocks, sliced and haematoxylin-stained, to evaluate their maturation stages (Byrne, 1990).

2.4 | Analysis of results

The results of the mentioned seven diet treatments were used to rank the general health conditions of sea urchins, based on the righting response test and the fattening of gonads, evaluated by means of their gonadosomatic indices, histology analyses and fecundity tests. In addition, the diameter of each sea urchin, in each treatment, was recorded by means of a metal calliper at 15-day intervals, to evaluate the growth increments promoted by each diet. The righting response tests were performed according to Hyman (1955) and Lawrence and Cowell (1996) and consisted in the measurement of the time required by individual sea urchins to turn back over, once they were positioned with the aboral face down. The percentage of survivorships was periodically recorded.

Evaluation of fertilization rates and larval morphology analyses was conducted using gametes collected from individuals sacrificed at 45 and 90 days. Mature eggs were counted and fertilized with *P. lividus* sperms at a ratio of 100 sperms: 1 egg (Romano, Miralto, & Ianora, 2010). In particular, collected eggs were allowed to settle and washed three times with 0.22-μm filtered seawater (FSW); eggs were then diluted to a final concentration of 3,000 eggs/ml. Concentrated sperm was collected "dry" and stocked undiluted at +4°C. Sperm mix was diluted (10 μl in 10 ml SW) just before insemination, and its concentration was measured using a counting cell and adjusted by further dilutions, up to 3 × e10^8 sperms/ml. An aliquot of 100 μl of this solution was added to 100 ml of egg suspension. About 4 min after sperm addition, eggs were checked for successful fertilization and excess sperm was removed by washing embryos with FSW.

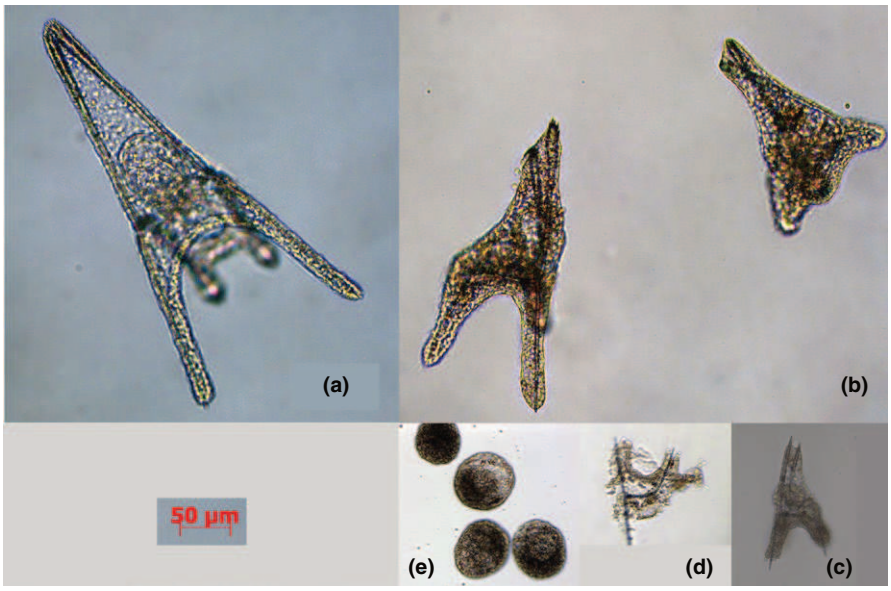

FIGURE 1 Shape of larvae and their possible abnormalities. (a) Normal pluteus; (b) deformities of the pluteus shape, with shortening of apical spiculae; (c, d) plutei deformities, abnormal growth of spicules and block of development; (e) blocked embryos and their blebbing

Multiwells containing 500 fertilized eggs were stored in a thermostatic chamber at 20°C and exposed to a 12:12-hr photoperiod. The test was ended after 48 hr, and *pluteus* larvae were fixed in paraformaldehyde and observed under the optical microscopy to assess (Figure 1) the percentage of abnormalities according to McEdward (1984) and Pagano et al. (1986). The significance of differences among treatments, for all of the above-mentioned records, was evaluated by one-way ANOVA. The significance of differences between the results obtained at 0, 45 and 90 days was assessed by means of *t* tests. Graphs and statistical analyses were computed using the software GraphPad Prism, version 6.00, for Macintosh (GraphPad Software, La Jolla, CA, USA, www.graphpad.com) and Statistica, version 10 (StatSoft Inc., Tulsa, OK, USA).

3 | RESULTS

3.1 | Health status and survival rates

Water quality parameters were kept consistent within the ranges usually measured in the field; any negative influence on survival and development of sea urchins should be excluded. The results of the righting response tests demonstrated good conditions of health of most individuals throughout the experiment. At the end of the test period (90th day), the average time to complete the righting tests was 1′ 22″ (±20″). Survival rates at 45 days (Figure 2a) ranged between 92% in the dietetic treatment with *U. rigida* and 100% in the dietetic treatment with *P. oceanica*. Other treatments exhibited intermediate values (Figure 2a). Survival rates decreased to 85% after 90 days in most formulated diets, and they were highest (Figure 2b) in the treatment with *P. oceanica* (100%) and *U. rigida* (89%). The differences in survival rates among diets were not significant (one-way ANOVA, $p > 0.05$), and the recorded mortality rates were due to individual variability.

3.2 | Size increments

Size increments during this short-term experiment were low, and they varied among diets. The size of animals, at the collection time, ranged between 38.03 and 40.33 mm (maximum diameter of thecae). The dietetic treatment with *P. oceanica* yielded the highest increments at 45 days (0.6 mm, corresponding to 1.0% of the initial size of thecae per month), while the lowest increments were produced by the treatments under the Diets a and b (Figure 3a), and they were, respectively, 0.22 and 0.20 mm, corresponding to 0.57% and 0.52% of the initial size of thecae per month. At the end of the experiment (Figure 3b), the dietetic treatment with the dry pellet yielded the highest size increments (1.38 mm in 90 days, corresponding to 1.17% of the initial size of thecae per month). The lowest increments were produced by the Diets a and b (Figure 3b), and they corresponded to about 0.4 mm in 90 days (0.34% of the initial size of thecae per month). The differences among diet treatments were significant (ANOVA, $p < 0.01$). Size increments were not constant during the experiment, but they were consistently proportional to the size of individuals (Figure 3). In fact, Diet c and *P. oceanica* produced a size increment of about 1.01% per month during the first 45 days and the same diets produced a size increment of 1.06% per month in the next 45 days.

3.3 | Gonadic indices

Sea urchins exhibited complex patterns of gonadic indices according to their diet treatments (Figure 4). Immediately after the collection, the first evaluation of GSI yielded an average value of 3.5% (±0.57%) and this represented the starting point of our experiments, to evaluate the effect of diet treatments. After 45 days of treatment, the formulated Diet d produced the best results (GSI 6.95%), followed by the Diet c (4.84%), while the formulated Diet b exhibited the worst performances for gonadic indices, with

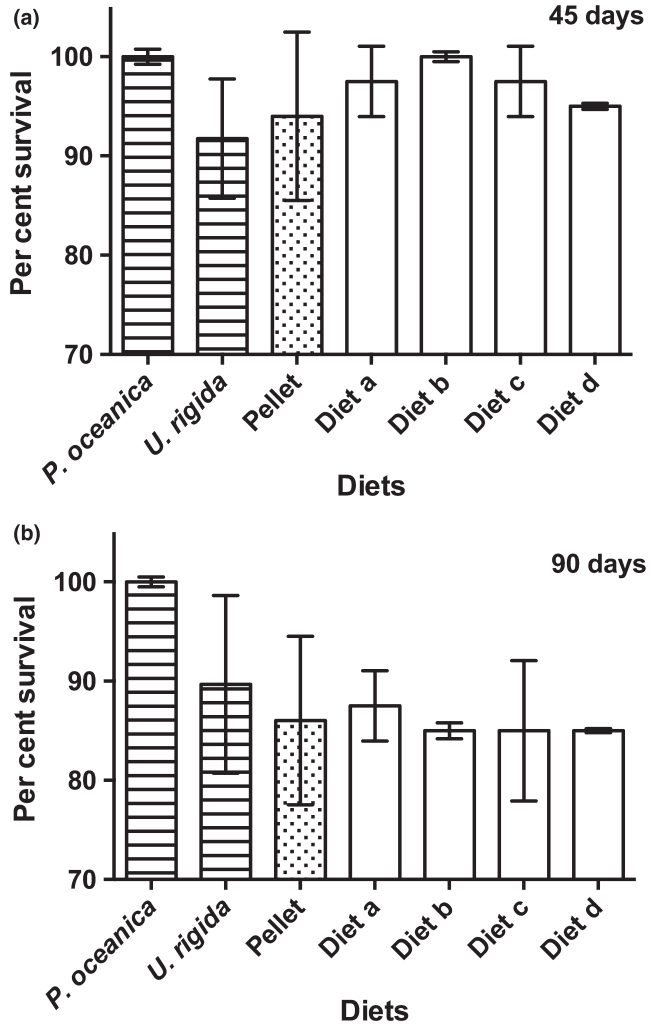

FIGURE 2 Survival rates obtained at 45 days (a) and 90 days (b) of the experimental treatments under various dietetic regimes. Fresh items are reported as horizontal banded bars. The control commercial pellet (Hendrix Classic K®) is indicated by a dotted bar. Four experimental diets evaluated in this study are indicated by white bars

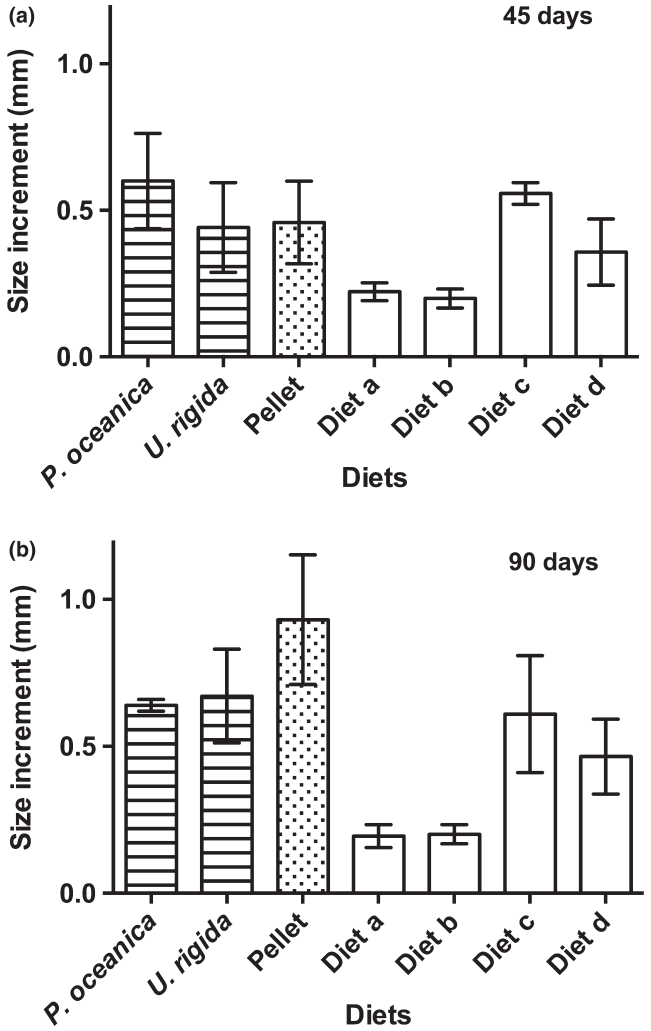

FIGURE 3 Size increments of theca diameter (in mm) in sea urchins measured at 45 days (a) and 90 days (b) of experiment. Fresh items are reported in horizontal banded bars. The control commercial pellet (Hendrix Classic K®) is indicated by a dotted bar. Experimental diets evaluated in this study are indicated by white bars

a reduction in the GSI down to 0.38% (Figure 4). The time progressions of GSIs were not homogeneous among diets, and they had contrasting trends, but the differences between the two testing periods were not significant for most diets (*t* test, $p > 0.05$). However, after 90 days of the experiments (Figure 4) the pellet prompted the highest gonadic indices (GSI 9.19%, $p < 0.01$), followed by the Diet c (5.30%; $p > 0.05$), *P. oceanica* (4.43%; $p > 0.05$) and Diet d (4.02%; $p > 0.05$). The lowest GSIs were obtained with *U. rigida* (2.13%), diet b (1.20%) and Diet a (0.43%). The overall differences in GSIs observed among dietetic treatments were significant (ANOVA, $p < 0.05$).

Variable levels of proteins characterized our dietetic treatments, fats and other feeding principles (Tables 1 and 2), and the protein abundance was significantly correlated to the GSI measured after 90 days of feeding (Figure 5) according to a linear relationships ($R^2 = 0.95$). In contrast, the contents in fats, fibres and ashes of various dietetic treatments (Tables 1 and 2) were not significantly related to the GSIs exhibited at the 90th day.

3.4 | Histological analyses

Gonadal stages were interpreted according to the reproductive cycles of *P. lividus* described by Byrne (1990). The analyses of gonads at various times of treatment indicated that they were in Stage V at Time 0 (partly spawned stage); the patterns of maturation were quite heterogeneous at 45 and 90 days (Figure 6); *U. rigida* treatment kept the reaching of Stage V (partly spawned stage) at 45 days (Figure 6a); the pellet treatment produced mature gonads (Stage IV) at the 90th day (Figure 6h); Diets b and d both conducted to Stage VI (spent stage) at the end of the experiment (Figure 6i, k); Diet c induced a growing stage (Stage II) at the end of the experiment (Figure 6j); and a recovering spent stage

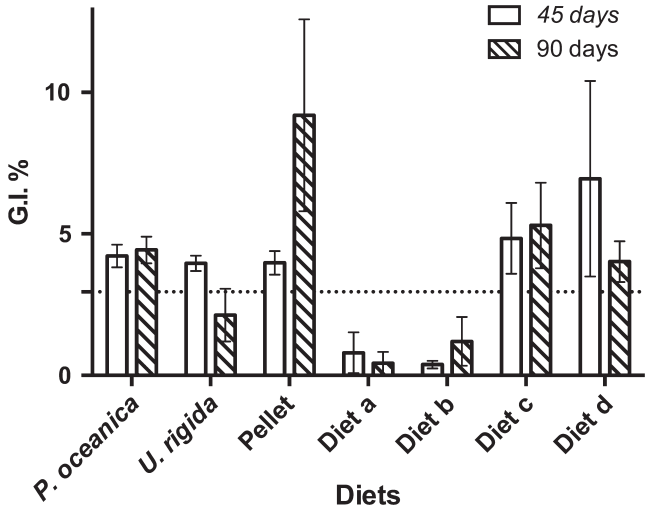

FIGURE 4 Gonadic indices recorded at 45 (white bars) and 90 (banded bars) days of the experiment under various dietetic treatments. The horizontal dotted line indicates the average GSI % measured at the start of the experiment

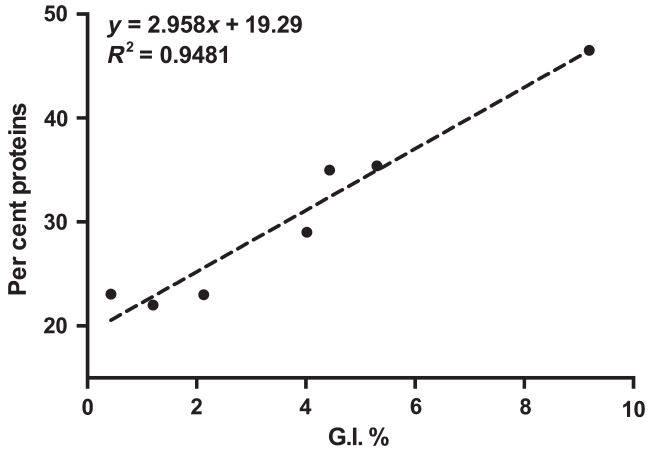

FIGURE 5 Relationship between protein contents of various dietetic treatments here tested and the gonadic indices (GSI %) recorded at 90 days of the experiment in each of them

was exhibited by *P. oceanica* samples at 45 days (Figure 6f), while oocytes were present in the ovaries at the 90th day (Figure 6l). Diet a was insufficient to allow gonadal growth, and, consequentially, the maturation stage could not be evaluated.

3.5 | Fertilization rates and larval morphology tests

The diet treatments produced no significant effects on the fertilization rates (Table 3) as all diets permitted rates of fertilized eggs ranging from 98% to 100% and these values were not different from controls (at $p > 0.05$). In contrast, the results of larval morphology tests were divergent with respect to the GSIs obtained under the considered dietetic regimes. In fact, the commercial pellet, exhibiting the highest GSI, also produced 85.47% of abnormal plutei, while the fresh diets, *P. oceanica* and *U. rigida*, produced 10.00% and 9.41% of abnormalities (ca. 90% and 91% of normal plutei), respectively (Figure 7). The percentages of abnormal plutei were higher in the Dietetic treatments c and d (14.06% and 27.46%, respectively), while no larvae were produced at all by individuals under the Dietetic regimes a and b (Figure 8), whose fertilized eggs developed into atretic embryos.

4 | DISCUSSION

The culture of sea urchins was conducted according to procedures devised by Sartori et al. (2016) and Azad, Pearce, and McKinley (2011), and the experimental conditions fitted the physiologic requirements of *P. lividus*, although the experiments were carried out in small aquaria. The righting response tests (Sherman, 2015) indicated that the survivorships exhibited sufficient health status at the end of the experimental period. Thus, the mortality rates observed at the end of experiments were due to stochastic events and the life in captivity conditions, as also confirmed by the absence of significant differences among treatments. *P. oceanica* and four artificial diets achieved higher survival rates both at 45 and 90 days, and none of diets (including the artificial diets and the pellet Classic K) produced mortalities higher than 15% at the end of tests. Similar survival rates were obtained by previous authors (Azad et al., 2011), adopting analogous experimental conditions. In addition, the resistance of animals to external stresses tends to increase during the development (Sartori & Gaion, 2016), thus assuring higher survival trends in older individuals.

The evaluation of size increments promoted by various diets was not the primary aim of this study, as previous research (Frantzis et al., 1992; Lawrence, 1975) revealed average size increments in the theca diameter of this species, both in nature and in the laboratory, comprised between 0.4 and 2 mm/month. The growth rates of this species were demonstrated to be slow (Boudouresque & Verlaque, 2007) and largely dependent on the amount and quality of feed ingested (Frantzis et al., 1988). However, our results were in the range reported for laboratory studies (Sartori et al., 2015), whose artificial diets promoted increases of the theca diameter of 0.5–1 mm/month and in agreement with those obtained in the field (Grosjean, Spirlet, & Jangoux, 1996). Thecae increments were slightly lower during the first 45 days of experiment, even when proportioned to the body size of animals. Thus, the period of acclimation to dietetic regimes produced slow growth, and succeeding periods yielded faster growth along with the maturation of gonads (Fabbrocini et al., 2012).

This study aimed at finding general rules for the formulation of artificial diets, able to maximize gonadic productions and maturation of gametes, as they represent the actual product of echinoculture practices (Fabbrocini & D'Adamo, 2010). In this perspective, the pellet Classic K confirmed its contribution in the production of large gonads (Sartori & Gaion, 2016), and after an acclimation period, when most feeds yielded similar results, it favoured gonadic indices as high as 9.19%, compared to the best performing formulated Diet c (GSI 5.3%) and the natural diet based on *P. oceanica* (GSI 4.4%). Other

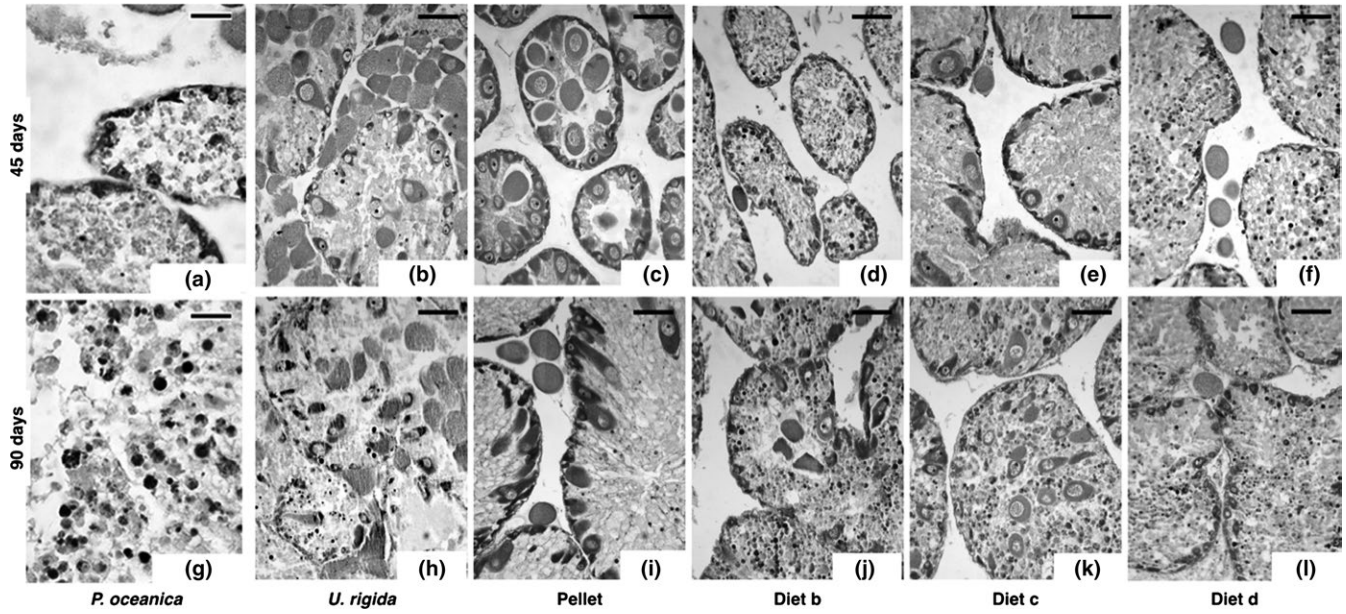

FIGURE 6 Ovaries of individuals after 45 days of culture (a–f) and 90 days of culture (g–l); a and g, under treatment with *Posidonia oceanica*; b and h, under treatment with *Ulva rigida*; c and i, under treatment with pellet Classic K feed; d and j, under treatment with formulated diet b; and e and k, under treatment with formulated Diet c; f and l, under treatment with formulated Diet d. Diet a did not produce evident gonadic masses to be histologically analysed. Scale bars correspond to 100 μm

TABLE 3 Average fertilization rates recorded using gametes produced within individual treatments and standard deviations (*SD*) among replicates

Treatment	Avg. fertilization rate	*SD*
Ulva rigida	99.2	1.35
Posidonia oceanica	100	1.55
Pellet	98.2	1.60
Diet A	100.0	0.13
Diet B	100.0	0.21
Diet C	97.9	2.05
Diet D	98.6	1.89

dietetic treatments, as in the case of *U. rigida* and the formulated Diet d, apparently produced slight reductions in the GSI, with respect to the check at 45 days. In fact, *U. rigida* yielded a GSI 3.95% at 45 days and 2.13% at 90 days. Therefore, Diet d yielded a G.I 6.95% at 45 days and 4.02% at 90 days. However, the differences between GSIs in the two test periods were not significant in several treatments and the most remarkable result was represented by the high efficiency of pellets, followed by the performances of Diets c and d.

It is interesting that a linear relationship was found between the protein contents of diets and the gonadic indices reached at the end of the experiment. This relationship is striking and quite significant, and it should be taken into account in the formulation of feeds for echinoculture practices. According to this relationship, the two formulated Diets a and b that contained the lowest percentage of proteins (230.6 and 220.2 g/kg, respectively), produced GSIs lower than 1.2%, both at 45 and 90 days. Also *U. rigida* that is considered as a control food for sea urchins and contained only 230 g/kg of proteins in our case, promoted a GSI of 2.13% at the 90th day. This result is comparable with the effect of the formulated Diet b. Results are in agreement with those by Fabbrocini et al. (2015), indicating that *P. lividus* fed on agar biocomposites for 14 weeks showed a modest increase in GSIs and a slight progression in the reproductive stages. This effect is not negative when echinoculture productions are directed to human consumption.

Previous research also took into account the role of fatty acids on body growth and gonad maturation. Several diets devised for various species of sea urchins, as *Strongylocentrotus* spp. (González-Durán, Castell, Robinson, & Blair, 2008) demonstrated the role of fatty acids to support their lipid metabolism. However, sea urchins demonstrated a clear ability for elongation and desaturation of shorter chain (18 C) polyunsaturated fatty acids to the longer chain (20 C) n-3 and n-6 HUFA (Castell et al., 2004). Thus, alternative sources of fatty acids may be feasible to obtain sufficient growth and gonadal maturation. This might explain why the dietary content of proteins was the most important factor to assure high GSIs. Our results indicate the absence of significant relationships between GSIs and the total contents of lipids in the tested diets, while there is a strong relationship between the gonadic indices and the diet contents of proteins.

The lipid contents of diets were coherent, in their turn, with the percentages of abnormal plutei. In fact, diets based on natural items (*P. oceanica* and *U. rigida*), containing lowest amounts of lipids, yielded the lowest rates of abnormalities and the commercial pellet, containing the highest percentages of lipids, yielded the highest rates of abnormal plutei. These data indicate that an ideal diet for *P. lividus* should contain relatively low quantities of lipids (as in

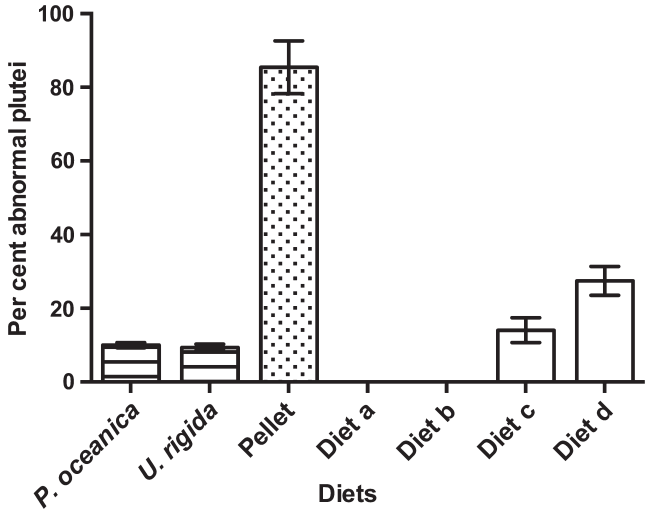

FIGURE 7 Percentage of abnormal plutei obtained from gametes collected, under each of the considered dietetic treatments, at the end of the experiment (90 days) and after in vitro fertilization. Diets a and b did not produce viable larvae

the case of Diets c and d, with 60 and 90 g/kg of fats, respectively) and high quantities of proteins to produce high gonadic indices (high weight of "roe") and low percentages of larval abnormalities. To a remarkable degree, the diet treatments had an effect on gonadic indices and influenced further larval development, but the fertilization was not influenced by the quality of feeds. In the same way, in various marine invertebrates the development of larvae was found to be more sensitive to maternal influences than the egg fertilization (Williams & Bentley, 2001).

Previous research mainly focused on the effects of diets on the abundance and taste of roe (Barker, Keogh, Lawrence, & Lawrence, 1998). However, modern applications of sea urchins for scientific purposes (Santella & Chun, 2011) and for the aims of ecological conservation (Abadie, Pace, Gobert, & Borg, 2018) claim for the need to assure good larval development and success of reproductive bursts. Similar studies performed on *Strongylocentrotus droebachiensis* (Pearce et al., 2002c) for a comparable experimental period, examined the effect of artificial diets with increasing levels of proteins (i.e., 190, 240 and 290 g/kg of their dry weight), and their results showed a positive effect of proteins on the gonadal increase

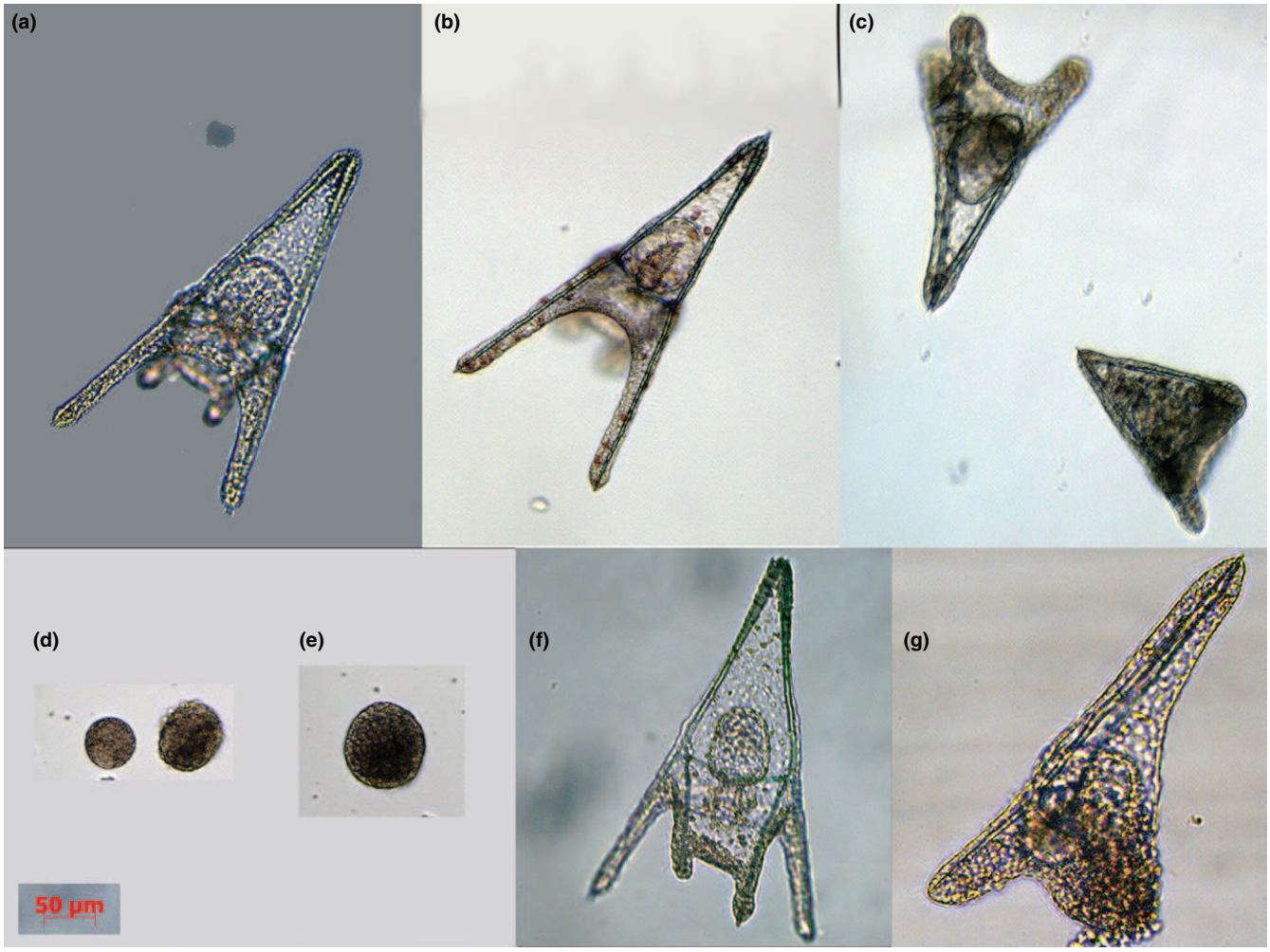

FIGURE 8 Larval shapes observed in: (a) *Posidonia oceanica* treatment; (b) *Ulva rigida* treatment; (c) pellet treatment; (d, e) diet treatments a and b, respectively, producing only atretic embryos showing blebbing; and (f, g) diet treatments c, d, respectively

of weight. As well, de Jong-Westman, March, et al. (1995) reported significantly higher gonad yields in adults of *S. droebachiensis* fed the prepared diets containing 200 g/kg protein (ground wheat, condensed fish soluble, and albumin) than in those given similar diets with only 100 g/kg of proteins.

Other investigations (Akiyama, Unuma, & Yamamoto, 2001) indicated, in contrast, that diets with protein concentration above 200 g/kg did not significantly alter somatic growth rates and GSI of various species of sea urchins (Akiyama et al., 2001; de Jong-Westman, Qian, et al., 1995; Kennedy, Robinson, Parsons, & Castell, 2000; McBride, Lawrence, Lawrence, & Mulligan, 1998). Nevertheless, the source of proteins may influence gonadic indices and various studies (e.g., Fernandez & Boudouresque, 1998, 2000) found that highest gonadic indices were achieved with diets exhibiting intermediate levels of animal proteins (about 100 g/kg of total proteins deriving from fish meals). In a consistent manner, GSIs were significantly lower in *P. lividus* fed the diets without animal proteins and in those with the highest level of animal proteins.

The dietetic effects herein demonstrated are modulated by ecological influences, as temperature, photoperiod and feed rations regulate the gonadic indices in various sea urchins (Garrido & Barber, 2001). The effects of environmental factors may be contrasting in different species, according to their life strategies and the features of their habitats. In the case of *P. lividus* that is a Mediterranean species with winter reproduction, high feed rations are necessary to produce gonadic tissues and long periods of starvation bring the gonads to a spent stage, due to the consumption of tissues to obtain energy (Sartori et al., 2016). In addition, conditions of high protein availability (predominant in winter, in local food webs) and low temperatures favour reproductive output in its populations. In fact, various vegetated ecosystems of the Mediterranean (e.g., *P. oceanica* meadows, one of the elective environments for this species) offer abundant resources for opportunistic herbivores and mesocarnivores in winter months (Zupo, 1993; Zupo et al., 2017). Thus, the winter reproduction of *P. lividus* is in accordance with its trophic requirements (abundance of protein-rich feeds, available as plant epiphytes) for the development of gonads.

Taking into account these considerations and the need to keep low the costs of feeding for productive purposes, a feed containing high proportions of carbohydrates (e.g., deriving from corn, wheat middling, soybean, algae) is suggested in the first phases of growth, up to the initial period of sexual maturation. The protein content of diets should be then increased up to the highest levels of 400–450 g/kg (assuring that most of it is provided by plant sources; Table 2), to facilitate the increase in gonadic tissues, while keeping relatively low (<90 g/kg) the abundance of lipids, to promote the production of viable gametes. In the case of aquaculture products directed to human consumption, a finishing may be considered, in the last period prior to collections, using agricultural wastes such as banana peels and pumpkins (Luo, Zhao, Chang, Feng, & Tian, 2014) to improve colour and taste of gonads.

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ORCID

Valerio Zupo http://orcid.org/0000-0001-9766-8784

Marina Paolucci http://orcid.org/0000-0002-1784-3843

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PLOS ONE

RESEARCH ARTICLE

High-quality RNA extraction from the sea urchin *Paracentrotus lividus* embryos

Nadia Ruocco[1,2,3], Susan Costantini[4], Valerio Zupo[5], Giovanna Romano[6], Adrianna Ianora[6], Angelo Fontana[3]*, Maria Costantini[1]*

1 Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Villa Comunale, Napoli, Italy, **2** Department of Biology, University of Naples Federico II, Complesso Universitario di Monte Sant'Angelo, Via Cinthia, Napoli, Italy, **3** Bio-Organic Chemistry Unit, Institute of Biomolecular Chemistry-CNR, Via Campi Flegrei 34, Pozzuoli, Naples, Italy, **4** CROM, Istituto Nazionale Tumori "Fondazione G. Pascale", IRCCS, Napoli, Italy, **5** Center of Villa Dohrn Ischia-Benthic Ecology, Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, P.ta S. Pietro, Ischia, Naples, Italy, **6** Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Villa Comunale, Napoli, Italy

* maria.costantini@szn.it (MC); afontana@icb.cnr.it (AF)

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Abstract

The sea urchin *Paracentrotus lividus* (Lamarck, 1816) is a keystone herbivore in the Mediterranean Sea due to its ability to transform macroalgal-dominated communities into barren areas characterized by increased cover of bare substrates and encrusting coralline algae, reduced biodiversity and altered ecosystem functions. *P. lividus* is also an excellent animal model for toxicology, physiology and biology investigations having been used for more than a century as a model for embryological studies with synchronously developing embryos which are easy to manipulate and analyze for morphological aberrations. Despite its importance for the scientific community, the complete genome is still not fully annotated. To date, only a few molecular tools are available and a few Next Generation Sequencing (NGS) studies have been performed. Here we aimed at setting-up an RNA extraction method to obtain high quality and sufficient quantity of RNA for NGS from *P. lividus* embryos at the pluteus stage. We compared five different RNA extraction protocols from four different pools of plutei (500, 1000, 2500 and 5000 embryos): TRIzol®, and four widely-used Silica Membrane kits, GenElute™ Mammalian Total RNA Miniprep Kit, RNAqueous® Micro Kit, RNeasy® Micro Kit and Aurum™ Total RNA Mini Kit. The quantity of RNA isolated was evaluated using NanoDrop. The quality, considering the purity, was measured as A260/A280 and A260/230 ratios. The integrity was measured by RNA Integrity Number (RIN). Our results demonstrated that the most efficient procedures were GenElute, RNeasy and Aurum, producing a sufficient quantity of RNA for NGS. The Bioanalyzer profiles and RIN values revealed that the most efficient methods guaranteeing for RNA integrity were RNeasy and Aurum combined with an initial preservation in RNAlater. This research represents the first attempt to standardize a method for high-quality RNA extraction from sea urchin embryos at the pluteus stage, providing a new resource for this established model marine organism.

Competing interests: The authors have declared that no competing interests exist.

Introduction

NGS technologies have found broad applications in functional genomic research, including gene expression profiling, genome annotation, small ncRNA discovery and profiling, determination of DNA sequences associated with epigenetic modifications of histones and DNA, and profiling DNA methylations [1–6].

"Omic" approaches are powerful instruments not only for genomic studies but also for many other research fields. For example starting from 2007, ecologists used this approach to address several important ecological questions, introducing the new discipline of "ecological genomics" and/or "molecular ecology" [7]. Advances in DNA technologies have had a very strong impact on molecular ecology studies, also providing new tools for understanding the response of organisms to environmental stress [8–10]. The study of stress response in animal species has represented a predominant subject in the research, as a consequence of global warming, ocean acidification and increased pollution [10]. Echinoids have been considered ideal models for monitoring marine environmental hazards [11], because they are often key herbivore species, having a major role in structuring and controlling macroalgal assemblages, thereby shaping the benthic seascape, and also playing an important role in coastal food webs throughout the world [12–16]. They have traditionally been used as model organisms to study reproduction and early cell differentiation, sperm-oocyte interactions and apoptosis [17–19]. These organisms have been also proposed as valuable bioindicators for detecting environmental perturbations [20–23].

Among the echinoderms, the sea urchin *Paracentrotus lividus* is considered a suitable organism to study the ecotoxicological responses to xenobiotics and the physiological reactions to physical stressors [24–29]. It has also been used to test the effect of marine natural toxins, such as diatom-derived secondary metabolites, which have also apoptotic and anti-cancer activity [30,31].

The species represents a useful test organism for several reasons: it is an important component of benthic communities in the Mediterranean Sea and Atlantic Ocean; extraction and maintenance of gametes are easy; the embryos grow rapidly and synchronously (pluteus stage is reached 48 hours post-fertilization) and embryos are transparent and suitable for microscope detection of sub-lethal effects of pollutants on development; they have a sufficiently long reproductive season (from October to May). Although *P. lividus* represents a well-established model organism, to date the complete sequence of its genome is still not available [19]. Thanks to NGS approaches, it would be possible to fill this genome gap by increasing the amount of molecular information on the sea urchin *P. lividus*. However, high-throughput NGS of genomes and transcriptomes requires high-quality, clean, and concentrated DNA/RNA.

This study compares five RNA extraction protocols using embryos of the sea urchin *P. lividus* to define the best method for obtaining high-quality RNA for NGS applications. We fertilized four pools of eggs and performed RNA extractions from embryos at the pluteus stage (at 48 hour post-fertilization) preserved in RNA*later*®, using five different protocols: a guanidinium-thiocyanate-phenol-chloroform (GTPC) extraction protocol with TRIzol®, and four widely-used Silica Membrane kits, namely GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), RNAqueous® Micro Kit (Ambion from Life Technologies), RNeasy® Micro Kit (Qiagen) andAurum™ Total RNA Mini Kit (Biorad). The quantity and quality of isolated RNA was evaluated taking into account the purity, measured as A260/A280 and A260/230 ratios, and the integrity, measured as RNA Integrity Number (RIN). The results yielded a first protocol for high-quality RNA extraction from sea urchin plutei, increasing the resources for such a well-established model organism.

Materials and methods

Ethics statement

P. lividus (Lamarck) were collected from a site in the Bay of Naples that is not privately-owned or protected in any way, according to Italian legislation (DPR 1639/68, 09/19/1980 confirmed on 01/10/2000). Field studies did not include endangered or protected species. All experimental procedures on animals were in compliance with the guidelines of the European Union (Directive 609/86).

Sample collection and preservation

Adult sea urchins were collected during the breeding season by scuba-diving in the Gulf of Naples, transported in thermic box to the laboratory within 1 hour after collection and maintained in tanks with circulating sea water until testing. Sea urchins were injected with 2 M KCl through the peribuccal membrane to obtain the emission of gametes. Eggs were washed with filtered sea water (FSW) and kept in FSW until use. Concentrated spermatozoans were collected, dried and kept undiluted at +4˚C until use.

Different quantities of eggs (500, 1000, 2500 and 5000) from five females were fertilized, using sperm-to-egg ratios of 100:1, and embryos were then collected at the pluteus stage (48 hpf) by centrifugation at 1800 rcf for 10 min in a swing out rotor at 4˚C. Immediately after harvesting, the embryos were placed in at least 10 volumes of the RNA*later*®, an RNA Stabilization Reagent (Qiagen, Hilden, Germany), and then frozen in liquid nitrogen and kept at -80˚C.

RNA extraction

Five different methods of RNA extraction were compared.

i) TRIzol® RNA extraction method

Total RNA was extracted using TRIzol (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, homogenizing with TissueLyser (Qiagen, Austin, TX, US) with 3 mm sterile aluminium beads at 20.1 Hertz (Hz) for 5 minutes. Extractions with chloroform/isoamyl alcohol (24:1) were performed, followed then by RNA precipitation by adding glycogen and isopropyl alcohol. Total RNA was suspended in 0.1% v/v diethylpyrocarbonate (DEPC)-treated water. Contaminating DNA was degraded by treating each sample with a DNase RNase-free kit (Roche, Milan, Italy) according to the manufacturer's instructions. The samples were then stored at -80˚C.

ii) GenElute™ Mammalian Total RNA Miniprep Kit

Embryo lysis was performed in 500 μl Lysis Solution/2-ME mixture and then RNA was extracted following the manufacturer's protocol. An additional step with DNase RNase-free kit (Roche, Milan, Italy) was used to remove contaminating DNA. Finally, RNA was eluted with 50 μl Elution Solution provided by the manufacturer. The samples were then stored at -80˚C.

iii) RNeasy® Micro Kit

Embryos were lysed with 350 μl Buffer RLT/2-ME (10 μl β-mercaptoethanol for each ml of Buffer RLT) and homogenized with TissueLyser (Qiagen, Austin, TX, US) using 3 mm sterile aluminium beads at 20.1 Hz for 5 minutes. RNA was extracted following the manufacturer's protocol. DNA contaminations were avoided using the RNase-Free DNase Set, provided by the kit. RNA was eluted with 14 μL RNase-free water. The samples were then stored at -80˚C.

iv) RNAqueous® Micro Kit

Pellets were suspended in 500 μl Lysis Solution by vortexing vigorously. Elution was performed in two steps, adding 10 μl Elution Solution each time, and preheating at 75˚C. Finally a

DNase treatment was performed adding 1/10 volume 10X DNase I Buffer and 1 μL of DNase I to the sample. DNase was finally blocked using a DNase Inactivation Reagent (1/10 of total volume). The samples were then stored at -80˚C.

v) Aurum™ Total RNA Mini Kit

Samples were disrupted with 350 μl Lysis Solution (already supplemented with 1% β-mercapto-ethanol), pipetting up and down several times to lyse cells thoroughly. After treatment with 80 μl of diluted DNase I (75 μl DNase Dilution Solution and 5 μl DNase I), RNA was eluted with 40 μl of Elution Solution. The samples were then stored at -80˚C.

Determination of RNA quantity and quality

The amount of total RNA extracted with the five methods was estimated by measuring the absorbance at 260 nm and the purity at 260/280 and 260/230 nm ratios, by a Nanodrop (ND-1000 UV-Vis Spectrophotometer; NanoDrop Technologies, to exclude the presence of proteins, phenol and other contaminants [32]. The integrity measurements of RNA were finally assessed by running 100–200 ng of RNA samples in each line of a 6000 Nano LabChip in an Agilent Bioanalyzer 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, US). RNA integrity was measured using the RIN value, which was calculated based on the comparison of the areas of 18S rRNA and 28S rRNA [33]. RIN values over a threshold of 8 were considered to indicate non-degraded RNA extraction methods.

Statistical analysis

The total number of sample extractions from four different amounts of embryos is reported in Table 1. RNA quantity and quality (A260/280 and A260/230 ratios, and RIN values) using different RNA extraction procedures were tested using One-way Variance Analysis (ANOVA). We first checked whether measures of RNA quality, absorbance ratios, A260/230 and A260/280, and RIN values were correlated in our data by a nonparametric Spearman's correlation coefficient. Moreover, we verified that their differences were statistically different using the t-test using whereby p-values lower than 0.05 were considered significant. Distribution of RIN values was graphically represented by boxplots for different treatments of sea urchin embryos. Statistical analyses were performed using GraphPad PRISM v.4 software (San Diego, CA, US).

Results

Increasing amounts of sea urchin *P. lividus* eggs (500, 1000, 2500 and 5000) were fertilized and embryonic development was followed until the pluteus stage, which is reached at about 48 hpf. The results of different RNA extractions are summarized in Table 1. Concerning RNA quantity, similar results were obtained using TRIzol, RNeasy and RNAqueous methods for 500 and 1000 embryos. In the case of 2500 and 5000 embryos, significantly higher quantity of RNA was extracted using the GenElute, RNeasy and Aurum methods (One-way ANOVA $p<0.0001$), whereas no significant differences were detected between 2500 and 5000 embryos versus 500 and 1000 embryos with the RNAqueous method ($p>0.05$). All extraction procedures from 2500 and 5000 embryos yielded sufficient amounts of total RNA as requested for NGS approaches (~2–3 μg of total RNA). We did not detect significant differences in RNA purity (A260/230 and A260/280 ratios) using the five considered methods. The Spearman's coefficients did not reveal a correlation between RNA quality variables based on absorbance ratios (A260/280 and A260/230) and RIN values (ρ ranged from 0.40 to 0.52; p values from 0.0833 to 1.0; for further details see S1 Table). These results were also confirmed by t-test, showing that RNA quality and RIN trends were statistically different ($p < 0.0001$).

Table 1. Extraction number, total RNA quantity (μg), purity (A260/280 and A260/230) and integrity (RIN values) from different number of *P. lividus* embryos using five different extraction methods: TRIzol, GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), RNAqueous® Micro Kit (Ambion from Life Technologies), RNeasy® Micro Kit (Qiagen) and Aurum™ Total RNA Mini Kit (Biorad). Values represent mean ± SD. Values in bold in the RIN column are the average values among the RIN values obtained at the different number of *P. lividus* embryos. N/A indicates samples in which no values are assigned.

Extraction method	Extraction number	Embryos	RNA quantity (μg)	A260/230	A260/280	RIN
TRIzol	4	500	2.4 ± 0.23	1.96 ± 0.44	1.81 ± 0.24	N/A
	7	1000	3.5 ± 0.08	1.68 ± 0.18	1.42 ± 0.37	4.20 ±1.13
	7	2500	8.1 ± 0.16	1.85 ± 0.51	1.91 ± 0.42	4.60 ± 0.89
	7	5000	8.9 ± 0.09	1.83 ± 0.67	1.86 ± 0.30	3.90 ± 0.67
						4.20
GenElute	4	500	3.2 ± 0.56	0.86 ± 0.87	2.00 ± 0.03	8.10 ± 0.03
	7	1000	4.2 ± 2.05	1.21 ± 0.05	1.98 ± 0.05	7.80 ± 0.05
	7	2500	12.8 ± 8.16	1.83 ± 0.03	1.93 ± 0.03	7.95 ± 0.35
	7	5000	25.9 ± 17.96	1.97 ± 0.03	1.96 ± 0.03	7.95 ± 0.07
						7.95
RNeasy	4	500	1.0 ± 0.27	0.50 ± 0.41	2.15 ± 0.25	9.20 ± 0.01
	7	1000	1.8 ± 0.14	0.78 ± 0.61	1.92 ± 0.26	9.45 ± 0.49
	7	2500	8.9 ± 0.05	2.35 ± 0.07	1.97 ± 0.01	9.30 ± 0.14
	7	5000	18.7 ± 7.91	2.17 ± 0.08	1.98 ± 0.05	9.20 ± 0.15
						9.30
RNAqueous	4	500	1.6 ± 2.1	0.46 ± 0.71	1.90 ± 0.34	7.90 ± 0.08
	7	1000	2.9 ± 3.6	0.72 ± 1.07	1.88 ± 0.48	8.10 ± 0.27
	7	2500	3.0 ± 3.9	2.37 ± 0.29	1.83 ± 0.28	10.0 ± 0.17
	7	5000	4.2 ± 5.3	2.35 ± 0.17	1.93 ± 0.14	8.90 ± 0.09
						8.70
Aurum	4	500	1.6 ± 0.71	1.96 ± 1.14	2.04 ± 0.25	9.65 ± 0.50
	7	1000	5.4 ±1.90	1.93 ± 0.36	2.13 ± 0.08	9.45 ± 0.35
	7	2500	13.3 ± 2.49	1.65 ± 1.18	2.08 ± 0.07	9.95 ± 0.07
	7	5000	23.0 ± 1.47	1.94 ± 0.75	2.05 ± 0.07	9.85 ± 0.07
						9.70

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Representative Bioanalyzer Agilent electrophoresis runs showed low or high quality of total RNA extracted from *P. lividus* embryos (Fig 1).

The methods used for total RNA extractions consistently showed two bands, corresponding to 28S and 18S rRNA, with the only exception being the TRIzol method, that showed degraded RNA with several bands. These results were also confirmed by the representative Bioanalyzer Agilent profiles showing electropherograms with low or high quality of total RNA extracted from different numerical amounts of *P. lividus* embryos along with corresponding RIN values (Fig 2).

Total RNA extracted with TRIzol showed a single peak corresponding to 18S at 42 seconds (s), absence of a 28S peak, a high amount of small size RNA occurring between 25s and 42 s and RNA degradation with a very low RIN value (RIN = 3.90). The integrity of *P. lividus* plutei RNA significantly improved with the use of Silica Membrane methods. In fact, in all four

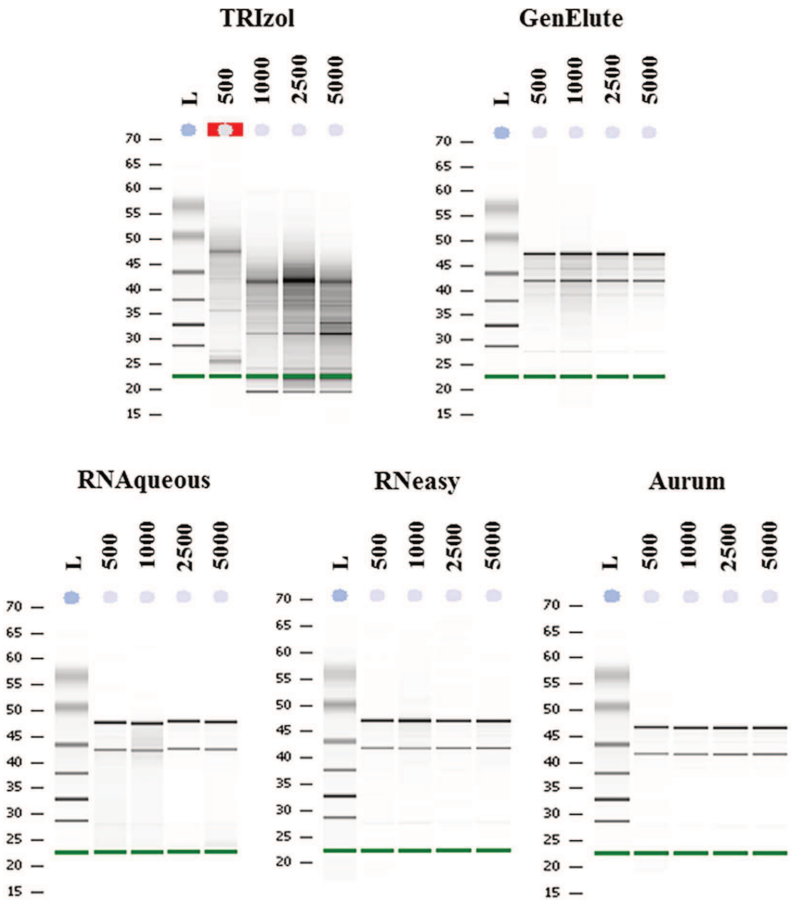

Fig 1. Bioanalyzer Agilent electrophoresis runs. Examples of representative Bioanalyzer Agilent electrophoresis runs for the five different methods applied for RNA extractions from *P. lividus* embryos: TRIzol, GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), RNAqueous® Micro Kit (Ambion from Life Technologies), RNeasy® Micro Kit (Qiagen) and Aurum™ Total RNA Mini Kit (Biorad). Four different numerical amounts of embryos were used for RNA extraction: 500, 1000, 2500 and 5000 embryos. The ladder (L) is reported in the first lane of each run. The green band at the bottom of each panel is the RNA 6000 Nano Marker (Agilent RNA 6000 Nano Kit, Agilent Technologies, Inc.). Red box in the 500 lane of TRIzol indicates that the Bioanalyzer software cannot calculate RIN values (reported as N/A in the Table 1) for this sample, because of very low concentration and high level of degradation of the RNA.

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commercial kits applied for RNA extraction, the electropherograms showed two peaks corresponding to 28S (42s) and 18S (47s) with very high average RIN values ranging from 7.9 for GenElute, 8.7 for RNAqueous, increasing to 9.3 for RNeasy and reaching a maximum value of 9.7 with the Aurum kit. Such high RIN values were considered suitable for NGS technologies.

In samples exhibiting A260/280 and A26030 ratios lower than 1.8 or over 2.0 (Table 1), we observed RIN values higher than 8. These samples also showed intact 28S and 18S. The distributions of RIN were also reported in the boxplot of RIN values (S1 Fig). Extraction by RNAqueous was the most unstable method based on RIN values (from 7.90 to 10.0). Moreover, even if GenElute and Aurum methods yielded comparable results producing higher quantities of RNA (Table 1), the boxplot clearly showed that they were not comparable in terms of RNA integrity. In fact, the GenElute method produced RNA with RIN values lower than those obtained with the Aurum method.

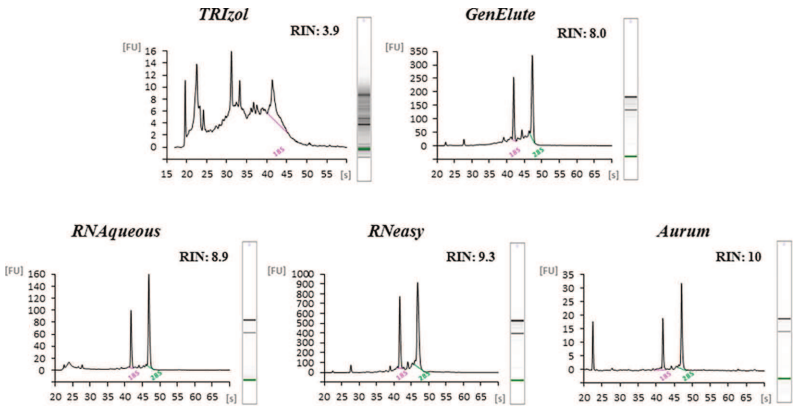

Fig 2. Agilent Bioanlyzer electropherograms. Examples of representative Agilent Bioanlyzer electropherograms of *P. lividus* RNA: for TRIzol, GenElute and RNAqueous RNA extraction from 5000 embryos extraction; for RNeasy and Aurum RNA extraction from 2500 embryos (see also Table 1). Relative Fluorescent Unit (FU) and seconds of migration (s) of RNA samples isolated according to the five different extraction methods are reported. RIN values are also reported.

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Discussion

In the present work we focused our attention on optimizing RNA extraction protocols from sea urchin *P. lividus* embryos at the pluteus stage. Since the sea urchin *P. lividus* is one of the most common sea urchins in the Mediterranean Sea, as well as in the Northeast Atlantic from Ireland to the coasts of Morocco, the Canary Islands and the Azores [34], setting up a feasible method of RNA extraction represents the first step to afford details of its trophic and reproductive biology. *P. lividus* is considered a keystone herbivore, able to transform communities dominated by macroalgae into barren areas thereby reducing biodiversity and altering ecosystem functions [35,36]. The sea urchin has been extensively used as an invertebrate model organism in developmental biology and in ecotoxicology studies to assess the effects of marine pollution on marine organisms [19].

Despite the well-recognized ecological significance of the sea urchin in marine environment, to date there are few molecular tools available for this species. For this reason, NGS approaches are increasingly being applied to this species, in order to increase the molecular data on *P. lividus*. To our knowledge, our work represents the first attempt to standardize a method for RNA extraction from sea urchin embryos at the pluteus stage. Our aim was to obtain high-quality RNA in sufficient quantities for NGS technologies. Firstly, we extracted RNA from different numbers of sea urchin *P. lividus* embryos at the pluteus stage, applying five different protocols. Our findings revealed that all extraction procedures from 500 and 1000 embryos did not yield sufficient amounts of total RNA as requested for NGS approaches (~2–3 μg of total RNA). Sufficient RNA quantity was obtained from 2500 and 5000 embryos. The most efficient procedures to produce such quantities were obtained with GenElute, RNeasy and Aurum, producing more than 10 and 20 μg of RNA, using 2500 and 5000 embryos, respectively.

Having established the number of embryos necessary to obtain the required quantity of RNA, we assessed RNA quality by measuring different features: A260/280 and A260/230 ratios and estimation of RIN values [32,37–39]. In our samples, the most efficient strategies for assessing RNA integrity was represented by the estimation of the RIN value using Agilent Bioanalyzer chips. The Bioanalyzer profiles showed that GenElute, RNeasy and Aurum procedures were only comparable for RNA quantity, not for RNA integrity. In fact, RIN values using the GenElute procedure were the lowest in comparison to the other Silica Membrane

procedures applied. RNAqueous was the most variable method whereas the most efficient for RNA integrity were RNeasy and Aurum.

Interestingly our findings demonstrated no significant correlations between A260/280 and A260/230 ratios and RNA integrity. A possible explanation for this may be due to the different stability of the RNAs. In fact, ribosomal RNA is more stable than mRNA [32, 40–43]. Moreover, the A260/280 ratio could have values of about two, because of the intact ribosomal RNA, even if the mRNA was degraded. This is, for example, the case of RNA samples after TRIzol extraction, suggesting that the method of preservation of our embryo samples represents a very important step to obtain high-quality RNA. Preservation in RNA*later* ensured greater stability for our samples since this a nontoxic storage reagent able to permeate cells and/or tissues and to stabilize and protect cellular RNA. This step minimizes the need for immediate processing of the samples, without jeopardizing the quality or quantity of RNA obtained after subsequent RNA isolation. RNA extraction from fresh cell/tissues has been used in many cases with success [23,41,43], but several laboratory conditions or field experiments do not allow for direct extraction upon collection. There are several indications that RNA*later* is a reliable preservative for RNA in a wide array of tissues improving the yield of total RNA [32,39].

This study fills a gap considering that no comparative studies for RNA extractions are available in the literature for sea urchin *P. lividus* plutei. A similar methodological analysis was performed by Pérez-Portela and Riesgo [43], although they did not use embryos at the pluteus stage (as in our case) from *P. lividus*, but tissues from another sea urchin, *Arbacia lixula*. In fact, their study aimed to optimize a preservation protocol for the isolation of high-quality RNA from three different *A. lixula* tissues: gonad, oesophagus and coelomocytes. Extractions of total RNA were performed with a modified TRIzol protocol for all tissues, applying four preservation treatments. The results showed high values of RNA quantity and quality for all tissues, indicating non-significant differences among samples. Insufficient RNA amount and great variability in RNA integrity were found in coelomocytes in RNA*later*. The most efficient system to stabilize RNA was the TRIzol method that produced high RNA quality and quantity. These data are comparable with our results (see above), because no correlation was found between RNA integrity and absorbance ratios, as in our experiments. Furthermore, the evaluation of RIN values by Agilent Bioanalyzer chips was the best approach to evaluate the RNA integrity (as in our case).

The extraction of high-quality RNA represents a fundamental step, considering that transcriptomic information and NGS approaches represent significant tools to acquire information regarding several biological processes. Several attempts, mainly for non-well-established model organisms, were recently made to establish methods for high-quality RNA extractions, because NGS represents the only way to address specific ecological and evolutionary questions [42,44].

Asai et al. [44] compared three commonly-used methods: TRIzol®, Aurum Total RNA Mini Kit and Qiagen RNeasy Micro Kit, in combination with preservation reagents TRIzol® or RNA*later*®, to obtain high-quality and large quantities of RNA from the copepod *Calanus helgolandicus*. Their results confirmed that the preservation of copepods in RNA*later*® and the extraction with Qiagen RNeasy Micro Kit were the optimal isolation method for high-quality and quantity of RNA for NGS studies of *C. helgolandicus*.

In conclusion, our results provide for the first time a standard protocol for isolating high-quality RNA from sea urchin plutei for high-throughput NGS studies thereby increasing the resources available for a well-established model organism such as *P. lividus*.

Supporting information

S1 Fig. Boxplot of RIN values obtained with the five different methods of RNA extraction. The boxes extend from the 25th to the 75th percentile, and the line in the middle is the median.

The error bars extend down to the lowest value and up to the highest. Dashed line at the RIN value of 8 is reported, because higher values than 8 are considered suitable for NGS analysis.
(PPT)

S1 Table. Correlation between RNA quality based on absorbance ratios (A260/280 and A260/230) and RIN values by Spearman's correlation coefficient and evaluation of their statistical difference by t-test, using the five different RNA extraction protocols for P. lividus embryos. We concern as statistically significant the p-values lower than 0.05.
(DOC)

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Author Contributions

Conceptualization: MC NR.

Data curation: MC NR.

Formal analysis: SC.

Funding acquisition: MC AF.

Investigation: MC NR.

Methodology: MC NR.

Project administration: MC AF.

Resources: MC AI VZ AF.

Supervision: MC.

Validation: MC NR SC.

Visualization: MC NR SC.

Writing – original draft: MC AF NR SC VZ GR AI.

Writing – review & editing: MC AF NR SC VZ GR AI.

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Toxigenic effects of two benthic diatoms upon grazing activity of the sea urchin: morphological, metabolomic and *de novo* transcriptomic analysis

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Nadia Ruocco[1,2,3], Susan Costantini[4], Valerio Zupo[5], Chiara Lauritano[6], Davide Caramiello[7], Adrianna Ianora[6], Alfredo Budillon[4], Giovanna Romano[6], Genoveffa Nuzzo[3], Giuliana D'Ippolito[3], Angelo Fontana[3] & Maria Costantini[1]

Diatoms are unicellular algae playing a key role as photosynthetic organisms in the world's ocean food webs. The chemical ecology of planktonic diatoms is well documented, but few studies have reported on the effects of benthic diatoms on their consumers, also due to difficulties in the collection, quantification and massive culturing of benthic species. Here for the first time we investigate the effects of feeding on two abundantly occurring benthic diatoms, *Nanofrustulum shiloi* and *Cylindrotheca closterium*, isolated from the leaves of the seagrass *Posidonia oceanica*, on the sea urchin *Paracentrotus lividus*. Adult *P. lividus* were fed for one month on diets of either one of the two diatoms and on the green alga *Ulva rigida*, used as a feeding control. By combining morphological, metabolomic and *de novo* transcriptomic approaches, we demonstrate toxigenic effect on embryos generated by females fed with these benthic diatoms. Furthermore, chemical analysis reveal the presence of polyunsaturated aldehydes only for *N. shiloi*, and a high production of other oxylipins (cytotoxic compounds on their grazers and on cancer cell lines) for both diatoms, including some additional peaks not correlated to the canonic oxylipins commonly observed in planktonic diatoms. These findings open new perspectives in the study of diatom secondary metabolites influencing their grazers.

Diatoms are unicellular eukaryotes, representing one of the largest and ecologically groups and exclusively depositing biogenic silica. The siliceous wall is transparent, allowing the entrance of the light, and perforated, making possible the diffusion and excretion of materials[1]. They contribute about 20% of global photosynthetic fixation of carbon (about 20 Pg carbon fixed per year), which is more than all the world's tropical rainforests, also playing important roles on earth and in oceans as oxygen synthesizers and biomass sources[2]. Functionally, diatoms are single cells but they can appear as filaments, chains, or colonies, and they are abundant in nearly all aquatic habitats, living either in the water column (planktonic species) or attached to any single substratum (benthic species). Moreover, diatom morphology can be considered as an additional environment assessment tool to the biological indices to evaluate for example the anthropogenic eutrophication effects[3].

Diatoms have been regarded as beneficial to the growth and survival of primary consumers such as planktonic and benthic filter feeders. However, many planktonic diatoms have been discovered to produce a wide range

[1]Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121, Napoli, Italy. [2]Department of Biology, University of Naples Federico II, Complesso Universitario di Monte Sant'Angelo, Via Cinthia, 80126, Napoli, Italy. [3]Bio-Organic Chemistry Unit, Institute of Biomolecular Chemistry-CNR, Via Campi Flegrei 34, Pozzuoli, Naples, 80078, Italy. [4]Unità di Farmacologia Sperimentale, Istituto Nazionale Tumori "Fondazione G. Pascale", IRCCS, Napoli, Italy. [5]Center of Villa Dohrn Ischia-Benthic Ecology, Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, P.ta S. Pietro, Ischia, Naples, Italy. [6]Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121, Napoli, Italy. [7]Unit Marine Resources for Research, Stazione Zoologica Anton Dohrn, Naples, Italy. Correspondence and requests for materials should be addressed to M.C. (email: maria.costantini@szn.it)

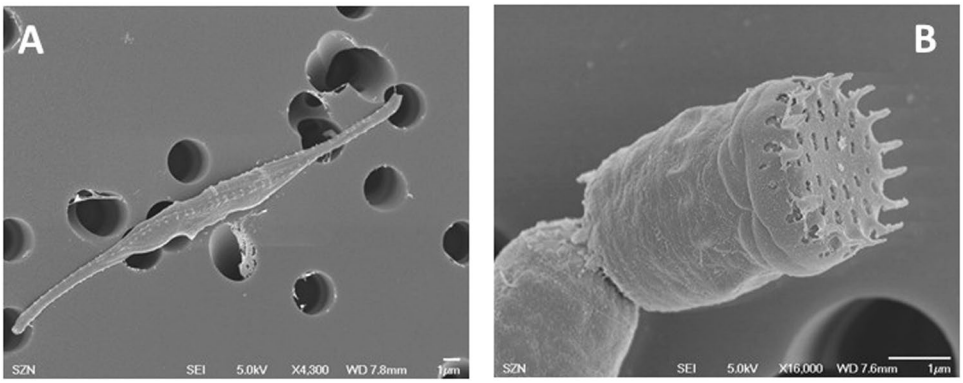

Figure 1. Scanning electron micrographs (SEM) of (**A**) *C. closterium* and (**B**) *N. shiloi* isolates. Scale bar = 1 μm.

of oxygenated fatty acid derivatives (called oxylipins)[4,5] that affect diatom growth[6,7] or have negative effects on the reproduction and development of several marine invertebrates, such as copepods[8,9], sea urchins[10–15] and sea stars[16], polychaete worms[17] and ascidians[16,18]. Moreover some oxylipins, the diatom derived polyunsaturated aldehydes, activated cell death in human cancer cell lines[19].

In contrast to the chemical ecology of planktonic diatoms that is well documented[20,21], few studies have investigated the chemistry of benthic diatoms also due to difficulties in their isolation, quantification and cultivation in plates, with respect to planktonic species[22]. Diatoms are characterized by rapid and extensive colonization of benthic substrate occurs through deposition of stalks, creating thick mats and exhibiting a strong dependence on hydrodynamic conditions and requiring stable substrates to establish a population[23,24].

Some *Cocconeis* species have been shown to induce sex reversal in the shrimp *Hippolyte inermis*, a benthic opportunistic herbivore, but the chemicals that induce sex reversal remain unknown[25–27]. Other benthic diatoms produce volatile organic compounds (VOCs) similar to those produced by planktonic diatoms that induce behavioural changes in several species of macrinvertebrates[28], but studies on the metabolic effects of fast-growing benthic diatoms on benthic invertebrates are still largely lacking[29–31].

Using morphological, metabolomic and *de novo* transcriptomic approaches here, for the first time, the effects of two abundantly occurring benthic diatoms, *Nanofrustulum shiloi* and *Cylindrotheca closterium*, isolated from the leaves of the seagrass *P. oceanica*, were investigated on the reproductive success of the sea urchin *Paracentrotus lividus*. Sea urchins were fed for one month on these two benthic diatoms in a microcosm, using the green alga *Ulva rigida* as a feeding control. The development of the embryos produced after feeding on these diets were then followed until the pluteus stage (48 hours post fertilization). In addition, gonadic tissues from these adults were analyzed by ^{1}H-NMR metabolomics. Finally, molecular approaches were applied to investigate the toxic effects of the benthic diatoms, by generating a *de novo* transcriptome assembly and annotation of *P. lividus* to identify differentially expressed genes. Fifty genes, belonging to different functional classes, were also followed using Real-Time qPCR to detect if the expression level of these genes was modulated by feeding on the benthic diatoms.

Results

Morphological and molecular characterization of benthic diatoms. SEM observation revealed that the first diatom isolated was about 50 μm in length, needle like and thin in shape, the two ends of the cell extended far from the centre of the cell; the cells showed spiral twist of the raphe system, which is characteristic for *Cylindrotheca closterium* (Fig. 1A). Molecular analysis of 18S rRNA gene amplified from the purified alga showed that it was closer (99%) to *C. closterium* than to other species. The second benthic diatom was characterized by rectangular frustules, forming chains linked by interlocking marginal spines characteristic of *Nanofrustulum shiloi* (Fig. 1B). This morphological result was also confirmed by 18S rRNA gene, showing 99% identity to *N. shiloi*.

Feeding experiments. The biomass of benthic diatoms fed to sea urchin replicates was calculated to be 1.6 pg C cell^{-1} for *C. closterium* and 1.8 pg C cell^{-1} for *N. shiloi*[32].

After one month of feeding, eggs and sperms were collected from sea urchins fed with *U. rigida*, *N. shiloi* and *C. closterium*. As soon as fertilization occurred, fertilization success and the time to reach first mitotic cleavage to obtain two blastomeres was measured in comparison with embryos deriving from sea urchins collected in the field at the beginning of the experiments (t0) (Table 1). At time 0 100% fertilization and first cleavage were obtained with all diets. Embryonic development was then followed until the *pluteus* stage; morphological observations showed that the percentage of abnormal embryos was higher in sea urchins fed on *C. closterium* and *N. shiloi* for one month ($p < 0.0001$) in comparison to the control diet (Fig. 2). In particular, both *C. closterium* and *N. shiloi* induced the same malformations, which principally affected the arms, spicules and apices, in comparison with control embryos (Supplementary Fig. S5). To confirm that sea urchins had really fed on diatoms, the content of fecal pellets was also analyzed by means of SEM. These observations showed the presence of *C. closterium* silica frustules in the fecal pellets (reported in the Supplementary Fig. S6 as an example), confirming that sea urchins have effectively eaten the diatoms.

	t0	*U. rigida*	*C. closterium*	*N. shiloi*
Fertilization	100	100	100	100
First cleavage	100	100	100	100
Normal plutei	90	89.4	45.3	60.9
Malformed plutei	10	10.6	54.7	39.1

Table 1. Percentage of fertilization, first cleavage (two blastomeres), normal plutei and malformed plutei in the embryos from sea urchins *P. lividus* collected in the field at the beginning (t0) and after one month of feeding with *U. rigida*, *C. closterium* and *N. shiloi*.

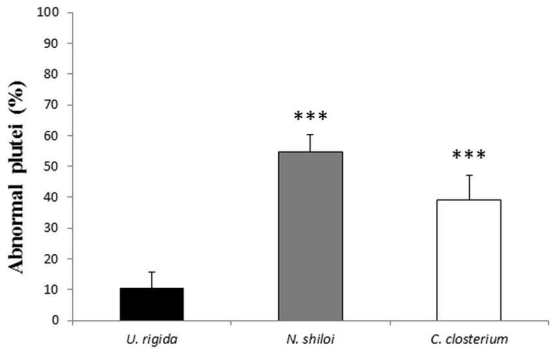

Figure 2. Percentage of abnormal plutei (%; at 48 hours post fertilization) of *P. lividus* sea urchin embryos spawned from adults fed for one month with *U. rigida*, *N. shiloi* and *C. closterium* (*** with a p-value < 0.001, Student's t-test, GraphPad Software Inc., San Diego, CA, USA).

^{1}H-NMR analysis of metabolites and lipids from sea urchin gonads. ^{1}H-NMR spectra were obtained from aqueous extracts of gonad tissues from five adult sea urchins fed with *U. rigida* (control), *N. shiloi* and *C. closterium*. The primary peaks in the spectra were assigned to individual metabolites (Supplementary Table S2).

Some metabolite classes were identified including acetoacetate, ATP, choline, lactate, glucose and amino acids (valine, leucine, isoleucine, alanine, threonine, arginine, lysine, glutamate, glutamine, aspartate, glycine, tyrosine, phenylalanine, tryptophan and histidine).

OPLS-DA plot (45% of the total variance) showed that the control and two treated groups clustered in separate classes, with a slight overlap between the two treated groups (Fig. 3A), suggesting the presence in these three groups of statistically different levels of metabolites (Fig. 3B). In particular: (i) the levels of acetoacetate and of six amino acids such as tyrosine, proline, valine, isoleucine, leucine and lysine, and acetoacetate were higher in the *N. shiloi* group when compared to the control group, and (ii) their levels were even higher in the *C. closterium* group when compared to the *N. shiloi* group. On the other hand, the levels of tryptophan decreased after feeding on both benthic diatoms and were lower in the *C. closterium* group compared to the *N. shiloi* group. Finally, the levels of arginine and alanine were higher in the treated groups when compared to the control group, and, higher in the *N. shiloi* group compared to the *C. closterium* group.

^{1}H-NMR spectra were also obtained from lipophilic extracts of gonad tissues. As shown in Supplementary Table S3, phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, linoleic acid, cholesterol and other unassigned lipids were found in the gonads of *P. lividus*. OPLS-DA plot (38.1% of the total variance) showed that the control and two treated groups clustered in separate classes with a slight overlap between the control group and the two treated groups (Fig. 4A), and a total overlapping of the *N. shiloi* and *C. closterium* groups. These data suggest the presence in these three groups of statistically different levels of metabolites between the control and the two treated groups, and metabolites with similar levels between the two treatments. In fact: (i) the levels of linoleic acid and cholesterol were higher in the *N. shiloi* group when compared to the control group, and (ii) their levels were even higher in the *C. closterium* group when compared to the *N. shiloi* group. The levels of phosphatidylcholine, sphingomyelin and other lipids decreased after feeding on both benthic diatoms and were lower in the *N. shiloi* group compared to the *C. closterium* group; whereas the levels of phosphatidylethanolamine and phosphatidylcholine $POCH_2$ were lower in the *N. shiloi* group compared to the *C. closterium* group (Fig. 4B). Considering only fatty acids, some fatty acids, such as linoleic acid and other fatty acids were higher in the *N. shiloi* group and even higher in the *C. closterium* group when compared to the *N. shiloi* group (Fig. 4C). On the other hand, other fatty acids decreased in *N. shiloi* and increased in *C. closterium* groups, with respect to the control group.

Transcriptomic assembly. The raw assembled transcriptome included almost 121 Mbp in 192493 transcripts grouped in 126941 genes. The mean GC content was 41.37%. The average and median contig lengths were 357 bp and 627 bp, respectively. The N50 was 920 bp. All analyses were conducted on *de-novo* assembled genes and the biological replicates clustered according to the experimental design.

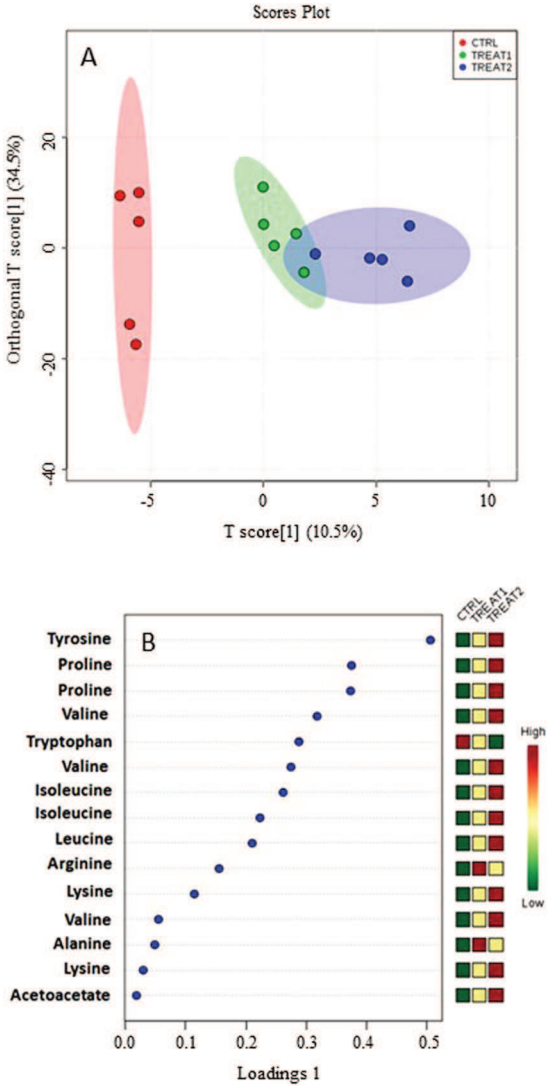

Figure 3. OPLS-DA (**A**) and Loading (**B**) plots (where the metabolites increased or decreased) of aqueous extracts from gonad tissues from adults sea urchin *P. lividus* after one month of feeding with *U. rigida* (used ad feeding control, reported as CTRL), *N. shiloi* (reported as TREAT1) and *C. closterium* (reported as TREAT2).

Figure 5 reports the BLASTx top hit species distribution of matches with known sequences and indicates that the majority of *P. lividus* contigs (reads) showed the highest homology with *Strongylocentrotus purpuratus* (18.6%). The other most represented species included *Exaiptasia pallida* (5.4%), *Acropora digitifera* (4.2%), *Crassostrea gigas* (2.2%), *Lingula anatina* (2.2%) and *Saccoglossus kowalevskii* (2.2%). All alignments were carried out setting the E-value thresholds at a value of $\leq$1e-5.

Differentially expressed genes in *P. lividus* plutei after feeding experiments (RNA-seq). Differentially expressed genes were identified between the three conditions: embryos at the pluteus stage spawned by adults fed for one month with *N. shiloi* and *C. closterium*, compared to those fed with *U. rigida* as control, with each diet including three biological replicates. Using the R Bioconductor package DESeq. 2, the number of differentially expressed (DE) genes were count in pairwise comparisons, discriminating those that were up and down regulated. The total number of DE genes were: (i) 1285 between plutei from sea urchins fed with *N. shiloi* compared to controls fed with *U. rigida*; (ii) 2386 between plutei from sea urchins fed with *C. closterium* compared to control; (iii) 303 between plutei from sea urchins fed with *N. shiloi* compared with those fed with *C. closterium*. For all these genes the following DE annotated genes were found, considering FDR $\leq$0.05 fold change $>$1.5 for up regulated and fold change <-1.5 for down regulated: (i) in the case of plutei spawned from sea urchins fed with *N. shiloi*, 217 total genes were identified, of which 113 were upregulated (with a range of fold-changes between 1.8 and 3.6) and 104 downregulated (with a range of fold-changes between -1.7 and -12) compared to the control. Of these, some genes showed very high values of fold changes, such as the two downregulated genes *sperm flagellar 2* and *centrosomal of 170 kDa B isoform X7*; (ii) in the case of plutei from sea urchins fed with *C. closterium*, 670 total DE genes were identified, of which 541 transcripts were upregulated (with a range of fold-changes between 1.7 and 20) and 129 downregulated (with a range of

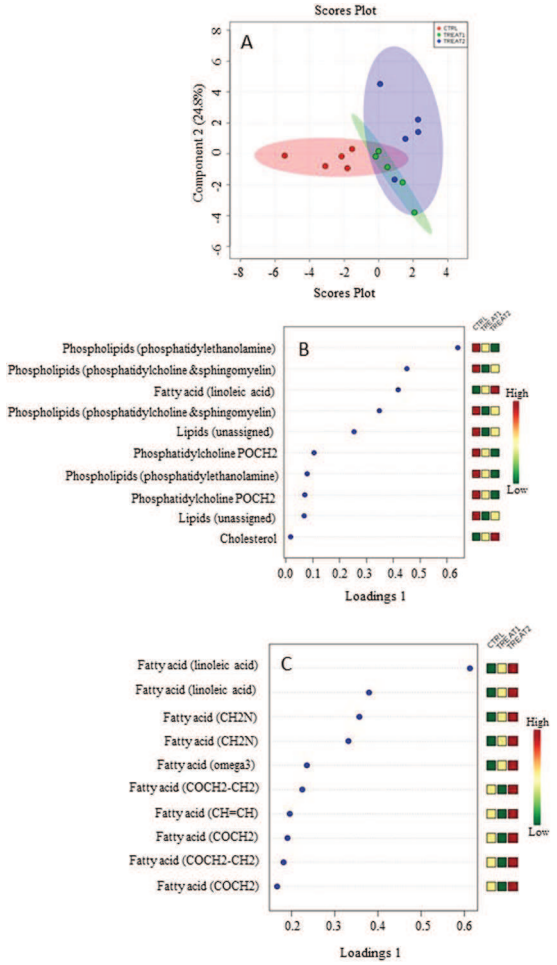

Figure 4. OPLS-DA (**A**) and Loading plots of (**B**) lipids and (**C**) fatty acids from lipophilic extracts of gonad tissues from adults sea urchin *P. lividus* after one month of feeding with *U. rigida* (used as feeding control, reported as CTRL), *N. shiloi* (reported as TREAT1) and *C. closterium* (reported as TREAT2).

fold-changes between −1.5 and −10). Also in this case, some genes showed very high values of fold changes: the downregulated genes *vacuolar sorting-associated 13C- partial*, *centromere W* and *uncharacterized protein LOC589705* and the upregulated genes *cleavage stimulation factor subunit 1*, *fibrocystin-L* and *cAMP-responsive element-binding -like 2*. Furthermore, comparing the two treatments with *N. shiloi* and *C. closterium*, 303 DE genes were found, of which 177 upregulated genes (with a range of fold-changes between −1.7 and −30) and 129 downregulated (with a range of fold-changes between −1.6 and −20). Two genes were strongly downregulated, *CD9 antigen* and *leukocyte elastase inhibitor-like isoform X3* and two strongly upregulated, *isocitrate dehydrogenase [NADP] mitochondrial-like* (35.8-fold) and *peptidyl-prolyl cis-trans isomerase E*.

To identify the pathways in which these genes were involved, a GO term enrichment analysis was performed using DE genes (Fig. 6). Twenty-seven GO terms were enriched including 12 in BP followed by 10 in CC and 5 in MF ($p < 0.05$). Overrepresented GO categories included binding, catalytic activity, cellular processes, metabolic processes and regulation of biological processes.

Comparison of *de novo* transcriptome of *P. lividus* with *S. purpuratus*. Since the majority of *P. lividus* contigs (reads) showed the highest homology with the sea urchin *S. purpuratus* (see Fig. 5), *de novo* transcriptome of *P. lividus* was aligned with that of *S. purpuratus* (available at http://www.spbase.org/SpBase/search/)[33]. In the case of plutei from sea urchins fed with *N. shiloi*, 18 DE genes were identified, of which 11 were upregulated (with a range of fold-changes between 1.5 and 2.2) and 7 downregulated (with a range of fold-changes between −1.5 and −1.8) compared to the control. In the case of plutei from sea urchins fed with *C. closterium*, 41 DE genes were identified, of which 17 were upregulated (with a range of fold-changes between 1.5 and 2.2) and 24 downregulated (with a range of fold-changes between −1.5 and −2.3).

Effects of *N. shiloi* and *C. closterium* on sea urchin embryos with Real time qPCR. Fifty genes, having key roles in different functional processes, were followed by Real Time qPCR (Supplementary Fig. S7)[11–13,34]. At the pluteus stage (48 hpf; Fig. 7, Supplementary Fig. S8 and Supplementary Table S4) *C. closterium* and *N. shiloi* had several common molecular targets.

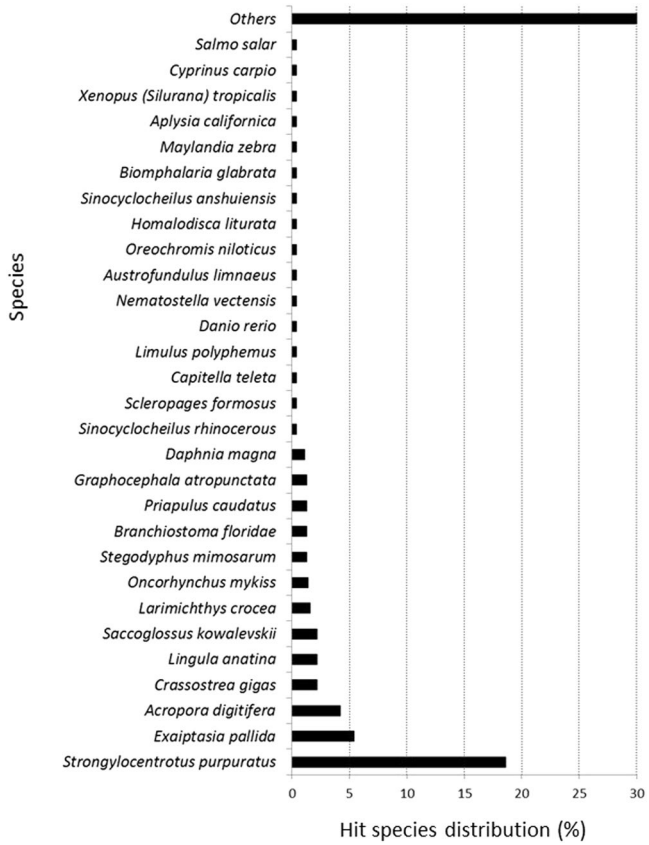

Figure 5. Blastx top hit species distribution of matches with known sequences.

- Stress: both benthic diatoms upregulated the expression levels of *hsp70*, *hsp60*, *GS*, *cytb*, *14–3–3ε* and downregulated *MTase*, *HIF1A* and *p53*. *C. closterium* and *N. shiloi* differentially affected the *hsp56* gene and *Nf-kB*. Moreover, *C. closterium* upregulated the genes *CASP8* whereas *N. shiloi* dowregulated *caspase 3/7* with respect to the control.
- Genes involved in skeletogenesis: both diatoms downregulated *SM30*, *BMP5–7* and *uni*; on the other hand, both species upregulated *Nec*, *p19* and *Jun*. Moreover, *C. closterium* increased the expression level of *SM50* gene.
- Development and differentiation: common molecular targets were *Blimp*, *Wnt6*, *nodal*, *FoxG*, *Foxo*, *One-Cut/Hnf6*, *FOXA*, *VEGF*, δ-2-catenin and *GFI-1*. These two diatoms differentially regulated other genes, as in the case *TCF7* and *TAK1* genes, upregulated by *C. closterium* and downregulated by *N. shiloi*. *C. closterium* also affected the expression levels of two other genes of this functional class, upregulating *sox9* and *Wnt8*.
- Detoxification: *C. closterium* and *N. shiloi* increased the expression levels of *MT*, *MT5*, *MT7*, *MDR1* and *CAT* and decreased that of *MT8*. Furthermore, the two diatoms differently expressed the *MT6* gene; *N. shiloi* also increased the expression level of *MT4*.

Discussion

Feeding experiments reported here revealed a noxious effect of two benthic diatom species (*C. closterium* and *N. shiloi*) on embryos spawned from adult sea urchins fed for one month on these diets. Embryos showed morphological malformations, affecting the arms, spicules and apex. These malformations were the same as those observed when sea urchin embryos were treated with some oxylipins, such as polyunsaturated aldehydes (PUAs, decadienal, heptadienal and octadienal) and hydroxyacids (5- and 15-HEPE), produced by planktonic diatoms[12,14]. These oxylipins have been shown to affect embryonic development in several marine invertebrates, including the sea urchin *P. lividus*[9,12–14,16]. Volatile organic compounds (VOCs), including PUAs, have been identified in several freshwater benthic diatoms, such as *Achnanthes biasolettiana*[35], *Gomphonema parvulum*, *Amphora veneta*, *Fragilaria* sp. and *Melosira varians*[36], but not much is known on the effects of these compounds on grazing invertebrates. Pezzolesi *et al.*[37] highlighted the production of PUAs in three diatoms commonly occurring in the microphytobenthic communities in temperate regions, *Tabularia affinis*, *Proschkinia complanatoides* and *Navicula* sp. The existence of a large family of PUAs, including some with four unsaturations, such as decatetraenal, undecatetraenal and tridecatetraenal, has been observed.

Feeding on these diatoms also increased the levels of some lipids in the gonads of *P. lividus*. In particular, we observed a strong increase in linoleic acid and cholesterol, compared to sea urchins fed an *Ulva* diet. Gonads of

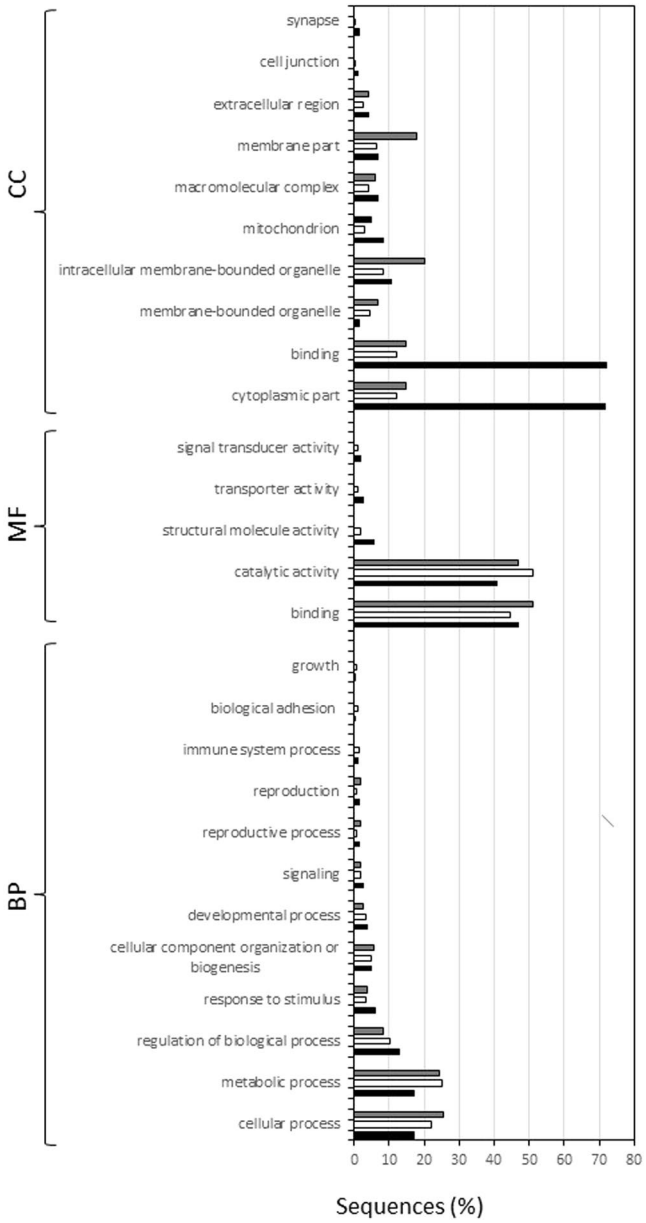

Figure 6. Overrepresented GO terms of sea urchin plutei after feeding experiments with the two benthic diatoms, *N. shiloi* and *C. closterium*, in comparison with *U. rigida* (feeding control), in the three major functional categories: biological process (BP), molecular function (MF) and cellular component (CC). The tree bars represent: black bar "control versus *N. shiloi*", white bar "control versus *C. closterium*" and grey bar "*N. shiloi* versus *C. closterium*".

this sea urchin are a rich source of PUFAs[38,39] and linoleic (18:2n-6) and α-linolenic (18:3n-3) acids are found in higher proportions in the gonads of *P. lividus*[40]. The increased lipids in *P. lividus* gonads in our experiments could be due to the diet they were fed upon since diatoms are known to be rich in lipids, mainly PUFAs, usually comprising up to 15–25% of dry biomass[41–43]. *C. closterium* is particularly rich in 16:0 and 16:1 (n-7) fatty acids that can comprise up to 64% of the total lipids[44]. These findings also indicate an increase in the aqueous (polar) phase of the amino acids tyrosine, proline, valine, isoleucine, leucine and lysine in the gonads of sea urchins fed with *C. closterium* with respect to those fed with *N. shiloi*. Lower levels of these amino acids were found in control gonads from adults fed with *U. rigida*. Sea urchin gonads are rich in essential amino acids such as phenylalanine, threonine, valine, lysine leucine, isoleucine and histidine (about 32.1% of total amino acids) which cannot be synthesized *de novo* by the organism and must thus be supplied by the diet. In addition, the level of acetoacetate increased in the gonads of adults fed with *C. closterium* with respect to *N. shiloi*. Diatoms, like other photoautotrophs synthesize a wide range of amino acids for building proteins and other compounds[45]. The increase in essential amino acids (valine, isoleucine, leucine and lysine) after feeding with both benthic diatoms could be ascribed to the two diatom diets. Diatoms are known to produce high levels of leucine and fairly abundant quantities of lysine[45,46]. Specific data on the amino acidic and proteic compositions of the two benthic diatoms used in

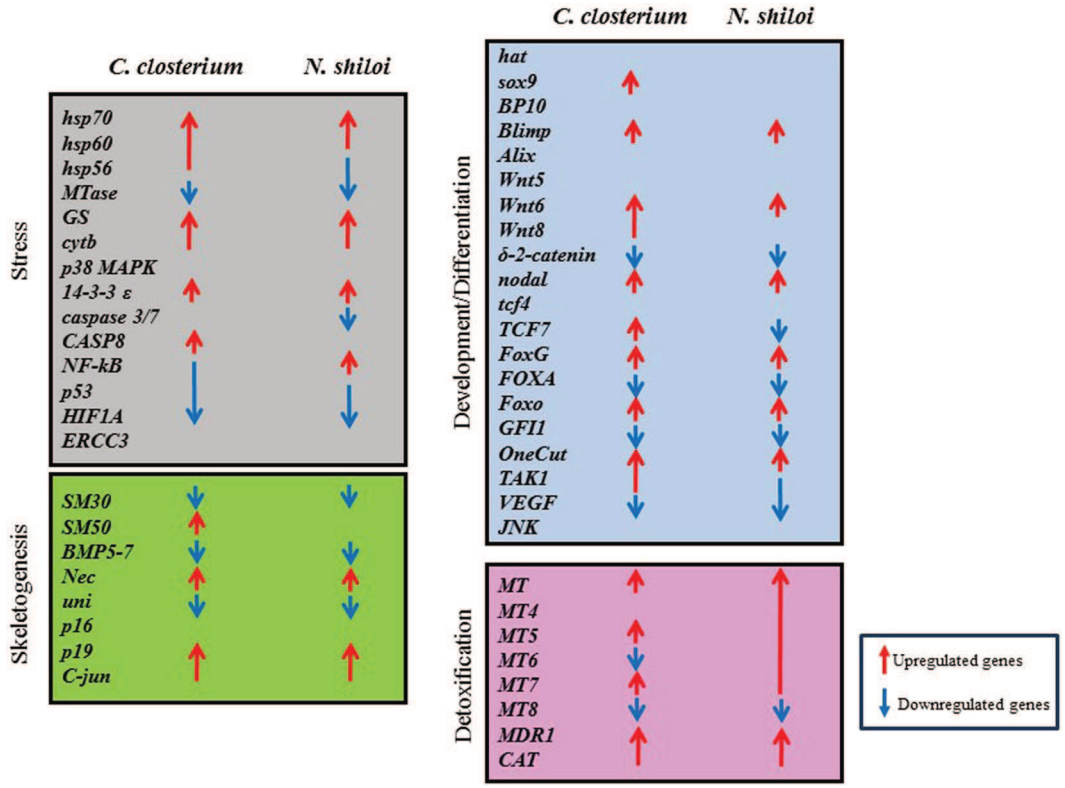

Figure 7. Synopsis of the patterns of up- and downregulation of different classes of genes in sea urchin embryos spawned from adults fed for one month with the two benthic diatoms *N. shiloi* and *C. closterium* (see also Supplementary Table S4 for the values and Supplementary Figs S7–S8).

this study are not available, but it is known that benthic diatoms belonging to the genus *Chylindrotheca* are rich in proteins[47]. To date, knowledge on proteins and amino acid composition of gonads, eggs and larvae of echinoids are scarce and the possibility to modify their profiles through diet manipulations is relatively unknown. For example, Gago *et al.*[48] found very few differences in the protein content of *P. lividus* eggs, prisms and pre-plutei and the amino acid composition of eggs from captive broodstock fed prepared diets or those obtained from wild broodstock. However, these results indicate that diet can significantly affect lipid, amino acid and protein levels of cultured sea urchins.

Another interesting result is the large-scale genomic information on *P. lividus* generated in this study. Analysis of differentially expressed (DE) genes indicated that metabolic, cellular, reproductive, developmental, immune system, biological regulation and response to stimuli and biological adhesion processes were all affected by benthic diatoms. The two diatoms targeted different genes and had a few common targets (Fig. 8 and Supplementary Table S5).

The interpretation of *de novo* transcriptomic results were also improved with the analysis by Real Time qPCR of a set of fifty genes, previously used to study the response of *P. lividus* embryos to oxylipins produced by planktonic diatoms. These genes have key roles in different functional processes such as stress response, skeletogenesis, embryonic development, cell differentiation, morphogenesis and detoxification processes (see Supplementary Fig. S7). New results show that all these genes were common molecular targets for *N. shiloi* and *C. closterium*, with the only exception of *p38 MAPK*, *ERRC3*, *hat*, *BP10*, *p16* and *Wnt5*. Some of these targeted genes were common to the genes from RNA-seq, confirming transcriptomic results (see Supplementary Fig. S9 for some examples). For example, using Real Time qPCR we found that *N. shiloi* upregulated the expression level of *FoxG* gene (see Supplementary Table S4). This gene was also upregulated by *N. shiloi* in the comparison of *de novo* transcriptome of *P. lividus* with *S. purpuratus*. Furthermore, Real Time qPCR revealed that *cytb*, *hsps60* and *hsp70* were upregulated by both *N. shiloi* and *C. closterium*. *De novo* transcriptome analysis showed that *metallotionein*, *cytochrome c*, *cytochrome P450* and several *heat shock proteins* (*heat shock cognate 71*, *DnaJ*, *heat shock partial*, *hsp90*) were also upregulated with both diatoms. The genes *catenin alpha-2* was down-regulated and *14-3-3 epsilon* upregulated in the *de novo* transcriptomic comparison between the two diatoms, and the data validated using Real Time qPCR.

All together these molecular results, supported by morphological findings, revealed that the majority of malformations affected the skeleton, the developmental plan and differentiation of sea urchin embryos. In fact, several genes belonging to the skeletogenic, developmental and differentiation classes were affected by the ingestion of benthic diatoms. Even if we did not observe differences between the two diatom diets at the morphological level, the molecular results (and mainly the *de novo* transcriptome) suggest that the toxic effect of feeding of *P. lividus* on the diatom *C. closterium* was higher than that of *N. shiloi*.

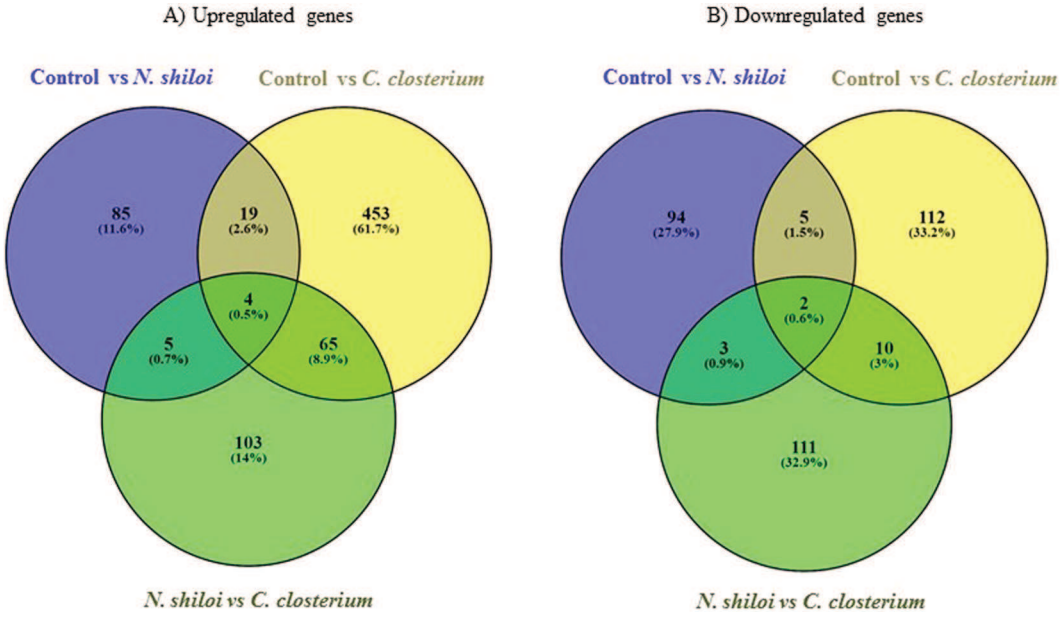

Figure 8. Venn diagrams considering upregulated genes and downregulated genes comparing the groups "control (*U. rigida*, feeding control) versus *N. shiloi*", "control versus *C. closterium*" and "*N. shiloi* versus *C. closterium*". *N. shiloi* and *C. closterium* induced an increase in the expression of 85 (11.6%) and 453 (61.7%) genes, respectively, compared to the control diet (*U. rigida*); they also induced the downregulation of 94 (27.9%) and 112 (33.2%) genes, respectively. The two diatoms had a few common targets (see also Supplementary Table S5 for the names of the common genes). In fact, in the case of up-regulated genes we found 19 common genes (2.6%) comparing the groups "control versus *N. shiloi*" and "control versus *C. closterium*"; 4 common genes (0.5%) comparing the groups "control versus *N. shiloi*", "control versus *C. closterium*" and "*N. shiloi* versus *C. closterium*"; 5 common genes (0.7%) "control versus *N. shiloi*" and "*N. shiloi* versus *C. closterium*"; 65 common genes (8.9%) comparing "control versus *C. closterium*" and "*N. shiloi* versus *C. closterium*". Considering the down-regulated genes, we found 5 common genes (1.5%) comparing the groups "control versus *N. shiloi*" and "control versus *C. closterium*"; 2 common genes (0.6%) comparing the groups "control versus *N. shiloi*", "control versus *C. closterium*" and "*N. shiloi* versus *C. closterium*"; 3 common genes (0.9%) "control versus *N. shiloi*" and "*N. shiloi* versus *C. closterium*"; 10 common genes (3.0%) comparing "control versus *C. closterium*" and "*N. shiloi* versus *C. closterium*".

This study is the first demonstration of the toxic effects of benthic epiphytic diatoms on embryos and larvae of the sea urchin *P. lividus* due to the feeding of adults during gonadal maturation. The effects may be considered comparable to those previously demonstrated in planktonic copepods fed on plankton diatoms[49]. Since there is few information available on chemical compounds from benthic diatoms, further studies are necessary to identify possible putative compounds responsible for the observed toxic effects on sea urchins. Preliminary investigations have been performed to test if these diatoms produce oxylipins, as in the case of planktonic species. GC-MS (Gas Chromatography-Mass Spectrometry) analysis revealed the presence of PUAs only for *N. shiloi* (data not shown). On the contrary, LC-MS (Liquid Chromatography Mass Spectrometry) profiles of methylated samples showed a high production of oxylipins, in particular hydroxy derivatives of eicosapentaenoic acid (EPA). In fact, MS spectra from both diatoms revealed the presence of two compounds: hydroxyeicosapentaenoic acids (HEPEs) and hydroxy-epoxy-eicosatetraenoic acids (HepETEs) (data not shown). In addition, both benthic diatoms showed some additional peaks that were not correlated to the canonic oxylipins commonly observed in planktonic diatoms, opening interesting perspectives of possibly finding new oxylipins or other compounds with possible cytotoxic effects.

Materials and Methods

Ethics Statement. *Paracentrotus lividus* (Lamarck) were collected from a site in the Bay of Naples that is not privately owned or protected in any way, according to the Italian legislation (DPR 1639/68, 09/19/1980 confirmed on 01/10/2000). Field studies did not include endangered or protected species. All experimental procedures on animals were in compliance with the guidelines of the European Union (Directive 609/86).

Isolation and morphological identification of two benthic diatoms. Epiphytes were isolated from leaves of *P. oceanica*, collected in Ischia, Naples (Italy) using a sterilized scalpel. Individual diatoms were aspirated by means of a Narishige syringe Syr-12, taking advantage of a Leica micromanipulator under inverted microscopy and transferred into 12-wells multi-wells in sterile seawater. The strains were collected and transferred daily to clean *f/2* medium until monoclonal cultures of the two benthic diatoms were obtained. Diatom cultures were grown in *f/2*medium (Sigma Guillard's) at 18 °C with a 12:12 photoperiod. Mother cultures of diatoms were transferred every 10 days in new multiwell plates.

Diatom samples from the mother culture were collected, fixed with 2.5% glutaraldehyde, filtered on cellulose Millipore filters and mounted on aluminum stubs for Scanning Electron Microscopy (SEM, Zeiss EVO MA LS). After three washings and treatment with osmium (1%), samples were dehydrated (25, 50, 75 and 100% ethanol) and coated with platinum for SEM observations and morphological identification.

Molecular characterization of benthic diatoms. Individual cell cultures were collected from the multi-well plates and concentrated by centrifugation for 20 minutes at 4500 rpm (revolution per minute) at 4 °C, then frozen in liquid nitrogen until use. Cell membranes were disrupted by lysis buffer containing Cetyltrimethylammonium bromide (CTAB) 2% and 2-Mercaptoethanol (2-ME, Sigma-Aldrich). RNAse was then added (final concentration 200 μg/ml) and digestion was performed at 65 °C for 45 minutes. Extraction with chloroform/isoamyl alcohol (24:1) was performed two times and 1 volume of ice-cold isopropanol (100%) was then added to the aqueous phase with glycogen for DNA precipitation at −20 °C overnight. After centrifugation, DNA was washed with 75% ethanol, centrifuged for 15 minutes, and air-dried. DNA was suspended in 20 μl sterile water. The amount of total DNA extracted was estimated by measuring the absorbance at 260 nm; purity was calculated using 260/280 and 260/230 nm ratios, using a NanoDrop spectrophotometer (ND-1000 UV-vis Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). The integrity of DNA was evaluated by agarose gel electrophoresis.

PCR was performed with specific primers for 18S rRNA (528 F and 1055R[50,51]; see Supplementary Table S1). Additional information is reported in the Supplementary Information file.

Preparation of cultures. To produce sufficient biomass of the two benthic diatoms, cultures were prepared (using as starting material the monoclonal mother-cultures) in 17-cm glass Petri dishes[22] and grown for one week. These cultures were then collected by aspiration by means of a sterile Pasteur pipette, and used to prepare massive cultures on a 2% vegetal substrate agar, which is harmless for sea urchins[52]. These massive cultures were grown for one week in a thermostatic chamber, up to the exponential growth phase to facilitate an even diffusion of the diatoms on the whole substrate. When agar massive cultures were inoculated, three glass slides were also deployed on the surface of the agar substratum and, at the end of the exponential phase (1 week), cells grown on the glass slides were counted under an inverted microscope using an ocular micrometer (20×). Based on these counts, the biomass of diatoms fed to sea urchins was then calculated as logC (quantity of intracellular carbon in picograms) $= -0.541 + 0.811 \times \log V$ (cell volume in μm^3)[32].

Feeding experiments, fertilization and morphological analysis of embryos. Twenty adult (12 females and 8 males) *P. lividus* were reared in each experimental tank of a continuous flow-through system (see Supplementary Information file for further details on the continuous flow-through system) and fed with *Ulva rigida* (3 control replicates) and the two benthic diatoms tested (3 replicates for each species). The daily amount of food (both *Ulva rigida* and the agar substrate incorporating the diatoms) given to sea urchins was 1 gram per sea urchin. After one month of feeding, eggs and sperms were collected from fed sea urchins. Eggs from each female were washed with filtered seawater (FSW) and kept in FSW until use. Concentrated 'dry' sperm was collected and kept undiluted at +4 °C until use. Eggs were fertilized utilizing sperm-to-egg ratios of 100:1. Fertilized eggs were kept at 20 °C in a controlled temperature chamber on a 12 h:12 h light:dark cycle. After 48 hours post-fertilization, morphological malformations in sea urchin *plutei* were determined for at least 100 plutei from each female (fixed in formaldehyde 4% in FSW) using a light microscope (ZeissAxiovert 135TV, Carl Zeiss, Jena, Germany). Statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA).

Fecal pellets were collected in the tanks during the feeding experiments, concentrated and processed for SEM observations (as described above) in order to detect and confirm the presence of diatoms ingested.

Embryo collection, RNA extraction, *de novo* transcriptome assembly and Real Time qPCR. After the feeding experiments on adults, about 5000 eggs (in 50 mL of FSW) from each female fed on *U. rigida* and on the two benthic diatoms were collected and fertilized. Embryos were then collected 48 hours post-fertilization (hpf) by centrifugation at 1800 relative centrifugal force (rcf) for 10 minutes in a swing out rotor at 4 °C. Embryos were placed in at least 10 volumes of the RNA*later*®, an RNA Stabilization Reagent (Qiagen, Hilden, Germany), and then frozen in liquid nitrogen and kept at −80 °C.

Total RNA was extracted using Aurum™ Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions[53] for RNA-seq experiments. Total RNA was extracted using RNAqueous Micro Kit (Ambion from Life Technologies), according to the manufacturer's instructions for Real Time qPCR experiments[53]. Samples were stored at −80 °C. For each sample, 600 ng of total RNA extracted was retrotranscribed with an iScript™ cDNA Synthesis kit (Bio-Rad, Milan, Italy), following the manufacturer's instructions.

RNA sequencing was performed (Genomix4Life Srl, Salerno, Italy) on nine samples, grouped in three experimental conditions: Control (*U. rigida*), 1st treatment (*N. shiloi*) and 2nd treatment (*C. closterium*), each one composed of three biological replicates.

Additional Information on preparation of cDNA libraries and raw assembled transcriptome are reported in Supplementary Information file, including Supplementary Figs S1–S4. The full dataset of raw data has been deposited in the SRA database (accession number: SUB2817153).

The expression levels of each gene by Real Time qPCR were analyzed and internally normalized against the control gene for *Pl-Z12-1*[54] using REST software (Relative Expression Software Tool, Weihenstephan, Germany) based on the Pfaffl method[55,56]. Variation of expression levels were calculated as relative expression ratios of the analyzed genes with respect to control embryos. Only expression levels greater than 1.5-fold with respect to

controls were considered significant. Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). See Supplementary Information for further details.

^{1}H-NMR Metabolomic analysis of the gonads. For each treatment gonad tissues were collected from five adult sea urchins after one month of feeding and stored at −20 °C. Gonads were re-suspended in 170 µl of H_2O and 700 µl of methanol and were sonicated for 30 sec. Then, 350 µl of chloroform were added and samples were mixed on an orbital shaker in ice for 10 min. 350 µl of H_2O/chloroform (1:1, v/v) were added to each cell suspension and centrifuged at 10000 rpm for 10 min at 4 °C. Thereafter, the aqueous (polar) and lipophilic (apolar) phases were collected separately, transferred to a glass vial and dried under nitrogen flow. Samples were analyzed using Nuclear Magnetic Resonance (NMR). The polar fractions were dissolved in 630 µl of PBS-D_2O with the pH adjusted to 7.20, and 70 µl of sodium salt of 3-(trimethylsilyl)-1-propanesulfonic acid (1% in D_2O) was used as the internal standard. On the other hand the lipophilic fractions were dissolved in 700 µl of deuterated chloroform. A 600-MHz Bruker Avance DRX spectrometer with a TCI probe was used to acquire ^{1}H spectra on the cellular polar fractions. All ^{1}H-NMR spectra were acquired at 300 K with the excitation sculpting pulse sequence to suppress water resonance. A double-pulsed field gradient echo was used, with a soft square pulse of 4 ms at the water resonance frequency and with gradient pulses of 1 ms duration, adding 128 transients of 64 k complex points, with an acquisition time of 4 s/transient. Time domain data were all zero-filled to 256 k complex points and an exponential amplification of 0.6 Hz was applied prior to Fourier transformation.

The assignments were based on the comparison of chemical shifts and spin-spin couplings with reference spectra present in the human metabolome database (HMDB)[57] and the Biological Magnetic Resonance Database (BMRB)[58].

Statistical and Pathway Analysis. The 0.50–8.60 ppm spectral region of the ^{1}H-NMR spectra was integrated in buckets of 0.04 ppm using the AMIX package (Bruker, Biospin GmbH, Rheinstetten, Germany). The water resonance region (4.5–5.2 ppm) was excluded during the analysis and the bucketed region was normalized to the total spectrum area using Pareto scaling. Orthogonal Projections to Latent Structures discriminant analysis (OPLS-DA) was used to compare the spectra obtained on the polar and apolar phases obtained from gonad tissues after feeding treatments.

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Author Contributions

N.R., V.Z., M.C. designed research; N.R. performed experiments of the research project; C.L. contributed to the molecular identification of benthic diatoms; D.C., V.Z. contributed to the design of the continuous flow-through system; S.C. performed metabolic analysis; M.C., A.I., S.C., A.B., V.Z. contributed new reagents or analytical tools; G.I., G.N., A.F. contributed for chemical experiments; N.R., A.I., G.R., V.Z., M.C. analyzed data; all the authors contributed to wrote the paper.

Additional Information

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