Towards characterization of lytic compound(s) produced by *Alexandrium tamarense*

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Abstract
We investigated optimal conditions for characterization of bioactivity of lytic compound(s) excreted by *Alexandrium tamarense* based on a cell-bioassay system. Allelochemical response of the cryptophyte *Rhodomonas salina* indicated the presence of lytic compound(s) in a reliable and reproducible way and allows for quantification of this lytic effect. The parameters tested were the incubation time of putatively lytic extracts or fractions with the target organism *R.salina*, different techniques for cell harvest from *A.tamarense* cultures and the optimal harvest time. A three hour incubation time was found to be optimal to yield a rapid response while accurately estimating effective concentration (EC_{50}) values. Harvest of *A. tamarense* cultures by filtration resulted in loss of lytic activity in most cases and centrifugation was most efficient in terms of recovery of lytic activity. Maximum yield of extracellular lytic activity of *A. tamarense* cultures was achieved in the stationary phase. Such optimized bioassay guided fractionation techniques are a valuable asset in the isolation and eventual structural elucidation of the unknown lytic substances.

Introduction:
The dinoflagellate genus *Alexandrium* (Halim) contains more than a dozen species capable of producing known marine phycotoxins. These well-described toxins – the saxitoxin group associated with paralytic shellfish poisoning (PSP) (Anderson 1998) and spirolides (Cembella *et al.* 2000), are potent neurotoxins in mammalian systems, but their ecological role and possible function as allelopathic agents is controversial and not well defined. In addition to the known neurotoxins, several *Alexandrium* produce allelochemical substances causing immobilization and lytic effects upon exposure to both planktonic heterotrophs and autotrophs. Toxic effects include (for flagellates) loss of flagellae, and for diverse taxa membrane disruption often resulting in cell lysis, or formation of temporary cysts (Tillman *et al.* 2008a). These lytic effects are clearly not attributable to either paralytic shellfish poisoning (PSP) toxins (PSP) or to spirolides.

The general objective of this study was to provide a basis and first steps towards a bioassay-guided characterisation of physico-chemical properties of lytic compound(s) excreted by *Alexandrium tamarense*.

Materials and Methods:
Cell culture:
One clonal culture (Alex2) from a large collection of *A. tamarense* isolates from the Scottish coast of the North Sea was chosen based on a quantitative comparison of lytic potency (Tillmann *et al.* submitted). This highly lytic clone was grown and harvested as described by Tillmann *et al.* (submitted)

Bioassay system:
A microalgal bioassay was used to quantify lytic activity based on the response of the autotrophic cryptophyte *Rhodomonas salina* (Kalmar culture collection strain KAC 30) as the target species. The *R. salina* strain was cultured under the same conditions as for Alex2. The bioassay was set up in a total volume of 4 ml in 6 ml glass vials. Each sample was spiked with 0.1 ml of *R. salina* culture which was adjusted (based on microscope cell counts) to 4 x 10^5 cells ml^{-1} to reach a start concentration of 1 x 10^4 ml^{-1} of the target cells. Culture medium in triplicate served as control. Samples were incubated for various incubation times in the dark (to restrict *R. salina* growth) at 15 °C. Subsequently, samples were fixed with 2% Lugol's iodine solution and cell concentrations of both *A. tamarense* and the intact target species were counted (from 0.5 ml) by inverted microscopy (Zeiss Axiovert 40C). A sub-area corresponding to at least 600 *Rhodomonas* cells in the control was counted. Percentage of intact cells of *Rhodomonas* was calculated as: Rho_{final}/ $Rho_{control}$*100% where Rho_{final} is the experimental final *Rhodomonas* cell concentration, and $Rho_{control}$ the final target cell concentration in controls.

Estimates of EC_{50}, i.e. the volume of Alex2 supernatant yielding a 50% decline in target cell concentration, were determined by fitting data points (log transformed percentage of sample as x values) to a sigmoid equation using the non-linear fit procedure of Statistica (Statsoft, Germany): $N_{final} = N_{control} / [1+(X/EC_{50})^{h}]$

where N_{final} is the experimental final target percent intact cell, $N_{control}$ the final target cell percentage in controls (100%), X the log-transformed amount of Alex 2 sample (as µl sample in the 4 ml test system), and EC_{50} and h are fit-parameters. Results are expressed as EC_{50} including 95% confidence intervals.

Experiments:
First, the optimal incubation time to quantify lytic extracellular compounds with *R. salina* as target organism was assessed. A supernatant of Alex2 culture was prepared by centrifugation (3220 × *g*, 15 °C for 10 min) of a dense stationary phase culture. The bioassay of supernatant effect on *R. salina* was performed for 1, 3, 6, 12, 24, 48 and 72h. For each incubation time, the bioassay was performed in duplicate, using a ten-point dilution series (3.9, 2, 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, and 0.005 ml).

Next, the optimal method to remove the cells from the medium containing the extracellular lytic compounds with the highest yield of lytic activity was evaluated. A dense culture in stationary phase was centrifuged as described above. In parallel, culture filtrate was prepared by gentle vacuum filtration of 25 ml culture volume upon either polycarbonate membranes (Millipore Isopore, 45 mm diameter) of 7 different pore sizes ranging from 8 to 0.1 µm or glass fibre filters (Whatman GF/C, nominal pore size 1.2 µm; 45 mm diameter). For each sample, the *Rhodomonas* bioassay was performed as described, using a seven-point dilution series (3.9, 2, 1, 0.4 0.1, 0.5, and 0.025 ml) in triplicate. Based on results of this experiment, all subsequent experiments were performed with supernatant prepared as described above, and stored at 4 °C until use.

As samples with high amounts of lytic compound(s) were desired for further isolation and characterization, the next step was to determine the optimal harvest time. The lytic potency of the cell culture under different growth phases was tested. Triplicate cultures were set up, and growth was monitored by cell counts after 0, 1, 3, 6, 10, 14, 17, 21, and 29 days. Simultaneously, on each sampling day, subsamples were removed from each culture and lytic activity was determined. As cell numbers increased during the experiment, the number of dilutions for each test series was increased from 3 to 10 (duplicate samples for each dilution).

Results and Discussion:
The final percentage of intact *Rhodomonas* cells strongly depends on the sample concentration (Fig. 1A). Although cell lysis is a fast process, occurring after only a few minutes at high doses (Tillmann *et al.* 2008b) the sigmoid dilution curve is different for changing incubation times. (Fig.1A). EC_{50} values calculated for each dilution curve showed a strong decrease for a 3 h compared to 1 h incubation, whereas longer incubation resulted in only minor further decreasing EC_{50} values (Fig. 1B). The effect of incubation time is most obvious at a low concentration of bioactive sample (i.e. at 50 µl, Fig. 1B) where intact *R. salina* cells decreased from 100% (1 h) to <10% by 72 h. This phenomenon may be related to the threshold-dependent mode of action of the compounds. When the concentration of lytic compounds is relatively low, an extended exposure time is likely required to achieve a critical encounter rate and accumulation at the putative target on the membranes of *Rhodomonas* cells, thereby inducing the lytic effect. In any case, as incubating for 3 h apparently allowed quantification of lytic activity, this incubation time was chosen for subsequent experiments. This incubation time allows a quick response (on the same day, an advantage for a bioassay) by reducing potential negative secondary effects of longer incubation time (like growth of the target or growth of bacteria).

When the pore size was ≥0.4µm, most of the lytic compounds passed through the polycarbonate filters (Fig.2). However, when pore size was ≤ 0.2 µm, no lytic activity was found in the filtrate. For glass fibre filters with a nominal pore size of 1.2 µm, a much larger loss compared to polycarbonate filters of comparable pore size was observed. This indicates that adsorption to the filter material is to a large extent responsible for loss of activity. This is supported by the observation that small amounts of lytic activity were found in filtrate of 0.2 µm

filters when the filtered volume was increased to > 100 ml (results not shown). Lytic activity in the supernatant was unchanged as compared to the whole cell culture. Centrifugation is the most convenient and accessible way to obtain cell-free samples with no obvious loss of lytic activity, therefore cell free supernatants will be collected for use in future separation and isolation steps.

In the exponential phase (Day 0-6, Fig 3), the lytic activity, represented by the EC_{50} value, remained unchanged at about 100-110 cells ml^{-1}. However, when cell growth decelerated and finally stopped in stationary phase, the lytic activity on a cellular basis increased (as indicated by a decreasing EC_{50} value) down to about 50 cells ml^{-1} and stayed almost constant even in old stationary cultures after 29 days (Fig 3).

We did not attempt to determine production rate of lytic compounds in these experiments, as this would require detailed knowledge of the rate of degradation of these extracellular compounds, and the structures and hence concentrations remain unknown. Nevertheless, the results indicate that during exponential growth, production rates are consistent with cell growth, whereas later on when growth slows, production of compounds continues, leading to a pronounced accumulation of lytic compounds in stationary phase.

To conclude, this study represents a preliminary effort to optimize the expression and measurement of lytic activity in *A. tamarense* by determining dose-responses under controlled conditions. In conjunction with a reliable cell bioassay (e.g. *Rhodomonas* test), further attempts to characterise and identify these lytic extracellular compounds will be follow bioassay guided fractionation techniques. The optimal bioactive fractions are obtained as cell free supernatant, harvested from dense cultures in stationary phase. With the optimization of the protocol for induction and harvest of lytic activity from *A. tamarense* we have made a significant advance in the ultimate objective of defining the mode of action and determining the chemical structures of these intriguing lytic compounds.

References

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Figure legends

Fig. 1 Percentage of intact cells of *R. salina* exposed to Alex2 cell-free culture supernatant. Target cell numbers after 1, 3, 6, 12, 24, 48, and 72h incubation were scaled to final control cell numbers. (A) Complete dilution series expressed as duplicate mean ± 1SD. (B) EC_{50} values (sample % in 4ml bioassay) and 95% confidence interval.

Figure 2 Percentage of intact cells of *R. salina* exposed to Alex2 cell culture, cell culture filtered by Millipore filters (pore size range from 8 to 0.1µm) and GF/C filters (pore size 1.2µm), or to cell-free supernatant. Target cell numbers after 3h incubation were scaled to final control cell numbers. Results expressed as triplicate mean ± 1SD.

Figure 3 Cell concentration (ml^{-1}) of *A. tamarense* and EC_{50} value (cell ml^{-1}) against time (d) of culture growth. Results expressed as triplicate mean ± 1SD.

Fig. 1

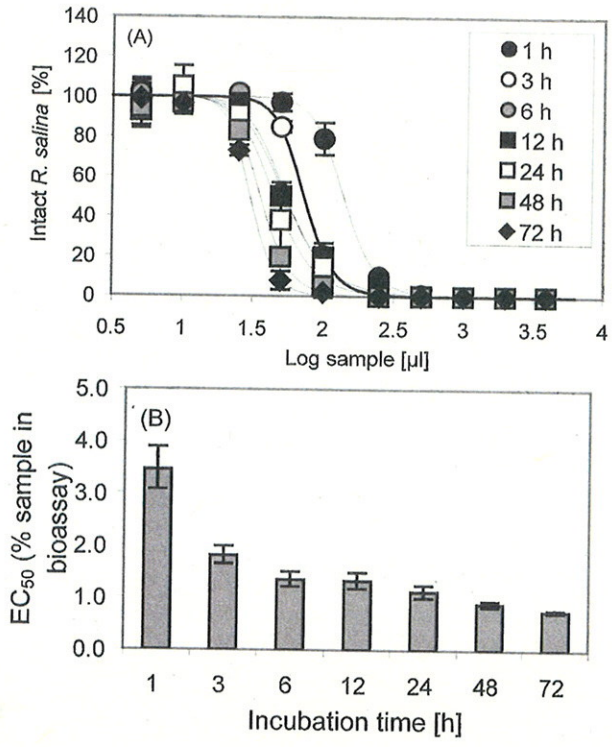

(A)
Intact R. salina [%]
Log sample [µl]
1 h
3 h
6 h
12 h
24 h
48 h
72 h
(B)
EC50 (% sample in bioassay)
Incubation time [h]

Fig. 2

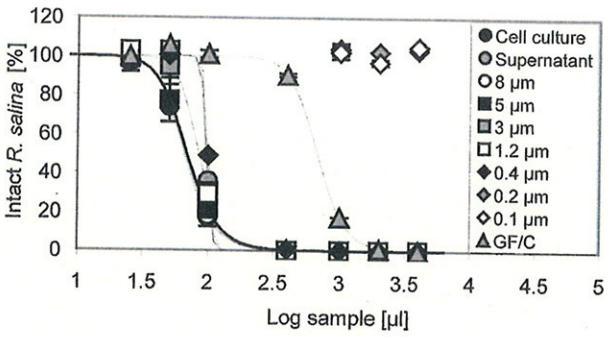
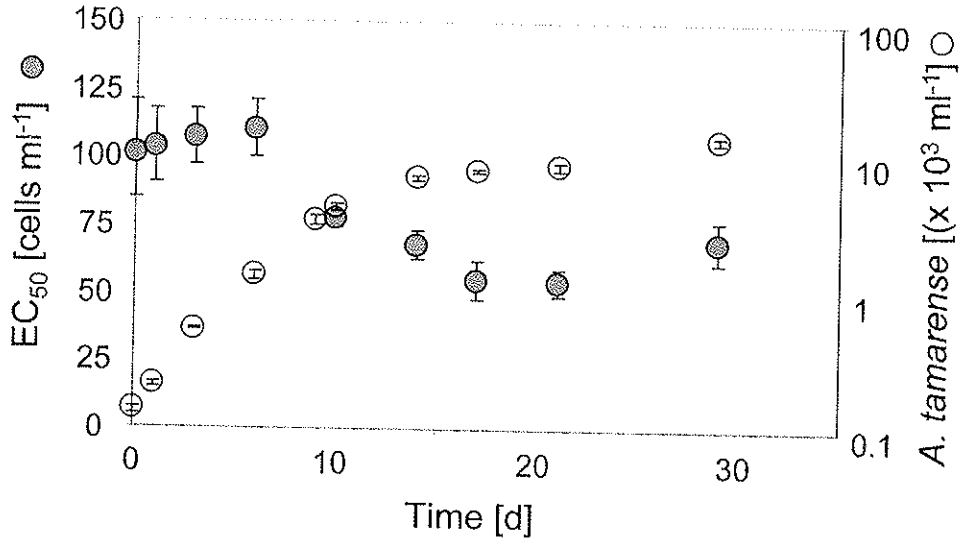

Intact R. salina [%]
Log sample [µl]
Cell culture
Supernatant
8 µm
5 µm
3 µm
1.2 µm
0.4 µm
0.2 µm
0.1 µm
GF/C

Fig. 3


150
125
100
75
50
25
0
EC_{50} [cells ml^{-1}] ●
0
10
20
30
Time [d]
100
10
1
0.1
A. tamarense [(x 10^3 ml^{-1}] ○