CELL DEATH AND VIABILITY IN MARINE PHYTOPLANKTON

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BACKGROUND

Active cell death or more commonly named "Programmed Cell Death" (PCD) or apoptosis was first employed by Kerr and colleagues (Kerr et al., 1972) to define the morphological processes leading to a controlled cell self-destruction, characterized by both morphological (cell shrinkage, membrane "blebbing", formation of apoptotic bodies, chromatin condensation, DNA fragmentation) and biochemical features (activation of certain proteolitic enzymes called caspases, alterations in membrane assimetry, releasing of cytochrome C by mitochondria) that affects individual cells (rev. Gewies, 2003). This process was in contrast to the necrosis mode of cell-death in which the cells suffer a passive accidental death, usually very rapid (Willingham 1999) showing a loss of plasma membrane integrity, swelling and then lysis, affecting the rest of the cells of the organism (Kerr et al., 1972). Recently, it has been demostrated that the core components of the active cell death machinery are conserved through evolution (Ameisen 2002, Richardson 2002).

Mortality of phytoplankton populations has often been attributed to external factors such as grazing, sedimentation, viral infection or environmental stress among others (Walsh 1983, Bratbak et al., 1999). However, recent studies suggest that cell death and mortality in phytoplankton could be a consequence of an active cell death process. Thus, it was found that the unicellular chlorophyta *Dunaliella tertiolecta* undergo cell mass cell death when placed in darkness (Franklin and Berges 1998) and it is inhibited by caspase inhibitors and shows DNA fragmentation (Segovia et al., 2003). In the same way, both the chlorophyta *Chlamydomonas reinhardtii* and the diatom *Thalassiosira pseudonana* have caspase orthologues (Bidle and Falkowski 2004) and the dinoflagellate *Peridinium gatunense* shows an active programmed cell death in response to CO_2 limitation and oxidative stress (Vardi et al., 1999).

This work try to study the use of some fluorochromes and stains to study viability and cell death in two dinoflagellate species, one armored (*A. minutum*) and the other naked (*K. brevis*), both bloom-forming. At the same time, some natural samples from Ría de Vigo and Pontevedra (NW Spain) were analyzed during the winter-spring of 2005.

MATERIAL AND METHODS

•**Laboratory cultures-** Cultures of the dinoflagellates *Alexandrium minutum* (Al 1V) from the COV-IEO culture collection (Spain) and *Karenia brevis* (CCMP 2281) from the CCMP culture collection (USA) were grown in 400 ml of L1 medium in a 14:10h LD cycles, at 19ºC. After three cycles of growing, a subculture of 200 ml from each one of the species were placed in dark (aluminum foil) at the 9th day of growing. Samples for counting cells (in triplicate by lugol´s technique), photosynthetic capability (by measuring Fv/Fm fluorescence emission in a PAM fluorimeter) and staining with different fluorochromes were taken every 2-3 days in both cultures, with and without light.

•**Field samples-** Net hauls (20 µm mesh) were collected in spring 2005 during proliferations of *Dinophysis* spp. in the Ría de Vigo and Pontevedra (Galicia, NW Spain) and observed lived under an inverted microscope Zeiss-Axiovert. Pictures were taken with a Canon D-60 camera at magnifications of 10x and 40x. Samples were processed as soon as they arrived to the lab (usually, 2 hours after collection).

•**Viability studies-** Several dyes were used to study viability and cell death in both laboratory and field samples:

- *SYTOX Green* (504/523nm, Molecular Probes): Dye was added at a final concentration of 5 µM, incubated for 15 min in dark and looked under the microscope with a blue filter.
- *Trypan Blue* (Sigma): Trypan Blue was added to the sample at 0.4% (w/w) and incubated for 30 minutes.
- *Fluorescein diacetate* (490/515nm, Sigma): Stock solution of 5 mg/ml was made in DMSO and stored at -20ºC. A final concentration of 5 µM and 30 min of incubation was employed for every sample.
- *Calcein AM* (496/520 nm, Molecular Probes): 1 mM stock solution was diluted in filtered sw and then added to the sample at a final concentration of 20 µM with an incubation time of 30 min.

•**Cell death studies:**:

- *AnnexinV-AlexaFluor 350* (346/442, Molecular Probes): AnnexinV-AlexaFluor 350 was prepared following manufacturer instructions. It was used in combination with SYTOX-Green, and added to the cultures at the end of the growth phase (early to very late stationary phase) and also to some of the field samples .Samples were looked under the microscope using a UV filter.

Alexandrium minutum Al 1V

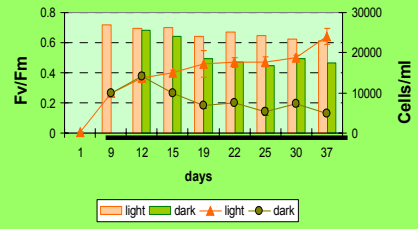

Figure 1- Growth (lines) and photosynthetic capacity (bars) of *A. minutum* Al 1V in both light (orange) and dark (green). Horizontal black line represents the dark period.

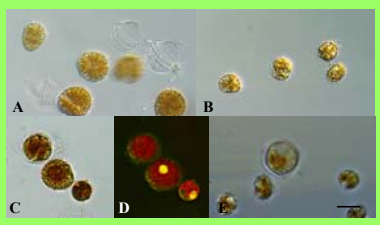

Figure 3- Light microscope images showing differences in Al 1V aspect. (A): cells from a 22 days-stationary phase culture in light. (B): cells from the same day as (A) but growing in dark. Note the smaller size. (C-D): C: cells from a 30 days culture in light, D: autofluorescence of the cells, showing a light spot. (E): cells from a 37 days-culture maintained in dark for 27 days; note sizes. Bar = 20 µm

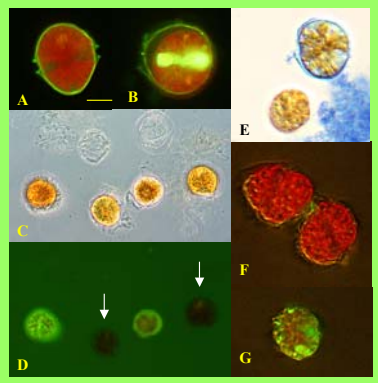

Figure 2- Al 1V stained with different dyes along the cell cycle. (A): single cell from a exponential phase culture (day 7th) in light stained with SYTOX-Green. (B): cell from exponential phase (7th) treated with SYTOX. Nucleus is stained with the dye. (C-D): cells from a very old culture (37 days) in light treated with FDA. Live cells show green fluorescence while dead cells (arrows) show only red autofluorescence. (E): dead (blue stained) and live cell (no staining) in stationary phase (22 days, 12 of them in dark) treated with Trypan Blue. (F): dead cells (only red autofluorescence) from an exponential culture (day 9) treated with Calcein-AM. (G): Live cell in exponential phase stained in green by Calcein-AM. Bar represents 10 µm except for C-D where represents 20 µm.

• After 37 days of being cultured in light and 27 in dark, Al 1V cells does not show a significant difference in photosynthetic activity (Fv/Fm).

• However, cells show a decrease in size as well as in cell number, specially in those cultures maintained in dark.

•The fluorochromes SYTOX-Green and Calcein-AM do not show clear results to be used to distinguish between live and dead cells.

•Fluorescein diacetate (FDA) appears to be the best dye to distinguish between live and dead cells in *A. minutum*. Cells continue swimming after adding of the dye for at least 24 hr.

•Trypan Blue appears to be as the non-fluorescent "dye" better to use to differentiate dead cells.

•No staining of AnnexinV-AlexaFluor were observed at all (data not shown).

Field samples

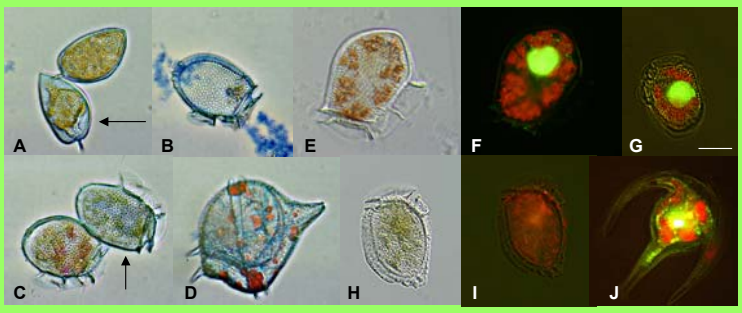

Figure 1: (A, C): Live field samples from Ría de Vigo (NW Spain) treated with Trypan Blue. Cells with an arrow show cells stained with TB, ie dead cells. (B, D): Dead cells from a dead field sample from Ría de Pontevedra. (E-G): Cells treated with SYTOX, (E-F): Live cell of *Dinophysis acuta* from a field sample treated with SYTOX. See the green color of the nucleus that would correspond to a dead cell, (G): dead cell of *Dinophysis acuminata* killed with sodium azida stained with SYTOX. (H-I): Live specimen of *D. acuminata* stained with Calcein-AM. Only red autofluorescence is observed. (J): Live cell of *Ceratium* stained with SYTOX. All pictures taken at 40x. Barr =20µm.

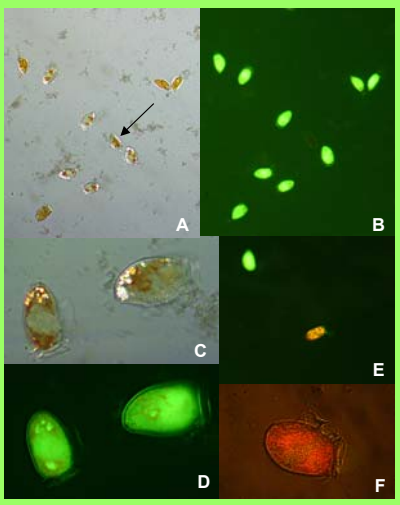

Figure 2: Field samples treated with Fluorescein Diacetate FDA. (A-B): field sample with abundance of *Dinophysis acuminata* and *Prorocentrum micans* from Ria de Pontevedra (NW Spain). Live cells have green colour while dead cells (arrow) only show autofluorescence. (C-D): Live cells of *D. acuminata* treated with FDA. (E): Live cell of *P. micans* (green) and dead cell (red) of *D. acuminata*. (F): Dead cell of D. acuminata treated with FDA. Pictures A, B and E taken at 10x, the rest at 40x.

• Fluorescein Diacetate is the fluorochrome that better distinguish among dead and live cells for field samples.

•Trypan Blue is also a good option to be used to stain dead cells, although the time of incubation is longer than with FDA, which can be of almost 1 minute.

• However, SYTOX and Calcein-AM are not able to differentiate between dead and live cells. SYTOX stain both, dead and live cells while Calcein-AM does not stain none.

Karenia brevis CCMP 2281

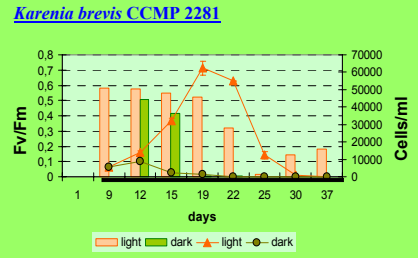

Figure 1- Growth (lines) and photosynthetic capacity (bars) of *K. brevis* CCMP 2281 in both light (orange) and dark (green). Horizontal black line represents the dark period.

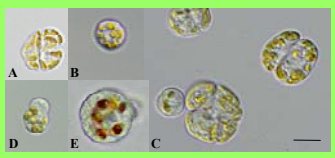

Figure 2- Light microscope images showing CCMP2281 morphology. (A): single cell from a 5-day exponential culture maintained in light. (B): cell from a 15-days culture, 5 of them maintained in dark. Note the smaller size, the vacuolation and the chlrorophyll condensation. (C): cells of different sizes from a 19-days culture in light. (D): cell from the same state-of-growh (19 days culture) but being maintained 9 days in dark. Cells become smaller and with "weird" morphologies. (E): cell from a 30 days-culture maintained in dark for 20 days, in a "semicyst" state. All pictures were taken at 40x. Bar = 20 µm

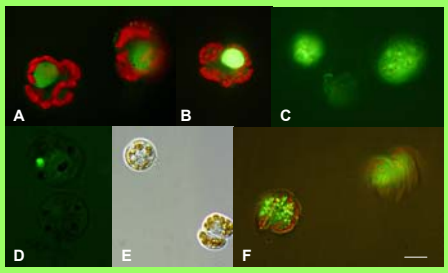

Figure 3- CCMP2281 stained with different dyes along the cell cycle. (A): cells from a exponential phase culture (day 5 th) in light stained with SYTOX-Green. (B): cell from a very old culture of 25 days treated with SYTOX. No difference is observed from the exponential phase(A). (C): cells from a late- exponential phase (15 days) in light treated with FDA. Live cells show green fluorescence. (D): cells from a culture in death-phase (37 days) treated with FDA. No fluorescence is observed at all. (E): "old" cells (15 days of culture, 5 of them in dark) treated with Trypan Blue. No blue color is observed. (F): cells from an exponential culture (day 12) treated with Calcein-AM, one of them swimming around. Bar = 20 µm.

• CCMP2281 appear to be very sensitive to dark, as only after 5 days of being in dark, cell number as well as Fv/Fm decay very fast. Cells show a decrease in size and a change in their morphology.

• SYTOX-Green and Trypan Blue do not show clear results to be used to distinguish between live and dead cells. Exponential cells as well as "very old" cells were stained with SYTOX, while TB did not stain them.

•As with Al 1V, FDA appears to be the best dye to distinguish between live and dead cells of *K. brevis*. Cells continue swimming after adding of the dye for at least 5 hr.

•Early apoptosis or, at least, traslocation of the phosphatidylserine residues to the outer face of the membrane, can be observed by using the fluorochome AnnexinV-AlexaFluor 350. This fact was only observed in very old cultures maintained in light (30 days) or in cultures maintained in dark (22 days of culture, 12 of them in dark).

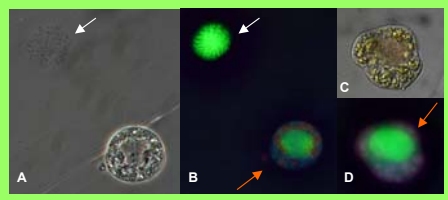

Figure 4- a very old CCMP2281 (30 days) treated with AnnexinV-Alexa-Fluor 350 and SYTOX-Green. (A): a single cell and a nucleus (arrow). (B): same field photographied with a UV filter. The nucleus brights green due to the SYTOX, while the blue colour is due to the AnnexinV-AlexaFluor350 bound to the membrane (orange arrow). (C-D): same cell photographied with and without fluorescence. All pictures were taken at 40x.

RESULTS AND DISCUSSION

•*A. minutum* showed an unusual type of growth, as even after 37 of culture in light or even after 27 days of being in dark, they have a good photosynthetic activity, with values of Fv/Fm of around 0.6 (light) or 0.46 (dark). Their morphology, however, changed a lot and thus, cells became smaller, vacuolated and adopted amorphous forms, while some of them became chlorotic and other showed yellow spots when observed under epifluorescence. This is in concordance with the majority of the studies related to mortality, where cells lost their "normal" morphology and become shrinkaged, vacuolated and less pigmentated.

•FDA has been the most useful dye to use with both lab cultures and field samples. Although is not a fluorescent molecule, when penetrates cells is cleaved by the esterases yielding a green fluorescent colour in the citoplasm (Coleman and Vestal 1987). This means that only live cells with active enzymes are capable of using the FDA. All the results were very consistent throughout all the repetitions of the experiments and the different field samples. This is also consistent with studies with *Amphidinium carterae* (Franklin and Berges 2004) although other authors were not able to differentiate dead from live cells of *Dunaliella salina* and *Chlamydomonas reinhardtii* (Markelova et al., 2000).

•SYTOX-Green is a DNA-specific dye that easily penetrates into cells with compromised membrane, staining cell nuclei in green (Veldhuis et al., 2001). It is considered a "mortal stain" in contrast to FDA. Although it has been previously used successfully with lab cultures (*A. carterae*, Franklin and Berges 2004 or *P. pouchetii*, Brussaard et al., 2001)) and also in phytoplankton communities (Veldhuis et al., 2001), it did not work with our lab cultures nor with the field samples. Even in field samples, observed just in the moment they were taken up, SYTOX stained all the nuclei of the cells even though they were swimming around. They were clearly alive but their nuclei was stained.

•Calcein-AM and Trypan Blue did not give consistent results. Calcein is vital dye used to stain live cells with metabolic esterase activity (Porter et al., 1995). Apparently, it can work well with cultures of *K. brevis* and *A. minutum*, but no results were obtained when working with natural samples. None of the samples observed during winter-spring 2005 (ca. once every two weeks) showed "positive" binding to Calcein even though cells were healthy and alive. Trypan Blue, a colorimetric stain that easily penetrates dead cells staining the citoplasm in blue, however, gave good results with natural samples and also with cultures of *A. minutum*, bur *K. brevis* was never stained with this dye.

•AnnexinV-AlexaFluor350 is a specific dye used to study apoptosis by detecting the phosphatidylserine traslocation that occurrs in early apoptotic cells, specially in mammalian cells (Vermes et al. 1995). Membranes of apoptotic cells appear in blue while normal cells does not. Promising results were obtained when using Annexin with *K. brevis* old/dark cultures, however no positive results were seen neither in *A. minutum* nor in field samples.

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