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Induzione di goniodomine e caratterizzazione dei composti bioattivi extracellulari nella dinoflagellata *Alexandrium pseudogonyaulax*

Goniodomin induction and Bioactive extracellular compounds characterisation in dinoflagellate *Alexandrium pseudogonyaulax*

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Vien’ne da me

Qualcùn sustién che so’ più bèl d’invèrne,
dicéva el mâr... pensànd déntra de lù...
vién’ne a ‘rcuntè ma mé le pén dl’infèrne
dla vita, di magón... chi en ne pòl più!

Qualcùn vién giù sóltànt per fé dó pas
c’è chi me guârda sól... senša fiaté
c’è quèl che gióca sai rimbàlš di sas...
ma tanti i basta d’respiré sa me!

El sènt’ne... ch’èn c’è bsóğn de tant paròl
ce se capisč al vòl... questión de pèl
s’arcòj le fòrś pr’argì tla vita vera...

tla luč dl’aurora quànt arnàsč el sól
o sal tramónt più rósč... ch’infòca el ciél...
dvènta elisìr sa la magìa dla sera

quànt l’onda canterina cminčia piàn
a fè da ninna nanna mai gabiàn!

Elvio Grilli
Poesia fanese sul mare

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List of abbreviations

Ap = *Alexandrium pseudogonyaulax*

Cop = copepods

GDs/GDA = goniodomins, goniodomin A

BECs = bioactive extracellular compounds

HABs = harmful algal blooms

Rs = *Rhodomonas salina*

Al = *Alexandrium limii*

At = *Alexandrium tamarense*

Pc = *Prorocentrum cordatum*

SC = single control (single incubator in goniodomin induction experiment)

C = control (double incubator in goniodomin induction experiment)

Ap/cop = *A. pseudogonyaulax* separated physically from copepods

Ap + cop = *A. pseudogonyaulax* in physical contact with copepods

Al Ap = *Alexandrium pseudogonyaulax* separated physically from *Alexandrium limii*

At Ap = *Alexandrium pseudogonyaulax* separated physically from *Alexandrium tamarense*

Pc ap = *Alexandrium pseudogonyaulax* separated physically from *Prorocentrum cordatum*

Abstract

Alexandrium pseudogonyaulax is a cosmopolitan dinoflagellate known for the production of goniodomins (GDs) and other unknown bioactive extracellular compounds (BECs) that may cause harmful algal blooms (HABs). However, this mixotrophic microalgae is recently expanding in Northern European waters, becoming one of the predominant species in abundance in the HABs of these waters. This expansion may pose a danger to coastal ecosystems and associated services, leading to both direct and indirect consequences not only for aquatic fauna and flora but also for human beings. This study tested whether there was a goniodomin induction in *A. pseudogonyaulax* when it was both in direct contact with and physically separated from copepods *Acartia tonsa* (one of its natural meso-zooplankton predators). In addition, *A. pseudogonyaulax* was also studied in partial connection with other closely related microalgae while being preyed upon by the same grazer, to test wheter goniodomins were expressed. Finally, this study attempted to quantify the *A. pseudogonyaulax* BECs lytic activity through *R. salina* bioassays. The results did not show any induction in both experiments with only *A. pseudogonyaulax* and with other microalgae. However, the *R. salina* bioassays showed consistent lytic activity by *A. pseudogonyaulax* BECs.

1. Introduction

Microalgae provide a multitude of services to marine ecosystems: they produce most of the atmospheric oxygen through photosynthesis, contribute significantly to the global carbon cycle and form the basis of the oceanic trophic network[1]. However, the proliferation of certain species can be harmful to ecosystems due to biotoxins production or because uncontrolled and excessive growth leads to oxygen depletion. These phenomena are called Harmful Algal Blooms (HABs). These blooms occur due to various phenomena such as poor water exchange, hurricanes, floods, and eutrophication, which is defined as excessive nutrient enrichment of aquatic environments usually due to anthropogenic influences, such as intensive use of fertilizers or waste water discharge[2].

HABs typically occur near the sea surface, depleting oxygen in the water below, which may result in the mortality of numerous marine organisms[3]. HABs can persist for weeks[2], causing significant economic losses in the seafood industry. For instance, in 2016, Chile experienced an extensive proliferation of *Pseudochattonella* sp. and *Karenia* sp., resulting in the death of 40,000 tons of fish and an estimated economic loss of 800 million USD[4]. Additionally, HABs may impact the tourism and recreation sector by causing water turbidity or producing foams[2].

Biotoxin production by specific microalgal species increases the damages caused by HABs and poses risks to human and animal health. These toxins can be harmful even at low microalgal concentrations, not necessarily involving any bloom.
The predominant group of microalgae responsible for frequent HABs and toxin production are dinoflagellates, especially from the genus *Alexandrium*[3,5].

The production of some classes of marine biotoxins is suggested to be a defense mechanism against grazers[6,7]. Copepodamides (Fig.1), released by copepods, one of the main consumers of dinoflagellates[8], lead to enhanced toxin contents in various microalgae[9–11]. This response is grazer-dependent, as demonstrated by different toxin cellular content levels in dinoflagellates exposed to different copepod genera[12–15].

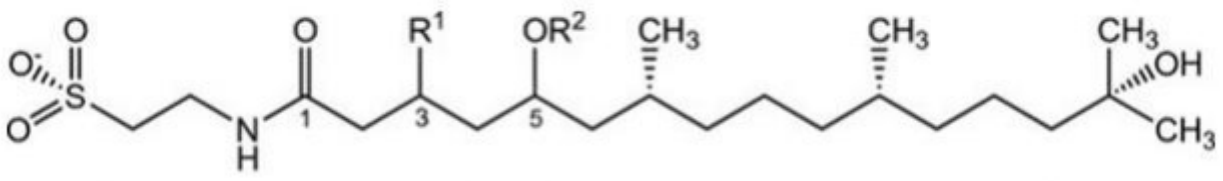

Fig. 1. *General structure of copepodamides. R1= -methyl or -methylene group R2= variable acyl group* [15]

Despite extensive research on paralytic shellfish toxin producing *Alexandrium* spp., there are still gaps in understanding the toxicity and environmental consequences of GD-producers, like *Alexandrium pseudogonyaulax*. This cosmopolitan dinoflagellate produces the phycotoxin goniodomin A (GDA) (Fig.2)[16–19], macrocyclic polyether with a wide range of biological effects, including antifungal and anti-angiogenic activity[17,20,21]. Goniodomins released by *Alexandrium* spp. have been associated with the mortality of marine invertebrates[22].

In addition, *A. pseudogonyaulax* is able to produce bioactive extracellular compounds (BECs). The chemical structure of these substances has not been elucidated yet, even though they cause various harmful effects (cytotoxic, haemolytic, allelopathic and ichthyotoxic)[23]. However, despite the harmful effects, the extent of GDA toxicity and the ecological impact of BECs remain poorly understood[24].

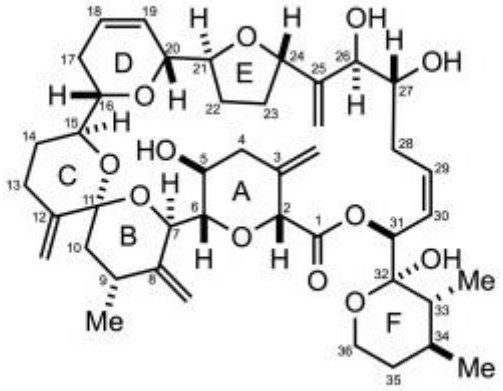

Fig. 2. *Chemical structurer of goniodomin A* [24]

Samples and records gathered in the North Sea and the Baltic Sea suggest that *A. pseudogonyaulax* is appearing in higher abundance and frequency [25–28].

Given the potential economic and environmental consequences of *A. pseudogonyaulax* blooms, further research is warranted.
To better understand the ecological role and the dynamics of GDA in *Alexandrium pseudogonyaulax,* one aim of this thesis was to verify if the goniodomin content of *A. pseudogonyaulax* increases when it is preyed upon by the calanoid copepod *Acartia tonsa*.

In the first part of the experiment copepods were either spatially separated by a mesh or sharing the same compartment as *A. pseudogonyaulax*.
The incubators used in the experiment were connected with a filter which allowed water, and copepodamides, exchange between them but prevented cell exchange.
Three conditions were tested in the experiment: *A. pseudogonyaulax* spatially separated from the copepods, *A. pseudogonyaulax* in physical contact with copepods and *A. pseudogonyaulax* separated from copepods while in the other side of the incubator copepods were feeding on *A. pseudogonyaulax*.
All tested conditions were compared to control which has only *A. pseudogonyaulax* without predators.

In addition, the second part of the experiment tested if a goniodomin induction by *A. pseudogonyaulax* also occurs when other closely related microalgae are preyed upon by the same grazer.
In this experiment copepods were feeding on different microalgae in one side of the incubator while in the other there is only *A. pseudogonyaulax*.

Finally, the lytic activity of *A. pseudogonyaulax* towards the cryptophyte *R. salina* was quantified.
All experiments were conducted with multiple strains of *A. pseudogonyaulax* in order to account for potential intraspecific variabilities.

2. Materials & methods

2.1. Study organisms

Acartia tonsa was chosen as representative of meso-zooplankton as it is a ubiquitous grazer of toxic planktonic algae cohabiting with *A. pseudogonyaulax* across northern Europe.
To perform experiments with single cohorts of copepods, eggs of *A. tonsa* were previously produced in 200-litre tanks. Daily, eggs were siphoned from the bottom of the tanks and stored in seawater at 4 °C for later usage.

For the experiments, *A. tonsa* eggs were incubated in fresh seawater collected from the German Bight in front of Helgoland (54°10'56"N, 7°53'17"E) at 20 °C and a salinity of 33.
Hatched nauplii were collected between 24- and 36-hours post-incubation to minimize age discrepancies among individuals.
Beginning 24 hours after hatching, *A. tonsa* was fed daily with *Rhodomonas salina* (strain KAC30, Kalmar culture collection), a well-established food source of *A. tonsa*.

R. salina cultures were maintained in F/2 medium under semi-batch conditions, at 20 °C and 150 PFDs of cool fluorescent light on a 16:8 hour light: dark cycle and provided with strong aeration.
To maintain *R. salina* culture in exponential growth phase ($2x10^5$ to $5x10^5$ cells/mL) cell densities were monitored using a Coulter Counter (Multisizer 4e, Beckman).

All three *A. pseudogonyaulax* strains (L2-D2 (A), L4-B1 (B), L4-B9 (C)) used in this study were established by micropipette isolation from live net tow samples under a M5A stereomicroscope (Wild, Heerbrugg, Switzerland) during an expedition with the R/V Uthörn in August 2020 in the western Danish Limfjord close to Thyborøn (56°42'00"N, 8°13'00"E).

Alexandrium limii (strain Atay99Shio-02) was collected in Shioya Bay (Okinawa, Japan) in July 1999 by S. Nagai. Both *Alexandrium tamarense* (strain NX-87-08) and *Prorocentrum cordatum* (strain 1-B3) were collected by Urban Tillmann at different times and locations; the first in Trodheimfjord (Norway) in August 2015, the second in North Atlantic in July 2018 (Fig. 3).

Fig. 3. Alexandrium limii, Alexandrium tamarense, Prorocentrum cordatum *and* Rhodomonas salina *pictures kindly provided by Urban Tillmann*

All microalgal strains used in the experiments were reared in polystyrene tissue culture flasks (TPP®) with "VENT" screw caps (made in PE), starting from the 25 cm^2/60 mL volume model then upscaled into higher volumes as required.

2.2. Culture medium preparation

All K/2 media (K-media[29] with half of the original nutrient concentrations, pH = 8.1, NO_3^-= 441 μM, NH_4^+=25 μM) employed in the present study was prepared from the same batch of aged seawater collected from the North Sea near Helgoland (Germany). The seawater was autoclaved, nutrients were added and then the seawater was additionally steril filtered (< 0.2 μm).
The original K-medium receipt was modified by replacing the organic phosphorus source with 3.62 μM Na_2HPO_4.
The salinity was adjusted to 25 by adding 1 L of deionised water in K/2 media 4 Liters tanks.

2.3. Goniodomin induction experiment

The induction experiments were performed in incubators consisting of two polystyrene tissue culture flasks (TPP®) (volume 750 mL / flask) in which the common wall consisted of a 10 μm mesh.

Copepods and microalgae were added to the incubators following different compositions depending on control and treatment conditions.
A. pseudogonyaulax strain C (L4-B9) was selected for conducting goniodomin induction experiments.

The bottles were always filled at maximum capacity with Filtered Sea Water (FSW) in case algae and copepods alone could not fill the bottles.
Once prepared, two 2 mL counting samples were taken out of them, then they were placed in the ROfre – 897 AC-INVERTER® (Fig. 4) and the rotating speed was set at 3.5 rpm.

Fig. 4. *Rotating device ROfre – 897 AC-INVERTER®*

The bottles were left in incubation for 24 hours in dim light. After that period water in the bottles was gently filtered over a mesh with different pore sizes (10, 20, 50 μm) in order to collect both copepods and algae.

At the end of the experiment, after the incubation time of 24 hours, copepods were then transferred into 15 mL tubes, fixed with 1% Lugol´s iodine solution and counted on a stereomicroscope Olympus SZX 12® in Bogorov chambers.
Microalgae were collected in 50 mL falcon tube, counting samples were taken out of each of them (250 μL), fixed with Lugol's iodine solution and counted on inverted microscope Zeiss Axiovert 40C® in counting chambers.
Algae left in the 50 mL falcon tube were centrifuged for 15 minutes at 10 °C at 16650 rcf in Allegra® X-15R Centrifuge (Beckman Coulter).

The supernatant was discarded manually, the remaining cell pellet was re-suspended in FSW, transferred into 2 mL Micro tube (REF Sarstedt AG & Co. KG) made in PP and centrifuged again (Centrifuge 5424 R® Eppendorf, Hamburg, Germany) at 10 °C for 15 minutes at 21120 rcf.
The supernatant was discarded and samples were stored at -20 °C.

2.4. Toxin extraction

Toxins were extracted from the samples by adding 500 μL methanol and Zirconium-silicate spheres (Matrix lysing D® MP Biomedicals, Fisher Scientific GmbH, Schwerte, Germany) (composition: ZrO2 64%, SiO2 33%) into the tube.
The tubes were homogenized (Fast prep®-24 MP Biomedicals, Eschwege, Germany) for 45 seconds then centrifuged for 15 minutes, at 10 °C at 16650 rcf.

The resulting supernatant was transferred into Nanosep® centrifugal filters (Cytica, Fisher Scientific GmbH, Schwerte, Germany), centrifuged with the previous conditions for 1 minute. Then, the filtered material was transferred into 2 mL Crimp vials® and sealed with Crimp caps® (Agilent Technologies, Waldbronn, Germany).

2.5. Mass spectrometry

Quantification of goniodomin A (GDA) was conducted using ultra performance liquid chromatography (UPLC, ACQUITY, Waters, Eschborn, Germany) coupled to triple quadrupole mass spectrometry (MS/MS, Xevo TQ-XS, Waters).
Separation was performed on HSS C10 column (2.1 mM X 50 mM, 2 μM, Purospher®, Merck, Darmstadt, Germany) and a pre-column (Acquity UPLC HSS C18 VanGuard Pre-Column (100 Å, 1.8 μM, 2.1 mM x 5 mM), Waters) at a column temperature of 40 °C.
Eluents used for liquid chromatography had the following composition:

- A1_WA: H_2O (500 mL) + FA (50 μL) + NH_4OH (250 μL)
- B1_WA: ACN/H_2O (450 mL + 50 mL) + FA (50 μL) + NH_4OH (250 μL)

Water was deionized and purified (Milli-Q, Millipore, Eschborn, Germany) to 18 M Ω/cM or better quality.

Formic acid 99 % (eluent additive for LC-MS) was purchased from Th. Geyer, Renningen, Germany and ammonium hydroxide (Fluka, Ammonium Hydroxide solution ≥ 25% in water for trace analysis) was purchased from Omnilab, Bremen, Germany.
The organic solvent Acetonitrile was purchased from Biosolve BV, Valkenswaard, Netherlands.

2.6. Copepods feeding on *Alexandrium pseudogonyaulax*

Three different copepod life stage were used during this experiment: N4 nauplii life stage (4-5 days after hatch), C4 copepodite life stage (9-10 days after hatch) and adult copepods (14 days after hatch). The experiment was repeated in three times using a different copepod life stage for each of them (see below).
Copepods used were fasting 24 hours before the start of the experiment to make sure that they feed during it.
Incubators were divided in: Single control (**SC**) (one single bottle with *A. pseudogonyaulax*), Control (**C**) (double incubator with *A. pseudogonyaulax* in one side and no organisms in the other one), Control with copepods (**Ap/cop**) (double incubator with *A. pseudogonyaulax* in one side and copepods in the other one) and finally the treatment incubators with copepods feeding on *A. pseudogonyaulax* in one side (**Ap+cop**) and only *A. pseudogonyaulax* in the other side (**Ap**) (Fig. 5).

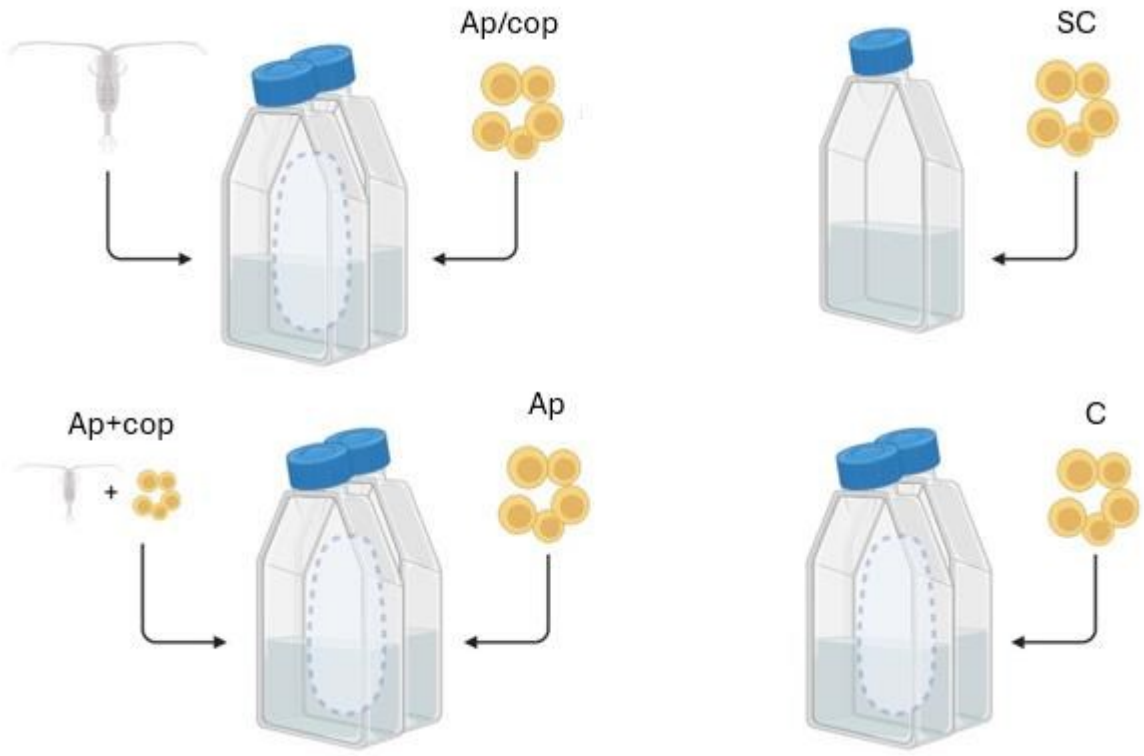

Fig. 5. *Incubator composition in copepods feeding on* A. pseudogonyaulax *induction experiment*

Each treatment and control incubators were prepared in four replicates.
A. pseudogonyaulax concentration in each incubator was 100 cells/mL while copepod concentration was different for each replicate based on the life stage (40 organisms in N4 nauplii, 35 organisms in C4 copepodites and 30 adult copepods in each bottle).

2.7. Copepods feeding on *Alexandrium limii*, *Alexandrium tamarense* and *Prorocentrum cordatum*

In this experiment only adult copepod life stage was used.
Copepods (30 adult for each treatment bottle) were feeding on *A. limii*, *A. tamarense* and *P. cordatum* while *A. pseudogonyaulax* was in the other side of the incubator.
Microalgal concentration inside the incubators for *A. limii*, *A. tamarense* and *A. pseudogonyaulax* was the same as the previous experiment: 100 cells/ml.

Since *P. cordatum* is approximately 200 times smaller than *A. pseudogonyaulax*, its concentration inside the bottles was adjusted in order to mimic roughly the same *A. pseudogonyaulax* biomass in the other bottles and thus guarantee the copepods the same prey biomass to feed on, i.e. 12 000 cells / mL.
Incubators were prepared in four replicates, in order to distinguish it, *A. pseudogonyaulax* in the different incubators was named after the other microalgae species in the other side: **Al Ap** (*A. pseudogonyaulax / A. limii*), **At Ap** (*A. pseudogonyaulax / A. tamarense*), **Pc Ap** (*A. pseudogonyaulax / P. cordatum*) (*Fig. 6*).

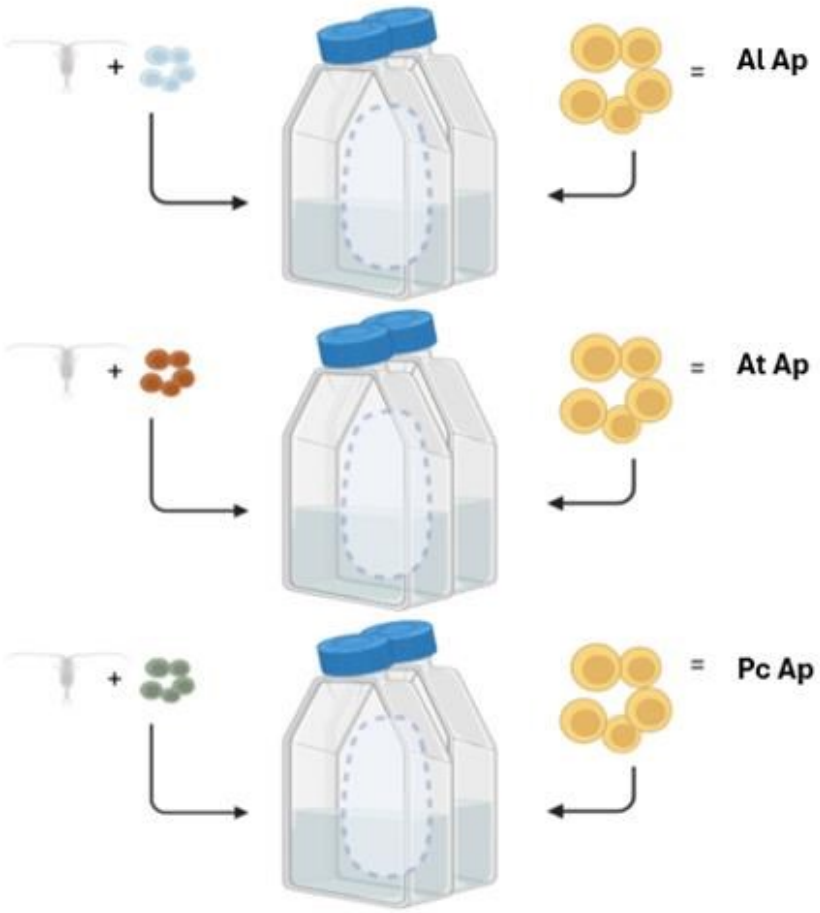

Fig. 6. *Incubator composition in Copepods feeding on* Alexandrium limii, Alexandrium tamarense *and* Prorocentrum cordatum *induction experiment*

Three 25 cM^2 /60 mL volume culture flasks with just *A. pseudogonyaulax* (100 cell/mL) were used as control during the experiment (**Ap C**).

A manual error occurred in the end of the experiment where the microalgae *A. tamarense* and *P. cordatum* were almost totally lost due to the use of an inappropriate gaze filter. For this reason, it is impossible to establish whether copepods actually fed on the selected preys during the experiment.

2.8. *Rhodomonas salina* bioassay

Microalgal bioassays using the cryptophyte *Rhodomonas salina* as target species served to quantify lytic activity in the three strains (A, B, C) of *A. pseudogonyaulax* studied.
Rhodomonas salina strain was cultured under the same conditions as for *Alexandrium pseudogonyaulax* strains.

The bioassay was set up in a total volume of 4 mL in 10 mL glass vials. Each sample was spiked with 0.1 mL of *R. salina* culture which was adjusted (based on microscope cell count) to 4 x 10^5 cells/mL to reach a start concentration of 1 x 10^4 cells/mL.
Every sample was prepared in triplicates. Culture medium with just *R. salina* served as control.

In each sample, except for the control, a variable dose of *Alexandrium pseudogonyaulax* supernatant was added (*Tab.1*).

Tab. 1. *A. pseudogonyaulax supernatant in the R. salina bioassay*

Supernatant (ml)	**K-media (ml)**	***R. salina* (µl)**
3.9	X	100
2	1.9	100
1	2.9	100
0.5	3.4	100
0.2*	1.9	100
0.1*	2.9	100
0.05*	3.4	100

*dilutions 1:10

In order to get *A. pseudogonyaulax* supernatant, an initial microalgal density (3/4 x 10^3 cells/mL) was adjusted in a 50 mL falcon tube and centrifuged for 10 minutes at 10 °C, 16650 rcf in Allegra® X-15R Centrifuge (Beckman Coulter) to separate the cells from the supernatant. Then the supernatant was manually separated from the pellet cells and added to the 10 mL vials with K-medium and *R. salina,* as shown in Table 1.

Samples were incubated at 20 °C in dark for 24 hours (to restrict *R. salina* growth). Subsequently, samples were fixed with 2% Lugol´s iodine solution and the intact target species were counted (from 0.5 mL) by inverted microscopy (Zeiss Axiovert 40C) in counting chambers.

A sub-area corresponding to at least 600 *Rhodomonas* cells in the control was counted and *R. salina* concentrations of the treatments were normalized to the control.
Estimates of LD_{50} values (= volume of *A. pseudogonyaulax* supernatant yielding a 50% decline in target cell concentration) were determined by fitting data points to a sigmoid equation.

2.9. Statistical analysis

For statistical analysis of the GDA cellular content obtained through previously described induction experiments, the program R. studio version 4.4.0 (2024) was used.
The packages installed were readr, car, ggplot2 and dplyr in order to perform diverse tests.
Normality was tested with Shapiro-wilks test, Fisher exact test allowed to assess the homoscedacity.

Finally, the t-test of student was performed in order to determine where there was a difference between control and treatment conditions.
For statistical analysis of ingestion rates (i.e. number of microalgae consumed by copepods per unit of time), Kolmogorov-Smirnov test was used to determine whether the copepods actually fed on the selected prey during the experiments.
All statistical tests were performed at a significance level of α=0.05.
Statistica Version 9.1 (Statsoft®, Tulsa, OK, USA) was used for the *R. salina* bioassays to determine LD_{50} values.

3. Results

3.1. Goniodomin induction experiments

3.1.1. Copepods feeding on *A. pseudogonyaulax*

In this experiment *A. pseudogonyaulax* did not show any induction by any of the tested copepod life stage (*Fig. 7*). Indipendent of a lack of GDA induction, across the treatments, the highest value of cellular GDA was obtained in the nauplii treatment, in the single control incubators (SC) (6.21 ± 0.93 pg / cell) whereas the lowest value was observed in the copepodite treatment incubators (Ap) (1.63 ± 0.31 pg/cell).
In general, GDA cellular content values were almost the same in every incubator of every replicate based on copepod life stage.
The only exception is represented by Ap incubators in the adult treatment which the mean of GDA value is significantly lower than the control (Students t-test, p = 0.0124).

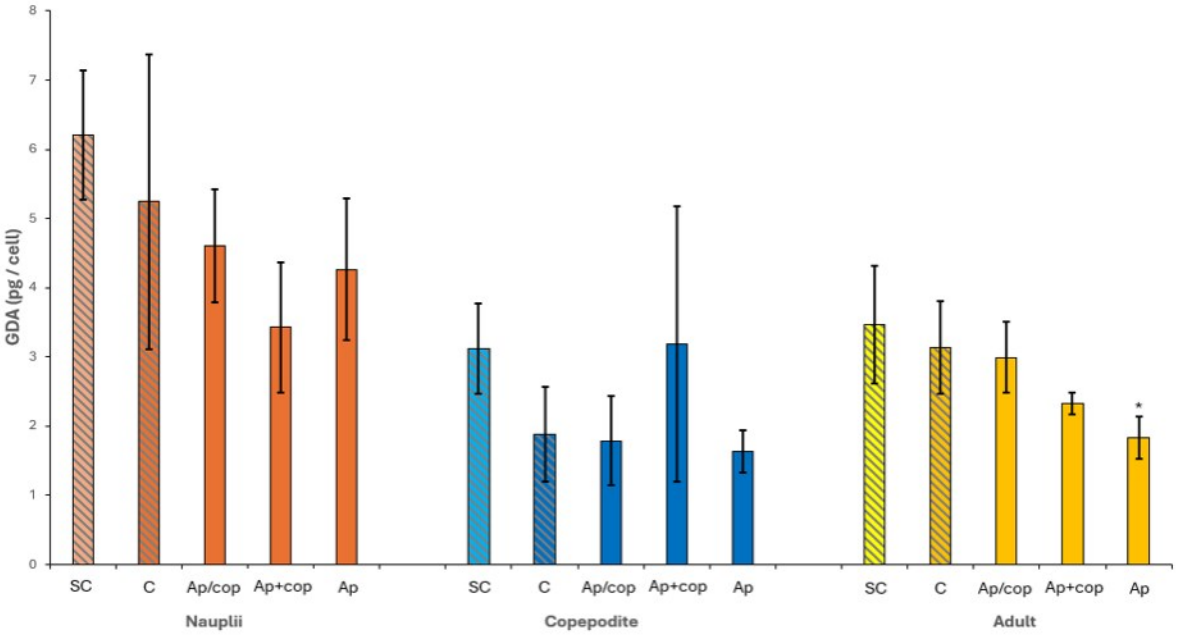

Fig. 7. *GDA cellular content (pg / cell) expressed in different incubators (SC, C, Ap/cop, Ap+cop, Ap) in the three different replicates (Nauplii N4, Copepodite C4 and Adult) in copepods feeding on* A. pseudogonyaulax *induction experiment. Treatment incubators (Ap/cop, Ap+cop, Ap) were compared to C incubators; values represent mean with 95% confidence intervals of four replicates. Note Ap incubator in adult replicate, it is the only value that is not equal to the control C but lower*

Ingestion rates statistically show an actual feeding of *A. pseudogonyaulax* by copepods for copepodite and adult life stages (Kolmogorov-Smirnov test, p-value copepodite = 0.02857, p-value adult = 0.02857) whereas, for nauplii life stage, this is not apparent (p-value nauplii = 0.1) (*Fig. 8*), however, the data still show clearly that the prey was consumed and this is also visible in the counting samples in the Appendices (pag. 23).

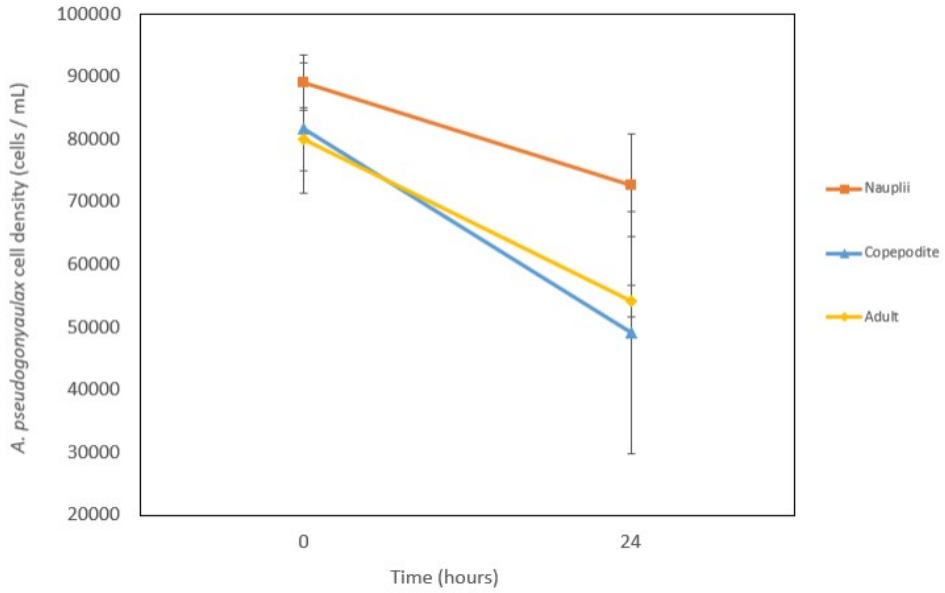

Fig. 8. *Cell densities (cells / mL) over 24 hours of* A. tonsa *feeding on monoalgal prey (*A. pseudogonyaulax *strain C); points represent mean with 95% confidence intervals from four biological replicates*

3.1.2. Copepods feeding on *A. limii*, *A. tamarense*, *P. cordatum*

No GDA induction is detected in the experiment (*Fig. 9*).All three replicates, based on different microalgae, show similar GDA cellular values (highest value: Pc Ap = 5.80 ± 0.19 pg/cell, lowest value: At Ap = 4.25 ± 0.28 pg/cell) (Student T-test, Ap Al p-value = 0.489, Ap At p-value = 0.161, Ap Pc p-value = 0.156, compared to control Ap C).

In addition, the only ingestion rate that could be represented, i.e. copepods feeding on *A. limii*, showed no detectable statistical difference in the number of preys at the beginning and end of the experiment (p-value = 0.2286) (*Fig. 10*).

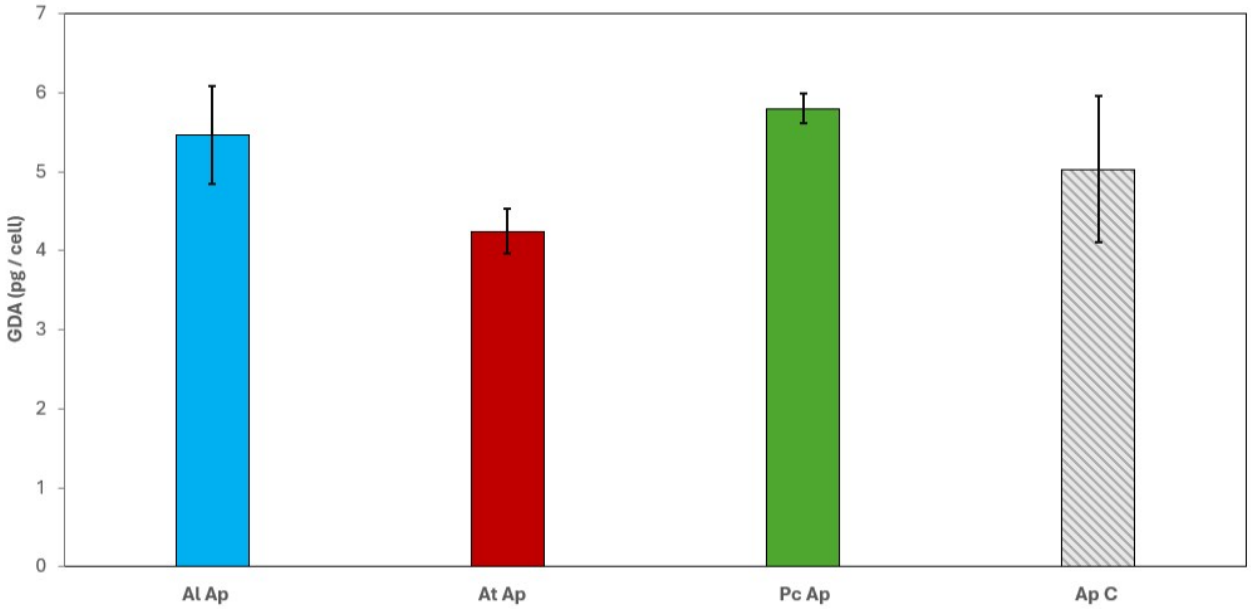

Fig. 9. *GDA cellular content (pg / cell) in* A. pseudogonyaulax *expressed in different incubators (Al Ap, At Ap, Pc Ap, Ap C) in "Copepods feeding on* A. limii*,* A. tamarense*,* P. cordatum *goniodomin induction experiment". Al Ap, At Ap and Pc Ap incubators compared to control Ap C; values represent mean with 95% confidence intervals of four biological replicates.*

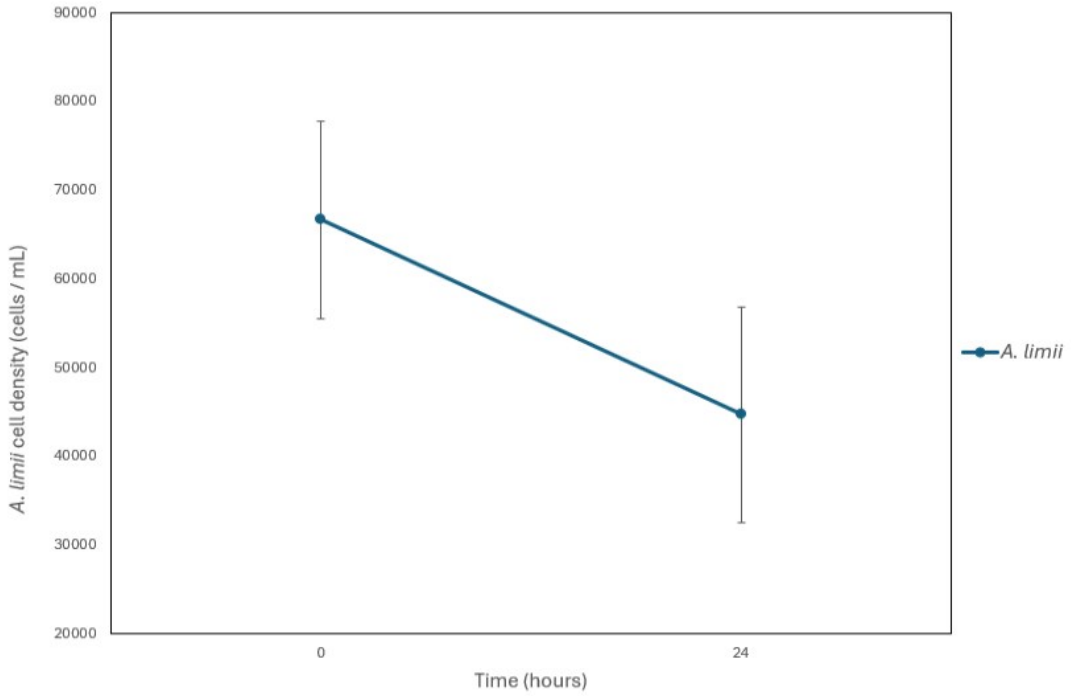

Fig. 10. *Cell densities (cells / mL) over 24 hours of* A. tonsa *feeding on monoalgal prey (*A. limii*); points represent mean with 95% confidence intervals from four biological replicates*

3.2. *Rhodomonas salina* bioassays

Alexandrium pseudogonyaulax strains A and B show a similar LD_{50} values (LD_{50} A = 1288 ± 136 cells / mL, LD_{50} B = 1175 ± 151 cells / mL) (*Fig. 11)* (*Fig. 12)*.
In contrast, strain C had a lower LD_{50} value (LD_{50} C = 229 ± 117 cells / mL) (*Fig. 13)*.

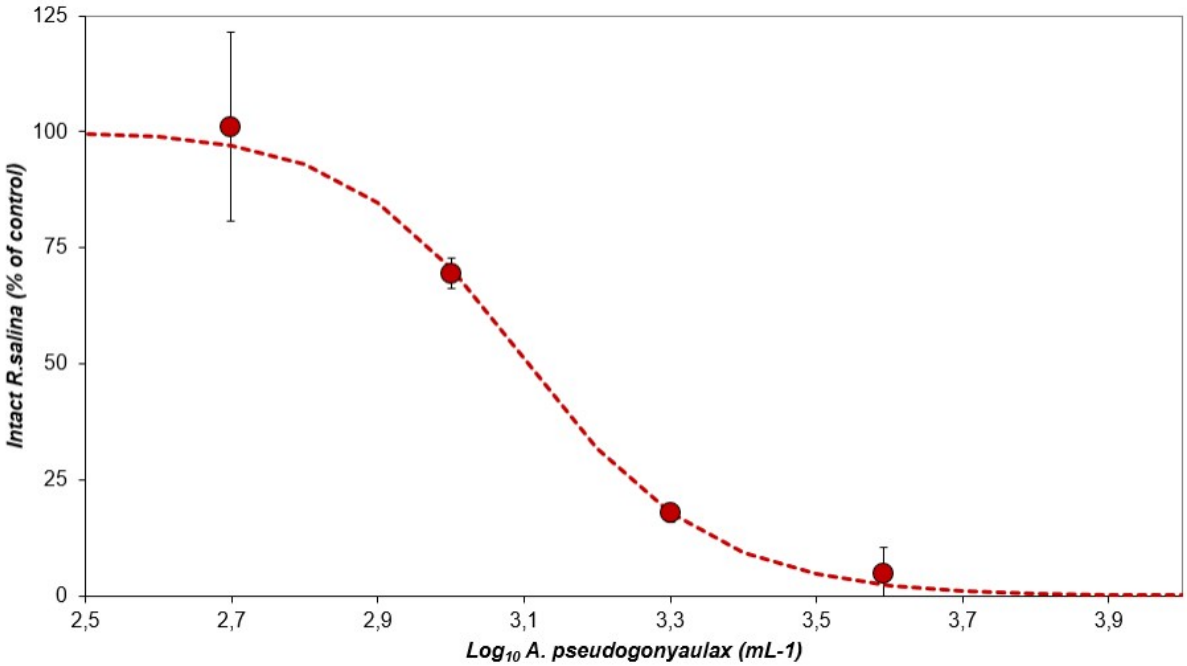

Fig. 11. *Dose response curve of the* Rhodomonas salina *bioassay over 24 hours with* A. pseudogonyaulax *strain A, LD_{50}A = 1288 ± 136 Ap cell density; points represent mean with 95% confidence intervals from three replicates, while line represent modelled dose-response curve*

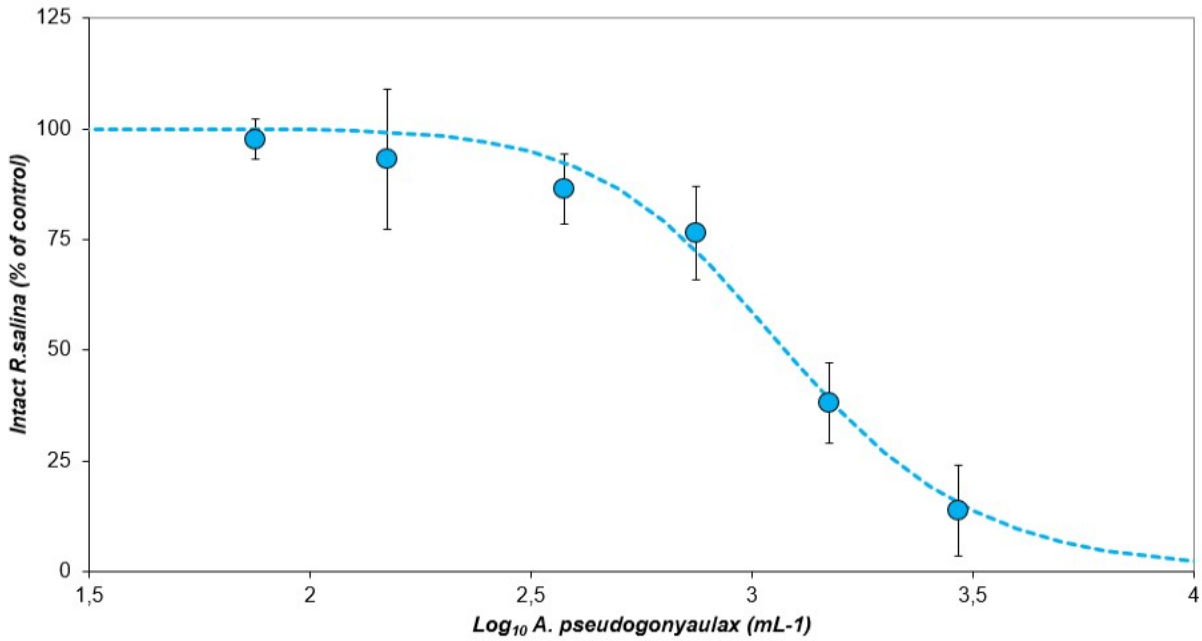

Fig. 12. *Dose response curve of the* Rhodomonas salina *bioassay over 24 hours with* A. pseudogonyaulax *strain B,* $LD_{50}B$ *= 1175 ± 151 Ap cell density; points represent mean with 95% confidence intervals from three replicates, while line represent modelled dose-response curve*

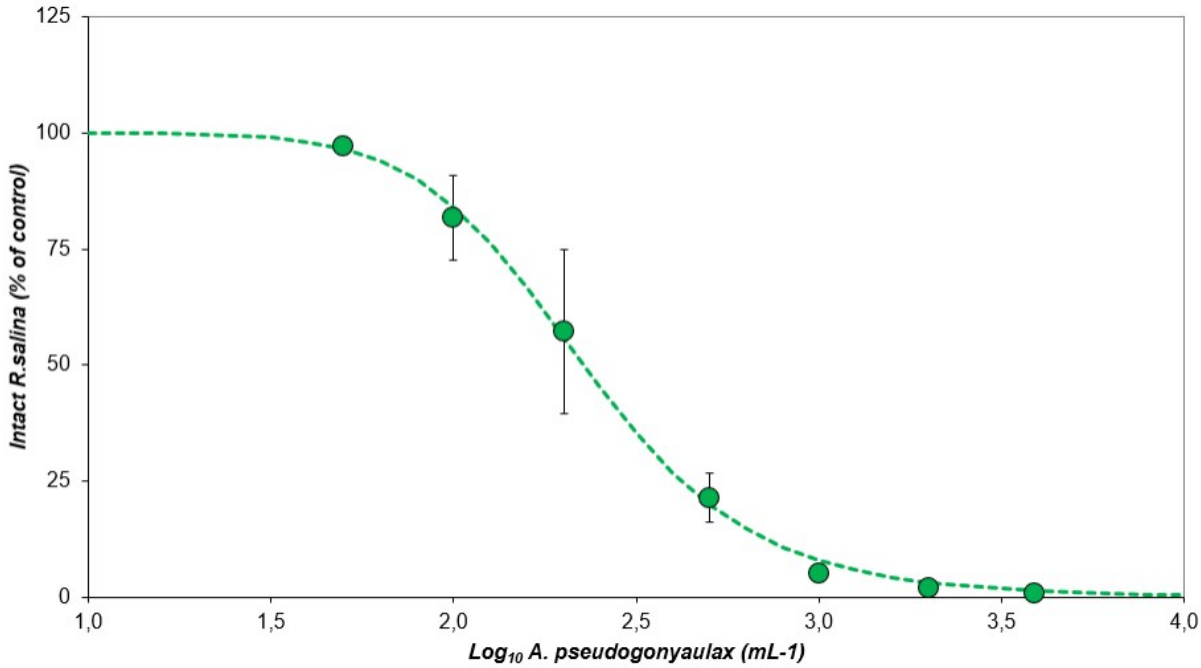

Fig. 13. *Dose response curve of the* Rhodomonas salina *bioassay over 24 hours with* A. pseudogonyaulax *strain C,* $LD_{50}C$ *= 229 ± 117 Ap cell density; points represent mean with 95% confidence intervals from three replicates, while line represent modelled dose-response curve*

4. Discussion

4.1. Goniodomin induction experiments

In this study, *A. pseudogonyaulax* did not show GDA induction from any of the copepod life stages tested in the experiment, irrespective of the microalgae used as food (i.e. *A. limii*, *A. tamarense*, *P. cordatum* or *A. pseudogonyaulax* itself).

It has been shown that toxin production is strongly influenced by the specific composition of the grazer community, making the response predator specific[15]. For example, the dinoflagellate *Alexandrium minutum,* when exposed to copepods of genus *Centropages*, showed a stronger response in terms of PSTs (Paralytic Shellfish Toxins) production than that obtained in similar experiments with *Acartia tonsa*[14]. On the other hand, exposure to copepods of the genus *Pseudocalanus* did not cause a detectable statistical effect on toxin cellular content in any of *A. minutum* tested strains[15].

In addition, it has been demonstrated that the chemical structure of copepodamides differ between freshwater and marine copepods species[30]. It is reasonable to assume, therefore, that copepodamides also differ from specie to specie belonging to the same environmental compartment. For the combination of these reasons, it is likely that, in this study, *Acartia tonsa* copepodamides could have a peculiar chemical composition such that they do not cause goniodomin A induction in *Alexandrium pseudogonyaulax*.

Another plausible reason that may explain the non-occurrence of toxin induction could be the copepods diet before the experiments. In the study from Grebner et al. [31], in fact, it is explained that copepodamide composition is strongly influenced by copepod diet, especially by the amount of available dietary fatty acids they can assume[31]. *R. salina* diet before the experiments may have led to the production of a certain type of copepodamides such that it did not trigger goniodomin induction in the studied dinoflagellate.

Perhaps the ability of copepods to effectively hunt their prey was influenced by the presence, albeit sparse, of goniodomin A.
The toxin could have caused limited motor and sensory capabilities in the grazers, which, as a result, were not perceived as a threat by *A. pseudogonyaulax*.

It is always important to keep in mind that goniodomin A could not be the specific microalgae defensive mechanism against grazers, which could explain the lack of GDA induction observed in this experiment under any conditions. In fact, this can be wrongly assumed only because of the known adverse effects of the toxin on mollusks and human and rat cell lines[17,20–22].

4.2. *Rhodomonas salina* bioassay

Since *Alexandrium pseudogonyaulax* is also producing bioactive extracellular compounds, these could be responsible for anti-grazer response. This hypothesis is supported by the fact

that BECs excreted in the aquatic environment, as demonstrated in this study, showed lytic activity. In this way, bioactive extracellular compounds would be an effective and direct response against grazers.

As already showed for other *Alexandrium* species in fact, BECs have anti-grazer properties along with allelopathic, ichthyotoxic and hemolytic effects[23]. For example, lytic compounds from *A. taylorii* caused immobilization and mortality to *Artemia salina* when exposed to the whole culture or just the supernatant[32].
The problem regarding anti-grazing compounds in *Alexandrium* spp. is the simultaneous presence of several classes of compounds and the inability to determine their specific bioactivity. Since BECs are not chemically identified it is impossible to establish if a specific group of compounds within them are responsible for observed adverse effects.

Further experiments are required to determine the effects of *Alexandrium pseudogonyaulax* BECs on a single group of compounds or a mixture of these and toxins. In addition, to confirm the bioactive extracellular compounds direct involvement in anti-grazing activities specific experiments involving copepods, or other microalgae consumers, are required.

As a first step, it is necessary to ascertain a real increase of BECs in *Alexandrium pseudogonyaulax* in presence of predators. Then confront the bioactive extracellular compounds lytic activity in samples with and without predators. An example of experiment that can be carried out for this purpose is shown in the figure (*Fig. 13)*.

In the proposed experiment copepods and *A. pseudogonyaulax* are placed in the same falcon tube, after an optimal incubation period, the content of the falcon is centrifuged in order to get the supernatant where the BECs are, separating it from pellet (*A. pseudogonyaulax* and copepods). With the obtained supernatant then perform a *Rhodomonas salina* bioassay as described in this same study. The difference between the control (without copepods) and treatment conditions in LD_{50} values could testify to an actual use of BECs in anti-grazing activities by *Alexandrium pseudogonyaulax*.

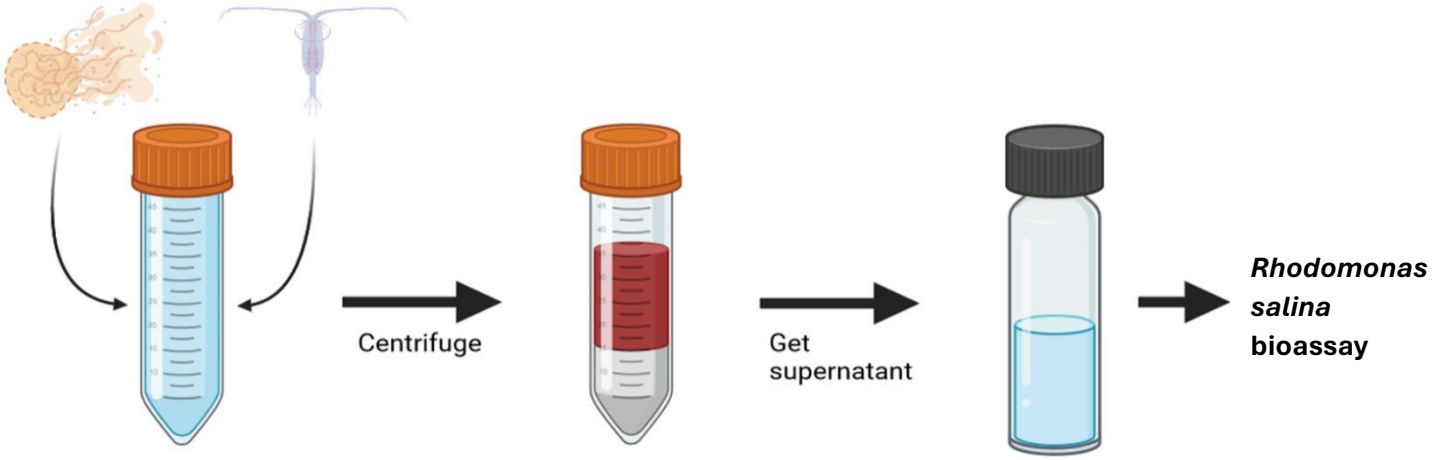

Fig. 13. *Proposed experiment to evaluate the lityc activity of* Alexandrium pseudogonyaulax *BECs in presence of predators.*

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6. Appendices

6.1 Goniodomin induction experiment cell counts

Cell counts represented a quality control of copepods actually feeding on the designated prey during induction experiments.

Tab. 2 *Nauplii feeding on* A. pseudogonyaulax *goniodomin induction experiment settings. "Copepod" and "Ap" lines indicate the starting conditions used later for the composition of the incubators for the experiment. The remaining lines indicate the concentration of* A. pseudogonyaulax *in the incubators at the start of the experiment.*

Settings	Cells counted	Sample volume (μL)	Density (cells/mL)
Copepod	18	5000	
Ap	135	100	1350
SC 1	390	4000	97,5
SC 2	473	4000	118,25
SC 3	346	4000	86,5
SC 4	372	4000	93
C 1	379	4000	94,75
C 2	503	4000	125,75
C 3	352	4000	88
C 4	352	4000	88
Ap/cop 1	327	4000	81,75
Ap/cop 2	320	4000	80
Ap/cop 3	350	4000	87,5
Ap/cop 4	332	4000	83
Ap+cop 1	518	4000	129,5
Ap+cop 2	503	4000	125,75
Ap+cop 3	473	4000	118,25
Ap+cop 4	456	4000	114
Ap 1	554	4000	138,5
Ap 2	562	4000	140,5
Ap 3	486	4000	121,5
Ap 4	436	4000	109

Tab. 3 *Numbers of* A. pseudogonyaulax *cells counted in nauplii feeding on* A. pseudogonyaulax *goniodomin induction experiment after incubation period.*

Results	Cells counted	Sample volume (μL)	Density (cells/mL)
SC 1	302	250	1208
SC 2	344	250	1376
SC 3	292	250	1168
SC 4	294	250	1176
C 1	356	250	1424
C 2	330	250	1320
C 3	320	250	1280
C 4	283	250	1132
Ap/cop 1	247	250	988
Ap/cop 2	253	250	1012
Ap/cop 3	250	250	1000
Ap/cop 4	262	250	1048
Ap+cop 1	483	250	1932
Ap+cop 2	388	250	1552
Ap+cop 3	318	250	1272
Ap+cop 4	389	250	1556
Ap 1	410	250	1640
Ap 2	457	250	1828
Ap 3	364	250	1456
Ap 4	365	250	1460

Tab. 4 *Copepods counted from each treatment incubator in nauplii feeding on* A. pseudogonyaulax *goniodomin induction experiment after the incubation period. In Ap+cop 1 incubator a manual error occurred: copepods were not added in the incubator. This specific incubator was not counted for the predation rate.*

Copepods	Organisms counted	Sample volume (μL)
Ap/cop 1	47	15000
Ap/cop 2	48	15000
Ap/cop 3	54	15000
Ap/cop 4	46	15000
Ap+cop 1	Ø	15000
Ap+cop 2	36	15000
Ap+cop 3	37	15000
Ap+cop 4	30	15000

Tab. 5 *Copepodite feeding on* A. pseudogonyaulax *goniodomin induction experiment settings.*

Settings	Cells counted	Sample volume (μL)	Density (cells/mL)
Copepod	38	6000	
Ap	177	100	1770
SC 1	497	4000	124,25
SC 2	446	4000	111,5
SC 3	488	4000	122
SC 4	499	4000	124,75
C 1	445	4000	111,25
C 2	461	4000	115,25
C 3	394	4000	98,5
C 4	548	4000	137
Ap/cop 1	435	4000	108,75
Ap/cop 2	456	4000	114
Ap/cop 3	441	4000	110,25
Ap/cop 4	392	4000	98
Ap+cop 1	487	4000	121,75
Ap+cop 2	415	4000	103,75
Ap+cop 3	370	4000	92,5
Ap+cop 4	480	4000	120
Ap 1	509	4000	127,25
Ap 2	454	4000	113,5
Ap 3	425	4000	106,25
Ap 4	437	4000	109,25

Tab. 6 *Numbers of* A. pseudogonyaulax *cells counted in copepodite feeding on* A. pseudogonyaulax *goniodomin induction experiment after incubation period.*

Results	Cells counted	Sample volume (μL)	Density (cells/mL)
SC 1	365	250	1460
SC 2	362	250	1448
SC 3	298	250	1192
SC 4	332	250	1328
C 1	264	250	1056
C 2	244	250	976
C 3	266	250	1064
C 4	289	250	1156
Ap/cop 1	210	250	840
Ap/cop 2	280	250	1120
Ap/cop 3	246	250	984
Ap/cop 4	248	250	992
Ap+cop 1	316	250	1264
Ap+cop 2	332	250	1328
Ap+cop 3	124	250	496
Ap+cop 4	215	250	860
Ap 1	402	250	1608
Ap 2	239	250	956
Ap 3	226	250	904
Ap 4	197	250	788

Tab. 7 *Copepods counted from each treatment incubator in copepodite feeding on* A. pseudogonyaulax *goniodomin induction experiment.*

Copepods	Organisms counted	Sample volume (μL)
Ap/cop 1	24	15000
Ap/cop 2	21	15000
Ap/cop 3	17	15000
Ap/cop 4	31	15000
Ap+cop 1	26	15000
Ap+cop 2	31	15000
Ap+cop 3	21	15000
Ap+cop 4	30	15000

Tab. 8 *Adult copepods feeding on* A. pseudogonyaulax *goniodomin induction experiment settings.*

Settings	Cells counted	Sample volume (μL)	Density (cells/mL)
Copepod	20	6000	
Ap	195	100	1950
SC 1	443	4000	110,75
SC 2	485	4000	121,25
SC 3	393	4000	98,25
SC 4	427	4000	106,75
C 1	500	4000	125
C 2	395	4000	98,75
C 3	440	4000	110
C 4	427	4000	106,75
Ap/cop 1	441	4000	110,25
Ap/cop 2	434	4000	108,5
Ap/cop 3	433	4000	108,25
Ap/cop 4	408	4000	102
Ap+cop 1	446	4000	111,5
Ap+cop 2	413	4000	103,25
Ap+cop 3	399	4000	99,75
Ap+cop 4	456	4000	114
Ap 1	409	4000	102,25
Ap 2	386	4000	96,5
Ap 3	390	4000	97,5
Ap 4	424	4000	106

Tab. 9 *Numbers of* A. pseudogonyaulax *cells counted in adult copepods feeding on* A. pseudogonyaulax *goniodomin induction experiment after incubation period.*

Results	Cells counted	Sample volume (μL)	Density (cells/mL)
SC 1	313	250	1252
SC 2	267	250	1068
SC 3	308	250	1232
SC 4	338	250	1352
C 1	294	250	1176
C 2	233	250	932
C 3	292	250	1168
C 4	277	250	1108
Ap/cop 1	283	250	1132
Ap/cop 2	261	250	1044
Ap/cop 3	236	250	944
Ap/cop 4	256	250	1024
Ap+cop 1	274	250	1096
Ap+cop 2	254	250	1016
Ap+cop 3	283	250	1132
Ap+cop 4	278	250	1112
Ap 1	211	250	844
Ap 2	256	250	1024
Ap 3	118	250	472
Ap 4	216	250	864

Tab. 10 *Copepods counted from each treatment incubator in adult copepods feeding on* A. pseudogonyaulax *goniodomin induction experiment.*

Copepods	Organisms counted	Sample volume (μL)
Ap/cop 1	18	15000
Ap/cop 2	15	15000
Ap/cop 3	16	15000
Ap/cop 4	22	15000
Ap+cop 1	21	15000
Ap+cop 2	16	15000
Ap+cop 3	18	15000
Ap+cop 4	20	15000

Tab. 11 *Copepods feeding on* A. limii*,* A. tamarense *and* P. cordatum *goniodomin induction experiment settings.*

Settings	Cells counted	Sample volume (μL)	Density (cells/mL)
Copepod	4	4000	
Ap	293	100	2930
Al	389	200	1945
At	1063	100	10630
Pc	3511	50	70220
Al 1	289	4000	72,25
Al 2	346	4000	86,5
Al 3	434	4000	108,5
Al 4	362	4000	90,5
Ap Al 1	445	4000	111,25
Ap Al 2	538	4000	134,5
Ap Al 3	518	4000	129,5
Ap Al 4	474	4000	118,5
At 1	255	4000	63,75
At 2	305	4000	76,25
At 3	250	4000	62,5
At 4	239	4000	59,75
Ap At 1	459	4000	114,75
Ap At 2	413	4000	103,25
Ap At 3	418	4000	104,5
Ap At 4	381	4000	95,25
Pc 1	566	50	11320
Pc 2	608	50	12160
Pc 3	613	50	12260
Pc 4	668	50	13360
Ap Pc 1	408	4000	102
Ap Pc 2	429	4000	107,25
Ap Pc 3	430	4000	107,5
Ap Pc 4	433	4000	108,25

Tab. 12 *Numbers of* A. limii*,* A. tamarense *and* P. cordatum *cells counted in adult copepods feeding on* A. pseudogonyaulax *goniodomin induction experiment after incubation period*.

Results	Cells counted	Counting volume (μL)	Density (cells/mL)
Al 1	157	250	628
Al 2	205	250	820
Al 3	303	250	1212
Al 4	234	250	936
Ap Al 1	174	250	696
Ap Al 2	229	250	916
Ap Al 3	193	250	772
Ap Al 4	240	250	960
At 1	19	250	76
At 2	73	250	292
At 3	11	250	44
At 4	14	250	56
Ap At 1	184	250	736
Ap At 2	140	250	560
Ap At 3	177	250	708
Ap At 4	185	250	740
Pc 1	165	50	3300
Pc 2	99	50	1980
Pc 3	117	50	2340
Pc 4	208	50	4160
Ap Pc 1	182	250	728
Ap Pc 2	195	250	780
Ap Pc 3	192	250	768
Ap Pc 4	176	250	704

Tab. 13 *Copepods counted from each treatment incubator in copepods feeding on* A. limii*,* A. tamarense *and* P. cordatum *goniodomin induction experiment*.

Copepods	Organisms counted	Sample volume (μL)
Al 1	39	15000
Al 2	17	15000
Al 3	37	15000
Al 4	43	15000
At 1	27	15000
At 2	34	15000
At 3	25	15000
At 4	17	15000
Pc 1	23	15000
Pc 2	10	15000
Pc 3	10	15000
Pc 4	14	15000

6.2 Cell counts of *Rhodomonas salina* bioassays

Tab. 14 *Settings and results of strain A* R. salina *bioassay, counts of settings were used to prepare the bioassay with the correct microalgal concentrations. In the results* R. salina *density was not calculate as they had no purpose for the experiment. Also* R. salina *cells were counted in the portion of the counting chamber represented by the diameter observable through the microscope.*

Settings	Sample Volume (µL)	Cells counted	Density (cells/mL)
R. salina	50	1139	710915
Ap A	100	296	2960
Results			
C 1	500	205	
C 1	500	201	
C 1	500	204	
C 2	500	196	
C 2	500	199	
C 2	500	194	
C 3	500	192	
C 3	500	200	
C 3	500	203	
3,9 S 1	500	31	
3,9 S 1	500	31	
3,9 S 1	500	27	
3,9 S 2	500	35	
3,9 S 2	500	37	
3,9 S 2	500	36	
3,9 S 3	500	16	
3,9 S 3	500	15	
3,9 S 3	500	17	
2 S 1	500	80	
2 S 1	500	63	
2 S 1	500	79	
2 S 2	500	96	
2 S 2	500	82	
2 S 2	500	79	
2 S 3	500	61	
2 S 3	500	66	
2 S 3	500	77	
1 S 1	500	160	
1 S 1	500	159	
1 S 1	500	155	
1 S 2	500	142	

1 S 2	500	133
1 S 2	500	146
1 S 3	500	165
1 S 3	500	155
1 S 3	500	157
0,5 S 1	500	178
0,5 S 1	500	177
0,5 S 1	500	186
0,5 S 2	500	170
0,5 S 2	500	165
0,5 S 2	500	159
0,5 S 3	500	189
0,5 S 3	500	164
0,5 S 3	500	161
0,2 S 1	500	206
0,2 S 1	500	208
0,2 S 1	500	186
0,2 S 2	500	174
0,2 S 2	500	156
0,2 S 2	500	176
0,2 S 3	500	175
0,2 S 3	500	199
0,2 S 3	500	191
0,1 S 1	500	193
0,1 S 1	500	193
0,1 S 1	500	191
0,1 S 2	500	186
0,1 S 2	500	188
0,1 S 2	500	201
0,1 S 3	500	185
0,1 S 3	500	213
0,1 S 3	500	202

Tab. 15 *Settings and results of strain B* R. salina *bioassay*

Settings	Sample Volume (μL)	Cells counted	Density (cells/mL)
R. salina	50	641	400085
Ap B	100	385	3850
Results			
C 1	500	191	
C 1	500	214	
C 1	500	194	
C 2	500	199	
C 2	500	225	
C 2	500	210	
C 3	500	184	
C 3	500	200	
C 3	500	196	
3,9 S 1	500	1	
3,9 S 1	500	2	
3,9 S 1	500	1	
3,9 S 2	500	1	
3,9 S 2	500	2	
3,9 S 2	500	4	
3,9 S 3	500	0	
3,9 S 3	500	0	
3,9 S 3	500	1	
2 S 1	500	5	
2 S 1	500	7	
2 S 1	500	3	
2 S 2	500	4	
2 S 2	500	3	
2 S 2	500	2	
2 S 3	500	5	
2 S 3	500	4	
2 S 3	500	3	
1 S 1	500	13	
1 S 1	500	9	
1 S 1	500	11	
1 S 2	500	7	
1 S 2	500	9	
1 S 2	500	11	
1 S 3	500	14	
1 S 3	500	8	
1 S 3	500	10	
0,5 S 1	500	35	

0,5 S 1	500	41
0,5 S 1	500	35
0,5 S 2	500	43
0,5 S 2	500	39
0,5 S 2	500	55
0,5 S 3	500	44
0,5 S 3	500	43
0,5 S 3	500	53
0,2 S 1	500	117
0,2 S 1	500	115
0,2 S 1	500	123
0,2 S 2	500	134
0,2 S 2	500	124
0,2 S 2	500	135
0,2 S 3	500	92
0,2 S 3	500	100
0,2 S 3	500	97
0,1 S 1	500	158
0,1 S 1	500	159
0,1 S 1	500	149
0,1 S 2	500	167
0,1 S 2	500	164
0,1 S 2	500	165
0,1 S 3	500	173
0,1 S 3	500	175
0,1 S 3	500	172
0,05 S 1	500	194
0,05 S 1	500	195
0,05 S 1	500	193
0,05 S 2	500	194
0,05 S 2	500	197
0,05 S 2	500	197
0,05 S 3	500	198
0,05 S 3	500	195
0,05 S 3	500	198

Tab. 16 *Settings and results of strain C* R. salina *bioassay*

Settings	Sample Volume (µL)	Cells counted	Density (cells/mL)
R. salina	50	671	418810
Ap C	100	430	4300
Results			
C 1	500	283	
C 1	500	242	
C 1	500	257	
C 2	500	248	
C 2	500	251	
C 2	500	248	
C 3	500	286	
C 3	500	273	
C 3	500	250	
3,9 S 1	500	22	
3,9 S 1	500	14	
3,9 S 1	500	21	
3,9 S 2	500	8	
3,9 S 2	500	8	
3,9 S 2	500	9	
3,9 S 3	500	10	
3,9 S 3	500	10	
3,9 S 3	500	9	
2 S 1	500	54	
2 S 1	500	39	
2 S 1	500	45	
2 S 2	500	42	
2 S 2	500	43	
2 S 2	500	60	
2 S 3	500	48	
2 S 3	500	42	
2 S 3	500	44	
1 S 1	500	177	
1 S 1	500	167	
1 S 1	500	209	
1 S 2	500	173	
1 S 2	500	188	
1 S 2	500	179	
1 S 3	500	176	
1 S 3	500	168	
1 S 3	500	189	
0,5 S 1	500	299	

0,5 S 1	500	320
0,5 S 1	500	238
0,5 S 2	500	217
0,5 S 2	500	256
0,5 S 2	500	289
0,5 S 3	500	265
0,5 S 3	500	224
0,5 S 3	500	254

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