

# SCIENTIFIC REPORTS



OPEN

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

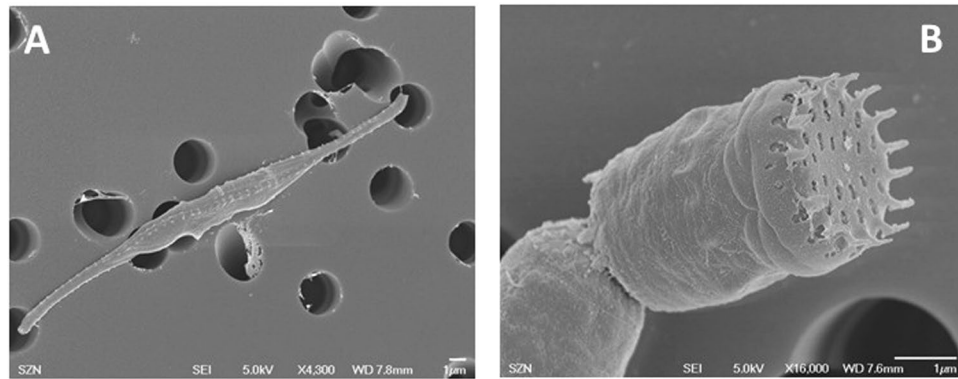
Nadia Ruocco<sup>1,2,3</sup>, Susan Costantini<sup>4</sup>, Valerio Zupo<sup>5</sup>, Chiara Lauritano<sup>6</sup>, Davide Caramiello<sup>7</sup>, Adrianna Ianora<sup>6</sup>, Alfredo Budillon<sup>4</sup>, Giovanna Romano<sup>6</sup>, Genoveffa Nuzzo<sup>3</sup>, Giuliana D'Ippolito<sup>3</sup>, Angelo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

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<sup>1</sup>. 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<sup>2</sup>. 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<sup>3</sup>.

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

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



**Figure 1.** Scanning electron micrographs (SEM) of (A) *C. closterium* and (B) *N. shiloi* isolates. Scale bar = 1  $\mu\text{m}$ .

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

In contrast to the chemical ecology of planktonic diatoms that is well documented<sup>20,21</sup>, 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<sup>22</sup>. 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<sup>23,24</sup>.

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<sup>25–27</sup>. Other benthic diatoms produce volatile organic compounds (VOCs) similar to those produced by planktonic diatoms that induce behavioural changes in several species of macroinvertebrates<sup>28</sup>, but studies on the metabolic effects of fast-growing benthic diatoms on benthic invertebrates are still largely lacking<sup>29–31</sup>.

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 <sup>1</sup>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

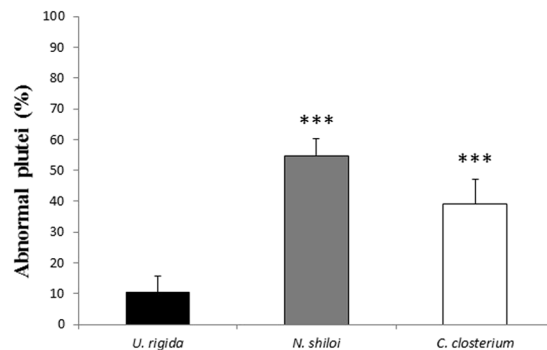
**Morphological and molecular characterization of benthic diatoms.** SEM observation revealed that the first diatom isolated was about 50  $\mu\text{m}$  in length, needle like and thin in shape, the two ends of the cell extended far from the centre of the cell; the cells showed spiral twist of the raphe 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  $\text{pg C cell}^{-1}$  for *C. closterium* and 1.8  $\text{pg C cell}^{-1}$  for *N. shiloi*<sup>32</sup>.

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	<i>U. rigida</i>	<i>C. closterium</i>	<i>N. shiloi</i>
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).

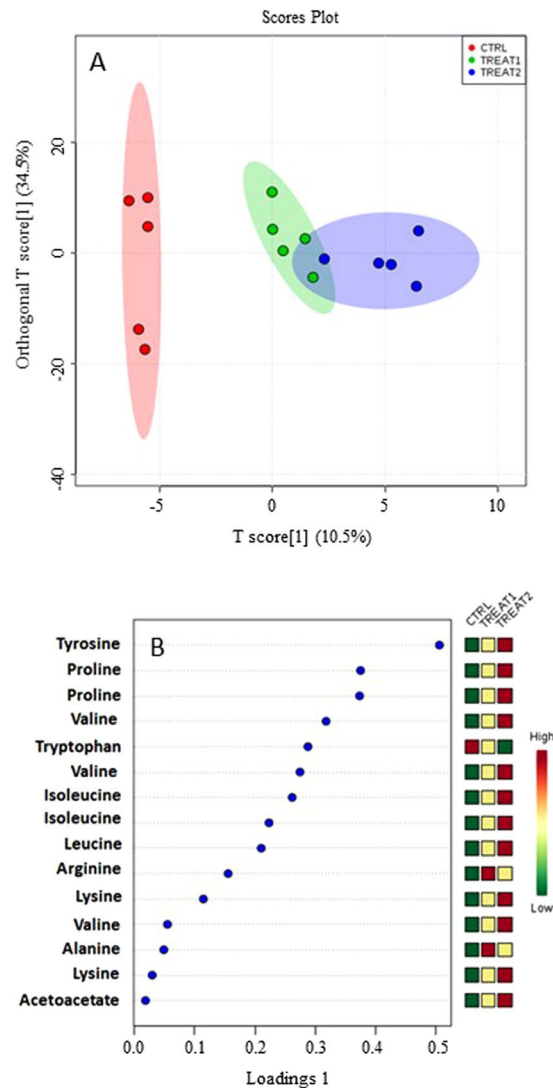
**<sup>1</sup>H-NMR analysis of metabolites and lipids from sea urchin gonads.** <sup>1</sup>H-NMR spectra were obtained from aqueous extracts of gonad tissues from five adult sea urchins fed with *U. rigida* (control), *N. shiloi* and *C. closterium*. The primary peaks in the spectra were assigned to 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.

<sup>1</sup>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<sub>2</sub> 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.

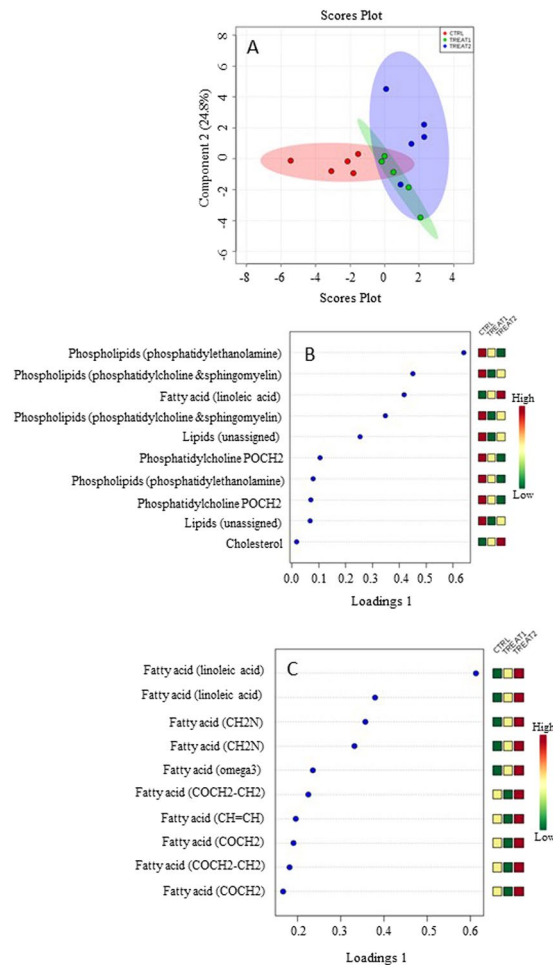
**Transcriptomic assembly.** The raw assembled transcriptome included almost 121 Mbp in 192493 transcripts grouped in 126941 genes. The mean GC content was 41.37%. The average and median contig lengths were 357 bp and 627 bp, respectively. The N50 was 920 bp. All analyses were conducted on *de-novo* 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 as 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 DESeq2, the number of differentially expressed (DE) genes were counted 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 170kDa 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



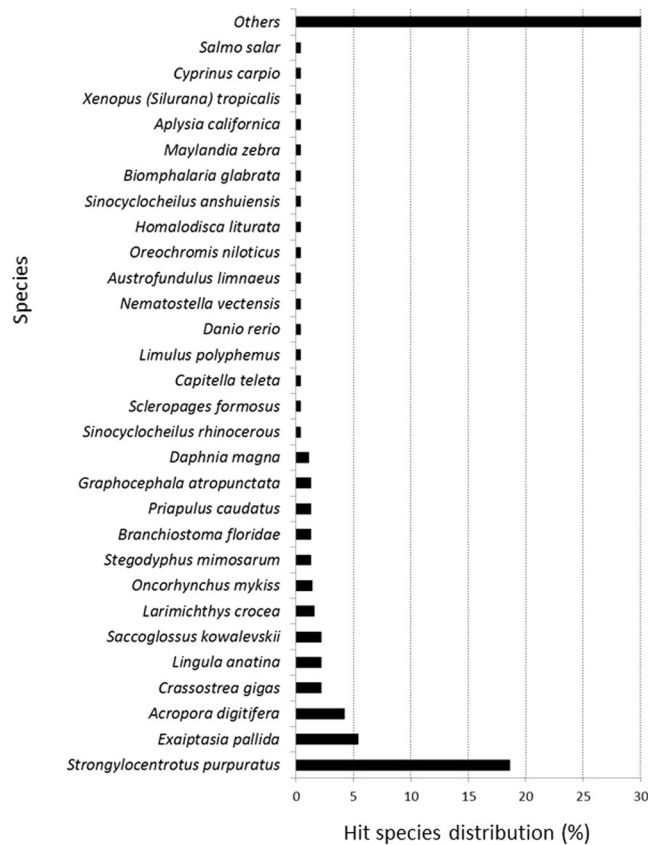
**Figure 4.** OPLS-DA (A) and Loading plots of (B) lipids and (C) fatty acids from lipophilic extracts of gonad tissues from adults sea urchin *P. lividus* after one month of feeding with *U. rigida* (used as feeding control, reported as CTRL), *N. shiloi* (reported as TREAT1) and *C. closterium* (reported as TREAT2).

fold-changes between  $-1.5$  and  $-10$ ). Also in this case, some genes showed very high values of fold changes: the down-regulated 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/>)<sup>33</sup>. 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 down-regulated (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)<sup>11-13,34</sup>. 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.



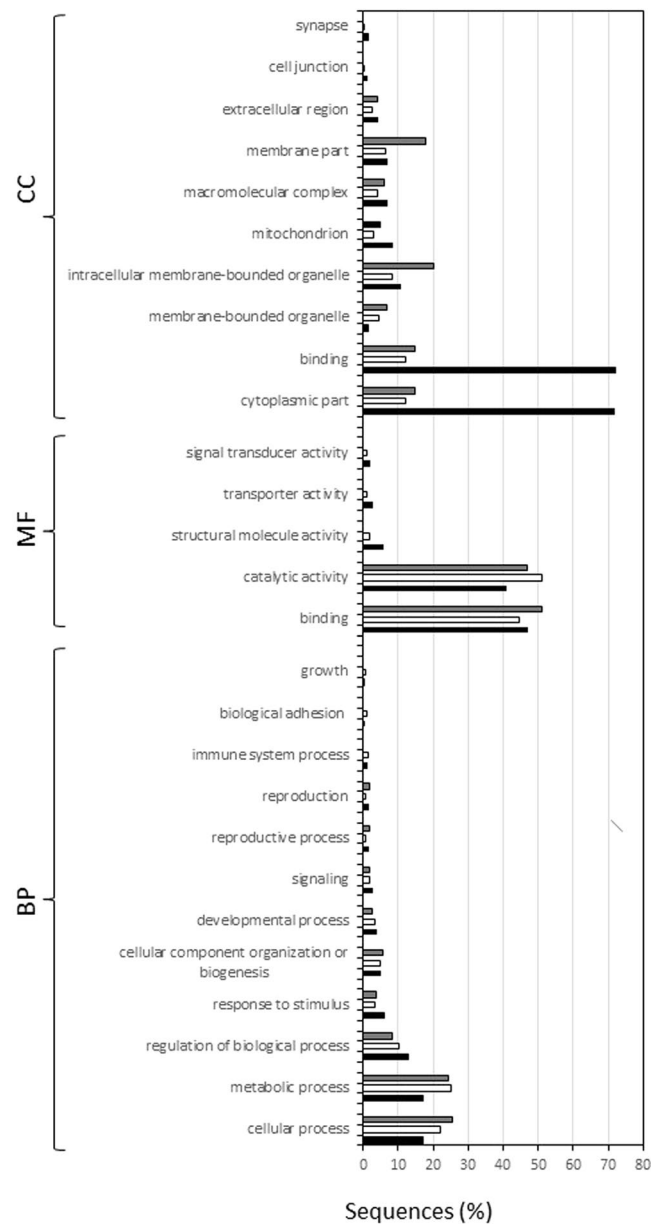
**Figure 5.** Blastx top hit species distribution of matches with known sequences.

- **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* downregulated *caspace 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*,  $\delta$ -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

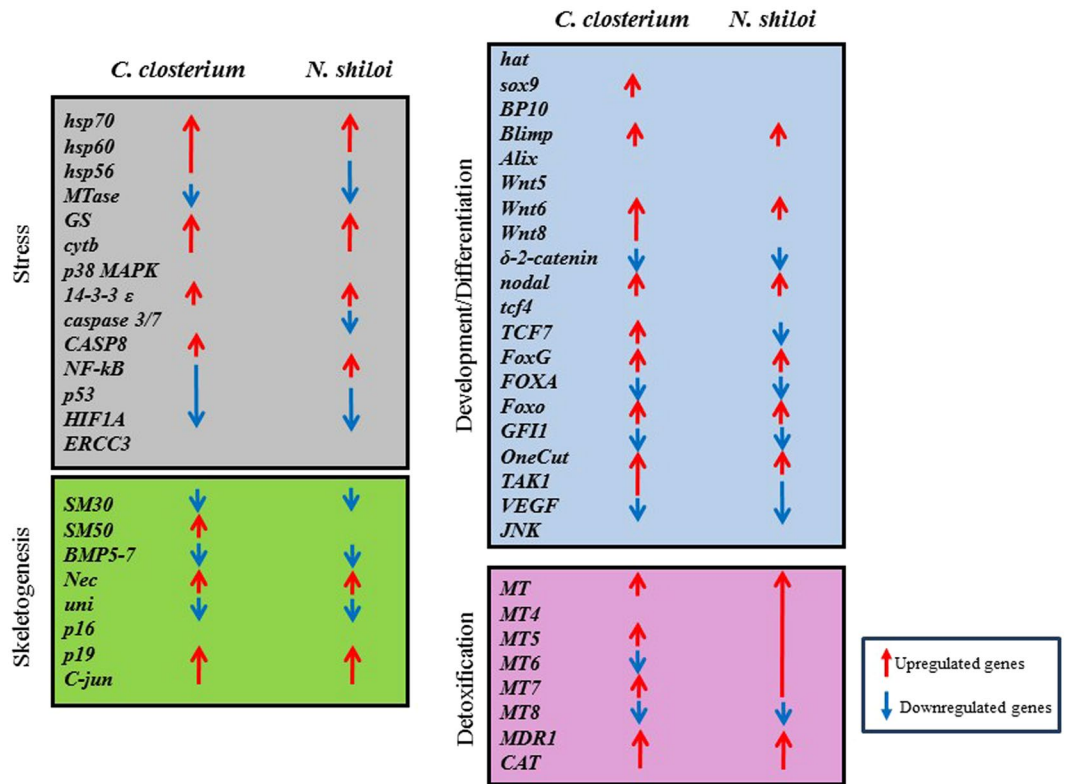
Feeding experiments reported here revealed a noxious effect of two benthic diatom species (*C. closterium* and *N. shiloi*) on embryos spawned from adult sea urchins fed for one month on these diets. Embryos showed morphological malformations, affecting the arms, spicules and apex. These malformations 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<sup>12,14</sup>. These oxylipins have been shown to affect embryonic development in several marine invertebrates, including the sea urchin *P. lividus*<sup>9,12-14,16</sup>. Volatile organic compounds (VOCs), including PUAs, have been identified in several freshwater benthic diatoms, such as *Achnanthes biasoletiana*<sup>35</sup>, *Gomphonema parvulum*, *Amphora veneta*, *Fragilaria* sp. and *Melosira varians*<sup>36</sup>, but not much is known on the effects of these compounds on grazing invertebrates. Pezolesi *et al.*<sup>37</sup> highlighted the production of PUAs in three diatoms commonly occurring in the microphytobenthic communities in temperate regions, *Tabularia affinis*, *Proschkinia complanatoidea* 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<sup>38,39</sup> and linoleic (18:2n-6) and  $\alpha$ -linolenic (18:3n-3) acids are found in higher proportions in the gonads of *P. lividus*<sup>40</sup>. 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<sup>41–43</sup>. *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<sup>44</sup>. 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<sup>45</sup>. 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<sup>45,46</sup>. 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).

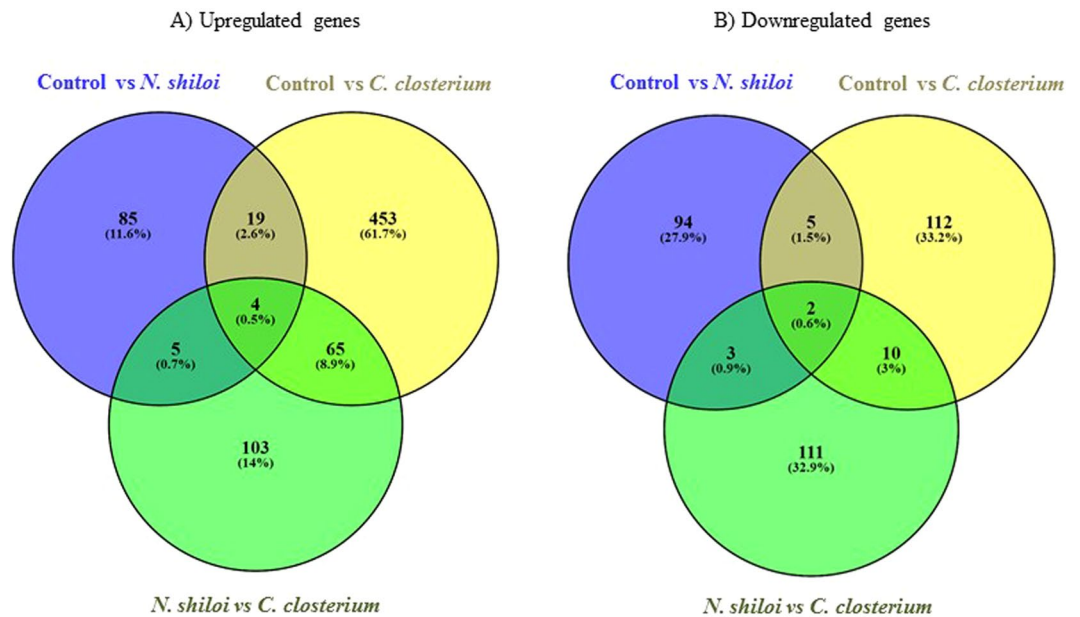
this study are not available, but it is known that benthic diatoms belonging to the genus *Chylindrotheca* are rich in proteins<sup>47</sup>. To date, knowledge on proteins and amino acid composition of gonads, eggs and larvae of echinoids are scarce and the possibility to modify their profiles through diet manipulations is relatively unknown. For example, Gago *et al.*<sup>48</sup> 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*, *hsp60* and *hsp70* were upregulated by both *N. shiloi* and *C. closterium*. *De novo* transcriptome analysis showed that *metallothionein*, *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<sup>49</sup>. 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<sup>50,51</sup>; 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<sup>22</sup> 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<sup>32</sup>. 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  $\log C$  (quantity of intracellular carbon in picograms) =  $-0.541 + 0.811 \times \log V$  (cell volume in  $\mu\text{m}^3$ )<sup>32</sup>.

**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 RNAlater<sup>®</sup>, an RNA Stabilization Reagent (Qiagen, Hilden, Germany), and then frozen in liquid nitrogen and kept at –80 °C.

Total RNA was extracted using Aurum<sup>™</sup> Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions<sup>53</sup> 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<sup>53</sup>. Samples were stored at –80 °C. For each sample, 600 ng of total RNA extracted was retrotranscribed with an iScript<sup>™</sup> 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*), 1<sup>st</sup> treatment (*N. shiloi*) and 2<sup>nd</sup> 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*<sup>54</sup> using REST software (Relative Expression Software Tool, Weihenstephan, Germany) based on the Pfaffl method<sup>55,56</sup>. 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.

**<sup>1</sup>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  $-20^{\circ}\text{C}$ . Gonads were re-suspended in  $170\ \mu\text{l}$  of  $\text{H}_2\text{O}$  and  $700\ \mu\text{l}$  of methanol and were sonicated for 30 sec. Then,  $350\ \mu\text{l}$  of chloroform were added and samples were mixed on an orbital shaker in ice for 10 min.  $350\ \mu\text{l}$  of  $\text{H}_2\text{O}$ /chloroform (1:1, v/v) were added to each cell suspension and centrifuged at 10000 rpm for 10 min at  $4^{\circ}\text{C}$ . 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  $630\ \mu\text{l}$  of  $\text{PBS-D}_2\text{O}$  with the pH adjusted to 7.20, and  $70\ \mu\text{l}$  of sodium salt of 3-(trimethylsilyl)-1-propanesulfonic acid (1% in  $\text{D}_2\text{O}$ ) was used as the internal standard. On the other hand the lipophilic fractions were dissolved in  $700\ \mu\text{l}$  of deuterated chloroform. A 600-MHz Bruker Avance DRX spectrometer with a TCI probe was used to acquire <sup>1</sup>H spectra on the cellular polar fractions. All <sup>1</sup>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)<sup>57</sup> and the Biological Magnetic Resonance Database (BMRB)<sup>58</sup>.

**Statistical and Pathway Analysis.** The 0.50–8.60 ppm spectral region of the <sup>1</sup>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.

## References

- Ehrlich, H. *et al.* Multiphase biomineralization: enigmatic invasive siliceous diatoms produce crystalline calcite. *Adv. Funct. Mater.* **8**, 2503–2510 (2016).
- Reinfelder, J. R., Kraepiel, A. M. L. & Morel, F. M. M. Unicellular C4 photosynthesis in a marine diatom. *Nature* **407**, 996–999 (2000).
- Vilmi, A., Karjalainen, S. M., Landeiro, V. L. & Heino, J. Freshwater diatoms as environmental indicators: evaluating the effects of eutrophication using species morphology and biological indices. *Environ. Monit. Assess.* **187**, 243 (2015).
- Fontana, A. *et al.* Chemistry of oxylipin pathways in marine diatoms. *Pure Appl. Chem.* **79**, 481–490 (2007a).
- Cutignano, A. *et al.* Lipoygenase products in marine diatoms: a concise analytical method to explore the functional potential of oxylipins. *J. Phycol.* **47**, 233–243 (2011).
- d'Ippolito, G. *et al.* 15S-Lipoygenase metabolism in the marine diatom *Pseudo-nitzschia delicatissima*. *New Phytol.* **183**, 1064–1071 (2009).
- Gallina, A., Palumbo, A. & Casotti, R. Oxidative pathways in response to polyunsaturated aldehydes in the marine diatom *Skeletonema marinoi* (Bacillariophyceae). *J. Phycol.* **52**, 590–598 (2016).
- Miralto, A. *et al.* The insidious effect of diatoms on copepod reproduction. *Nature*. **402**, 173–176 (1999).
- Lauritano, C. *et al.* New oxylipins produced at the end of a diatom bloom and their effects on copepod reproductive success and gene expression levels. *Harmful Algae*. **55**, 221–229 (2016).
- Romano, G., Miralto, A. & Ianora, A. Teratogenic effects of diatom metabolites on sea urchin *Paracentrotus lividus* embryos. *Mar. Drugs*. **8**, 950–967 (2010).
- Romano, G., Costantini, M., Buttino, I., Ianora, A. & Palumbo, A. Nitric oxide mediates the stress response induced by diatom aldehydes in the sea urchin *Paracentrotus lividus*. *PLoS One*. **6**, e25980 (2011).
- Varrella, S. *et al.* Molecular response to toxic diatom-derived aldehydes in the sea urchin *Paracentrotus lividus*. *Mar. Drugs*. **12**, 2089–2113 (2014).
- Varrella, S. *et al.* Toxic diatom aldehydes affect defence gene networks in sea urchins. *PLoS One*. **11**, e0149734 (2016a).
- Varrella, S. *et al.* First morphological and molecular evidence of the negative impact of diatom-derived hydroxyacids on the sea urchin *Paracentrotus lividus*. *Toxicol. Sci.* **151**, 419–433 (2016b).
- Ruocco, N. *et al.* Diatom-derived oxylipins induce cell death in sea urchin embryos activating *caspase-8* and *caspase 3/7*. *Aquat. Toxicol.* **176**, 128–140 (2016).
- Caldwell, G. S. The influence of bioactive oxylipins from marine diatoms on invertebrate reproduction and development. *Mar. Drugs*. **7**, 367–400 (2009).
- Simon, C. A., Bentley, M. G. & Caldwell, G. S. 2,4-Decadienal: Exploring a novel approach for the control of polychaete pests on cultured abalone. *Aquaculture*. **310**, 52–60 (2010).
- Lettieri, A., Esposito, R., Ianora, A. & Spagnuolo, A. *Ciona intestinalis* as a marine model system to study some key developmental genes targeted by the diatom-derived aldehyde decadienal. *Mar. Drugs*. **13**, 1451–1465 (2015).
- Sansone, C. *et al.* Diatom-derived polyunsaturated aldehydes activate cell death in human cancer cell lines but not normal cells. *PLoS One* **9**, e101220 (2014).
- Pohnert, G. & Boland, W. The oxylipin chemistry of attraction and defense in brown algae and diatoms. *Nat. Prod. Rep.* **19**, 108–122 (2002).
- Pohnert, G. Diatom/copepod interactions in plankton: the indirect chemical defense of unicellular algae. *ChemBiochem.* **6**, 946–959 (2005).
- Raniello, R., Iannicelli, M. M., Nappo, M., Avila, C. & Zupo, V. Production of *Cocconeis neothumensis* (Bacillariophyceae) biomass in batch cultures and bioreactors for biotechnological applications: light and nutrient requirements. *J. Appl. Phycol.* **19**, 383–391 (2007).
- Whitton, B. A., Ellwood, N. T. W. & Kawecka, B. Biology of the freshwater diatom *Didymosphenia*: a review. *Hydrobiologia* **630**, 1–37 (2009).
- Taylor, B. W. & Bothwell, M. L. The origin of invasive microorganisms matters for science, policy, and management: the case of *Didymosphenia geminata*. *BioScience* **64**, 531–538 (2014).
- Zupo, V., Messina, P., Carcaterra, A., Aflalo, E. D. & Sagi, A. Experimental evidence of a sex reversal process in the shrimp *Hippolyte inermis*. *Invertebr. Reprod. Dev.* **52**, 93–100 (2008).

26. Zupo, V. & Messina, P. How do dietary diatoms cause the sex reversal of the shrimp *Hippolyte inermis* Leach (Crustacea, Decapoda). *Mar. Biol.* **151**, 907–917 (2007).
27. Zupo, V. Effect of microalgal food on the sex reversal of *Hippolyte inermis* (Crustacea: Decapoda). *Mar. Ecol. Progr. Ser.* **201**, 251–259 (2000).
28. Maibam, C. *et al.* Ecological relevance of wound-activated compounds produced by diatoms as toxins and infochemicals for benthic invertebrates. *Mar. Biol.* **161**, 1639–1652 (2014).
29. Crenier, C. *et al.* Minor food sources can play a major role in secondary production in detritus-based ecosystems. *Freshw. Biol.* **62**, 1155–1167 (2017).
30. Shimeta, J., Cutajar, J., Watson, M. G. & Vlamis, T. Influences of biofilm-associated ciliates on the settlement of marine invertebrate larvae. *Mar. Ecol. Progr. Ser.* **449**, 1–12 (2012).
31. Jüttner, F., Messina, P., Patalano, C. & Zupo, V. Odour compounds of the diatom *Cocconeis scutellum*: effects on benthic herbivores living on *Posidonia oceanica*. *Mar. Ecol. Progr. Ser.* **400**, 63–73 (2010).
32. Menden-Deuer, S. & Lessard, E. J. Carbon to volume relationships for dinoflagellates, diatoms, and other protist plankton. *Limnol. Oceanogr.* **45**, 569–579 (2000).
33. Cameron, R. A., Samanta, M., Yuan, A., He, D. & Davidson, E. SpBase: the sea urchin genome database and web site. *Nucleic Acids Res.* **37**, D750–D754 (2009).
34. Ruocco, N. *et al.* New inter-correlated genes targeted by diatom-derived polyunsaturated aldehydes in the sea urchin *Paracentrotus lividus*. *Ecotoxicol. Environ. Safety.* **142**, 355–362 (2017b).
35. Fink, P., Von Elert, E. & Jüttner, F. Oxylipins from freshwater diatoms act as attractants for a benthic herbivore. *Archiv für Hydrobiol.* **167**, 561–574 (2006).
36. Wendel, T. & Jüttner, F. Lipoygenase-mediated formation of hydrocarbons and unsaturated aldehydes in freshwater diatoms. *Phytochem.* **41**, 1445–1449 (1996).
37. Pezzolesi, L., Pichierrri, S., Samori, C., Totti, C. & Pistocchi, R. PUFAs and PUAs production in three benthic diatoms from the northern Adriatic Sea. *Phytochem.* **142**, 85–91 (2017).
38. Archana, A. & Babu, K. R. Nutrient composition and antioxidant activity of gonads of sea urchin *Stomopneustes variolaris*. *Food Chem.* **197**, 597–602 (2016).
39. Kabeya, N. *et al.* Biosynthesis of polyunsaturated fatty acids in sea urchins: molecular and functional characterisation of three fatty acyl desaturases from *Paracentrotus lividus* (Lamarck 1816). *PLoS One.* **12**, e0169374 (2017).
40. Martínez-Pita, I., García, F. J. & Pita, M. L. The effect of seasonality on gonad fatty acids of the sea urchins *Paracentrotus lividus* and *Arbacia lixula* (echinodermata: echinoidea). *J. Shellfish Res.* **29**, 517–525 (2010).
41. Levitan, O., Dinamarca, J., Hochman, G. & Falkowski, P. G. Diatoms: a fossil fuel of the future. *Trends Biotechnol.* **32**, 117–124 (2014).
42. Mangas-Sanchez, J. & Adlercreutz, P. Highly efficient enzymatic biodiesel production promoted by particle-induced emulsification. *Biotechnol. Biofuels.* **8**, 58 (2015).
43. Yi, Z., Xu, M., Di, X., Brynjolfsson, S. & Fu, W. Exploring valuable lipids in diatoms. *Front. Mar. Sci.* **4**, 17 (2017).
44. Song, C. *et al.* Biodiesel production process from microalgae oil by waste heat recovery and process integration. *Bioresour. Technol.* **193**, 192–199 (2015).
45. Bromke, M. A. Amino acid biosynthesis pathways in diatoms. *Metabolites.* **3**, 294–311 (2013).
46. Werner, D. *The Biology of diatoms.* University of California Press: Berkeley and New York, vol 24, 200 pp. (1977).
47. Brown, M. R. & Jeffrey, S. W. J. The amino acid and gross composition of marine diatoms potentially useful for mariculture. *J. Appl. Phycol.* **7**, 521–527 (1995).
48. Gago, J., Martins, T. & Luis, O. J. Protein content and amino acid composition of eggs and endotrophic larvae from wild and captive fed sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea). *Aquacult. Res.* **47**, 114–127 (2016).
49. Ianora, A. *et al.* Aldehyde suppression of copepod recruitment in blooms of a ubiquitous planktonic diatom. *Nature.* **429**, 403–407 (2004).
50. Elwood, H. J., Olsen, G. J. & Sogin, M. L. The small-subunit ribosomal RNA gene sequences from the hypotrichous ciliates *Oxytricha nova* and *Stylonychia pustulata*. *Mol. Biol. Evol.* **2**, 399–410 (1985).
51. Kooistra, W. H. C. F., De Stefano, M., Mann, D. G., Salma, N. & Medlin, L. K. Phylogenetic position of *Toxarium*, a pennate-like lineage within centric diatoms (Bacillariophyceae). *J. Phycol.* **39**, 185–197 (2003).
52. Fabbrocini, A. *et al.* Agar-based pellet as feed for sea urchins (*Paracentrotus lividus*): rheological behaviour, digestive enzymes and gonad growth. *Aquacult. Res.* **43**, 32–331 (2012).
53. Ruocco, N. *et al.* High-quality RNA extraction from the sea urchin *Paracentrotus lividus* embryos. *PLoS One.* **12**, e0172171 (2017a).
54. Costa, C., Karakostis, K., Zito, F. & Matranga, V. Phylogenetic analysis and expression patterns of *p16* and *p19* in *Paracentrotus lividus* embryos. *Dev. Genes Evol.* **222**, 245–251 (2012).
55. Pfaffl, M. W., Horgan, G. W. & Dempfle, L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **30**, e36 (2002).
56. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RTPCR. *Nucleic Acids Res.* **29**, e45 (2001).
57. Wishart, D. S. *et al.* HMDB: the Human Metabolome Database. *Nucleic Acids Res.* **35**, D521–526 (2007).
58. Ulrich, E. L. *et al.* BioMagResBank. *Nucleic Acids Res.* **36**, D402–408 (2008).

## Acknowledgements

Nadia Ruocco was supported by a PhD (PhD in Biology, University of Naples Federico II) fellowship co-funded by the Stazione Zoologica Anton Dohrn and Bio-Organic Chemistry Unit of the Institute of Biomolecular Chemistry-CNR, Pozzuoli, Naples. We acknowledge the Genomix4Life S.r.l. (Spin-Off of the University of Salerno, Baronissi, Salerno, Italy) for support in RNA/seq and bioinformatics. We thank the Morpho-Funtional Analyses and Bioimaging Unit of our Institute for technical assistance in preparing stubs for Scanning Electron Microscope experiments and photos. Thanks are also due to the Fishery Service of the Stazione Zoologica and the Monitoring and Environmental Data Unit for providing sea urchins, Alberto Macina from the Marine Resources for Research Unit for his technical support in the setting up of the continuous open flow-through system for feeding experiments and Massimo Perna of the Integrative Marine Ecology Department for embryo fixation.

## 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

**Supplementary information** accompanies this paper at <https://doi.org/10.1038/s41598-018-24023-9>.

**Competing Interests:** The authors declare no competing interests.

**Publisher's note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2018