Review Article

A short review on the halotolerant green microalga *Asteromonas gracilis* Artari with emphasis on its uses

ABSTRACT

The halotolerant green single-celled *Asteromonas gracilis* isolated from the hypersaline saltern ponds of Messolonghi, Greece, was kept in laboratory cultures, grown effectively at various salinities and used for feeding rotifers, protozoa, copepods and *Artemia* sp. In all feeding trials all filter feeders accepted *A. gracilis* and grew. Additionally as of its morphology, movement and culture reliability *Asteromonas* is a valuable teaching tool for phycological studies, supreme to the other usual microalgae for this purpose. A lot of research awaits for the potential exploitation of *A. gracilis* in many sectors as data in the literature are scarce.

Comment [LP1]: The exploitation potential of *A. gracilis* in many sectors awaits research results, as literature data are scarce.

Key words: Halotolerance, green algae, *Asteromonas gracilis*

INTRODUCTION

There is a worldwide (especially during the previous decade) bursting of publications on applied phycology. This is partly due to the interest concerning the useful products of algae (biofuels, antioxidants, etc.) and partly to the role of algae and especially microalgae in their beneficial effect on environment (CO_2 sequestering, ~~bioremedetion~~bioremediation, etc.). Because of this there is no surprise that phycological studies are gaining momentum in university curricula. Additionally, experimentation with algae offers many advantages due to the rapid multiplication, rapid response to culture stimuli and ease of handling. However not all algae are the same, on the contrary, among their numerous species ~~—~~there are easily manipulated and very cumbersome ones. Considering ~~microalgae~~microalgae, I will try to highlight a very little studied one that is the chlorophyte *Asteromonas gracilis* ~~(~~Artari~~,~~ 1913~~)~~. This species can offer certain advantages to researchers and teachers in terms of ease of handling, isolation, culturing and enumeration. If the initial purpose of a phycology course is to intrigue the students to handle effectively microalgae, then *Asteromonas* is a perfect choice. In the present study I combine knowledge accumulated for *Asteromonas* from review of the literature with my personal experience on this species over the years.

There is paradoxically a big lack of research interest for the halotolerant green microalgae, *A. gracilis*. For the first time it was described from Crimean saline pools by Artari in 1913 and then it has been found in many other similarly saline environments worldwide. Artari's description (1913) and subsequent works of others (Wislouch, 1924; Smith, 1933; as quoted in Peterfi & Manton, 1968) elucidated beyond doubt that this species is what was first reported as *Stephanoptera fabreae* by Dangeard (1910, 1912; as quoted in Peterfi & Manton, 1968). After that time it was quite away in the past (1968) that Peterfi and Manton described in detail the morphology of this species and then its halotolerance became widely known by the pioneered work of Ben-Amotz & Grunwald (1981) and since then it fell in almost oblivion. Only recently Fawzy et al. (2014) and Fawzy (2017) studied *A. gracilis* as a potential source for β-carotene and lipids. Meanwhile Hotos and Avramidou (1995) and Hotos (2002 & 2003) studied its culture in various conditions and its use as food for the marine rotifer *Brachionus plicatilis* respectively. Life in the extreme hypersaline environment is harsh and only few halotolerant or halophilic~~halophilous~~ species can survive and even more, thrive by means of their physiological adaptation mechanisms. Microalgae have few representative species able to tolerate hypersalinity. Furthermore life in hypersalinity has not been studied adequately and particularly in Greece data are actually lacking.

A. gracilis (Family: Asteromonadaceae, Order: Chlamynomonadales, Class: Chlorophyceae, Division: Chlorophyta) occurs only as solitary cells of variable spindled shape from narrow to broad (Fig 1-A-C). Cell size varies greatly 12-22 μm in length and 8-16 μm in breadth. The widest part of the cell lies to about a third of the way from the anterior end. From the anterior part which is wider than the rear emerge two flagella laterally from the median slightly pointed papilla. The flagella are equal in size and their length is about 1.5 times that of the longitudinal cell length. The species is characterized by the absence of a call wall and because of this presents high flexibility-plasticity able to temporarily

change its shape depending on the medium salinity; swollen and round in dilution, compact and elongated in hypersalinity. The external of the cell lacks scales but according to electron microscopy described by Peterfi & Manton (1968) there are varied amount of fibril deposits on the flagella and body surfaces. Along its cell 6 longitudinal keel-like ridges offer a stellar appearance when looked transversely (Fig. 1-B, Fig. 2-B). In side view these keels look transparent giving the impression of wing like appendages. The color of the cell is green and only a faint small orange eyespot is seen on the anterior side.

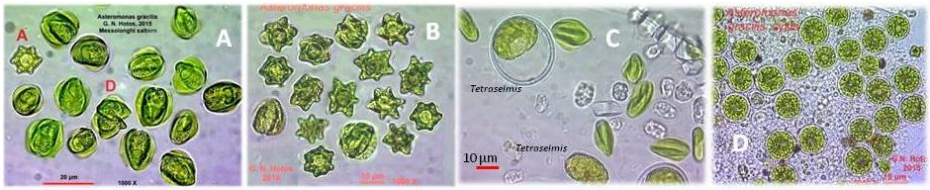

Figure 1. A- *Asteromonas gracilis* in its most usual form (A: indicates a stellate appearance, D: cell division). B- Stellate-shaped cells. C- Cells of *A. gracilis* among salt crystals, there also depicted 2 cells of *Tetraselmis marina* one in palmelloid stage and one vegetative in order to compare sizes. D- Cysts of *A. gracilis*.

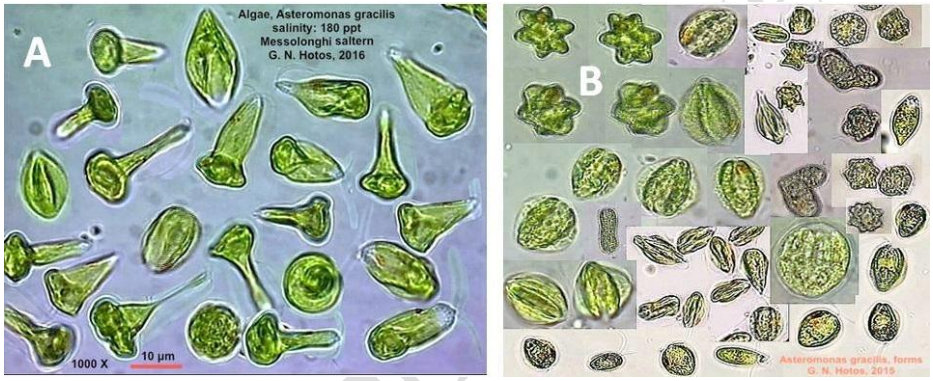

Figure 2. A- *A. gracilis* in nail-like form in very high salinity (180 ppt). B- A collage of *A. gracilis* various forms.

Figure 3. A- (1-5) advancement of cell division stages in *A. gracilis*. B- Swollen spherical cells of *A. gracilis* full of vesicles after lowering salinity, a faint orange colored eye spot can be seen, in the center of the picture a cell of *Dunaliella* can be seen for the sake of size comparisons. C-Typical pear-shaped cells of *Dunaliella salina* full of carotenoids.

A. gracilis as a flagellated chlorophyte possesses motility that is manifested in a unique way not to be confused with any other flagellate. The cell is moving around randomly rather slowly in a smooth trajectory while slowly rotates around its long axis and frequently tumbles temporarily and stops moving so as to appear as a stellate 6-radial cell in cross section or in end view. The overall movement of the cell is characterized by a tremor like vibration unique only to this species.

There is one only big basin-shaped thin and pale chloroplast occupying the biggest part of the cell interior up to the flagellar root. One asymmetrical pyrenoid is located close to the pointed hind end of the anteriorly located large nucleus which contains a centrally located nucleolus. Several starch plates cover the pyrenoid. In the cytoplasm can be seen various numbers of globules probably made of reserve metabolites that are stained positively with neutral red. In the anterior area around the flagellar root there are also many tiny vesicles probably derived from the nearby located Golgi bodies.

When conditions are worsening in terms of depletion of nutrients or probably accumulation of toxic metabolites and all these are clearly exhibited in old cultures of *Asteromonas*, then thick-walled spherical cysts are formed and are accumulated at the bottom of the vessel. The cysts are immobile as they have previously lost flagella and are massively deposited among a matrix of amorphous material (Fig. 1-D). Before the completion of metamorphosis to cysts cells are enlarged and accumulate large amount of stroma vesicles full of starch while the pyrenoid shrinks. Meanwhile the cell wall starts to form as a discontinuous layer around the cytoplasm and thickens gradually from the inside side of the cell. The outside of the wall seems rugosed at great magnification (1000 X). When fully formed cysts are 10-15 μm in diameter with the cell-wall thickness around 3 μm. There is no strong evidence that cysts are somehow associated with sexuality as Gorbunova (1961) claimed and this is strengthened by my observations as I never noticed any fusion of cells to form zygote neither zoospores ever been observed. Multiplication of *A. gracilis* is accomplished by simple cell division while the cells are mobile (Fig. 3-A). Only at a later stage near the end of the separation of daughter cells the couple stops moving for short periods.

MATERIALS AND METHODS

In the saltern ponds of Messolonghi (W. Greece) where the salinity seasonally reaches values around 300 ppt, only three species of green microalgae were found (Hotos, unpublished data). Most abundant is *Dunaliella salina* which contributes to the pink-red water coloring due to its profound accumulation of β-carotene in its turning red cell, an accessory and sunlight protection pigment (Fig. 2-C). Second in abundance is *Asteromonas gracilis* which although has the same mode of life as *Dunaliella*, is not found in the same ponds where *Dunaliella* prevails. Third is *Tetraselmis marina* that can be found in lower salinity ponds and although can endure also very high salinities exhibits a peculiar mode of life as apart from its very rapidly moving cells it also creates palmelloid stages (Fig. 1-C) that is, immotile bubble-like transparent envelopes inside which lies its green cell in various states (sole or in division).
These three species of halotolerant algae are members of division Chlorophyta and share some common characteristics. They are mostly green in color, they dont have a cell wall, they are flagellated (*Asteromonas* and *Dunaliella* with two flagella, *Tetraselmis* with four), highly motile, big in size with average length along long axis 18-30 μm, phototactic aided by their cell's red spot, transform to cysts when the water nutrients deplete, produce and accumulate glycerol as a compatible solute to cope with the osmotic pressure and exhibit shape polymorphism (*Asteromonas* the most impressive). The algae were cultured in the laboratory at 21-23 ^{o}C, in 500 – 1000 ml Erlenmeyer flasks, illuminated with white light of 4000 lux intensity, and set in various salinities in the range of 30-260 ppt using simple aeration and Walne's growth medium. Cell counting was performed regularly and samples were centrifuged frequently at 3000 rpm in order to get the algal paste which was used as food to various filter feeders.

RESULTS AND DISCUSSION

Concerning the culture of the above species in various salinities, they are able to multiply and grow from dilute sea water (~30 ppt) to brine (>350 ppt). From observations on laboratory cultures, *Asteromonas* proved the most stable in all salinities as it adapted rapidly to every salinity even after abrupt transition from lower to higher and vice versa. *Dunaliella* needed more time to adapt and presented a longer induction phase compared to *Asteromonas* and also shorter stationary phase. Concerning the stationary phase which is often of less concern, as the exponential phase of the culture attracts the main interest of the culturist, must be emphasized here that in the case of *Asteromonas*, a prolonged healthy and viable stationary phase makes it the "champion" of durability among all known cultured microalgae. In all trials, *Asteromonas* retained for impressively long time its healthy green color and faded out last among all cultures in the same conditions (*Dunaliella, Tetraselmis, Rhodomonas, Nannochloropsis, Isochrysis*). *Tetraselmis marina* as compared to *Asteromonas* and *Dunaliella* proved to be more difficult in culture as it demanded intense light, a narrower range of salinity (~80 – 130 ppt), a very long induction phase followed by a rapid exponential phase and a very short stationary phase, after which the culture always collapsed.
In all salinities above 40 ppt *Asteromonas* retains a vivid green color and its typical spindle shape of its vegetative cells while its motility seems restless. If however salinity is lowered the cell swells, its color becomes faint green and the beating of flagella slows down (Fig. 3-B). A lot of colorless globules accumulates all over the cell interior giving the appearance of a highly granulated mass. At 25 ppt where lies the lowest salinity tolerance level (Hotos & Avramidou, 1995), the cells seem almost spherical with no keels evident and quite motionless in spite the presence of flagella. If salinity is increased again then in a very short time 1-3 hours the cells regain their typical spindle shape.

Asteromonas forms cysts only in elevated salinities when nutrients in the medium have depleted and presumably toxic metabolites have accumulated. Sometimes in very old cultures left unattained for long, the water looks almost transparent devoid of cells and only a green mass is laid on the bottom of the vessel. Discarding the supernatant and introducing this sediment in freshly prepared medium, after some days the culture is restored as cysts in the sediment are germinated and a vivid green colored *Asteromonas* population is established again. Another interesting thing occurring in high salinity (>80 ppt) cultures in the late exponential and early stationary phases is the accumulation of white foam on the surface of the aerated vessel. This phenomenon present also in *Dunaliella* cultures is due to the release and accumulation of extracellular glycerin a molecule that is profoundly produced by these species in high salinities to be used as a compatible solute to balance osmotic stress. Apparently in highly active growing algal mass, the surplus of the produced glycerin is excreted creating the white foam. Contrary to the statement by Ben-Amotz & Grunwald (1981) that extracellular glycerin is initiated in *Asteromonas* (and *Dunaliella*) by very high temperatures (>47 ^{o}C) in my cultures this happened frequently at much lower temperatures in the order of 20-23 ^{o}C. Interestingly the presence of foam is not adversely affecting the viability of the culture, on the contrary as I have noticed, it is a sign of a healthy culture and the foam creating cultures can last for long even after the aeration has been cut off.

Concerning the studies in the biota of the hypersaline waters, that is not only the algae but also other protists (protozoa), crustaceans, rotifers and nematodes that live there, having in mind that only limited information is available on their biology in this particular environment, a lot of studies are required concerning their maintenance in laboratory conditions. A prerequisite for their culture (in high salinity) is a stable supply of their appropriate food which must be suspended in the water (as they are filter feeders), and of the appropriate nutritious value. This leads to the use of phytoplankton, and more specific a halotolerant phytoplankton. Such kind of food is the above mentioned three microalgae species which share with them the same ability to tolerate high concentrations of salt. *Asteromonas* proved to be by far the most convenient food. All tested organisms, protozoa (*Fabrea salina*, *Condylostoma* sp., *Euplotes* sp.), flagellates (*Colpodella* sp.), copepods (*Tisbe* sp., *Tigriopus* sp.), *Artemia* sp. and rotifers (*Brachionus plicatilis*) (Figures 4, 5 & 6) accepted *Asteromonas*, grew and multiplied intensively without trouble. The salinity of their medium varied from 35 ppt (all organisms) to 130 ppt (only for *F. salina* and *Artemia* sp.). On the contrary, little success achieved with *T. marina* as this microalga was unstable in culture vigor and for some unknown reasons seemed to inactivate the ability for intensive feeding of the filter feeders especially at lower salinities. A lot of research is required on this topic. The supremacy of *Asteromonas* primarily and *Dunaliella* secondly over *Tetraselmis* as an effective food for filter feeders, may be attributed to their cell size, cell motility, taste, or even several excreted substances. Special mention must be done here concerning *B. plicatilis*, the commercially sole cultured live food for fish larvae of marine fish in hatcheries. The literature abounds with studies using small sized phytoplankton as food for it. *Asteromonas* (tens of times bigger in volume over *Nannochloropsis*, *Isochrysis*, *Chlorella*, etc.) has no disadvantages compared to them. On the contrary, having in mind its ability for a stable and trouble-free culture, *Asteromonas* can be the ideal food for *B. plicatilis* at least in terms of practicability. Even if after the prolonged stationary phase the *Asteromonas* culture collapses, there are always living cells in a state of dormant cysts on the bottom of the vessel. After decantation of the clear supernatant and addition of new fertilized water and aeration, the culture almost immediately revitalizes. Additionally, if (which is often the case) the monoculture is invaded by unwanted algal species, the raise of salinity (e.g. above 70 ppt) will eliminate them leaving *Asteromonas* the sole species. In all studies and trials on *B. plicatilis* using *Asteromonas* as its food in salinities similar to sea water, growth rate and multiplication (of rotifers) proved more than satisfactory (Hotos, 2002; 2003). Comparing *Asteromonas* to *Tetraselmis* and *Dunaliella* it became obvious that it was possible to collect *Asteromonas* from any salinity that was cultured and after centrifugation to put its cells in every salinity in the region of 35 – 150 ppt without any adverse effect on the cell's viability and its acceptance by the cultured organisms that fed upon *Asteromonas*. This was not the case with *Tetraselmis* or *Dunaliella* as it takes a considerable time for their cells to adapt to the new salinity, a phenomenon which had adverse effect on the cultured filter feeders.

Another issue that is worth mentioning, is that of the use of microalgae as teaching tools in phycology and biology courses. Usually in experimental classes the microalgal material used consists of small immotile species (e.g. *Chlorella*, *Isochrysis*, diatoms, cyanobacteria, etc.). Observations on them in order to study the cell structure is rather difficult and losing of real interest is often the case. Intriguing the interest of students is of paramount importance for deepening in the field of phycology. *Asteromonas* in this respect is the ideal model species for the following reasons:

1. Is big celled (18-23 μm) easily observable from magnifications of 100X and higher. Its big size makes it also an ideal convenient tool for teaching the counting technique.
2. It moves in a fascinating slow shaking way like trembling, whipping its two flagella, in a forward slow movement changing direction frequently, becoming stationary from time to time, reversing position in space and all the above resulting in:
3. Polymorphism as it appears either as elongated with six greenish semitransparent keels along the long axis of the cell, either as a stellate cell, or a sphere. Occasionally it appears in a peculiar nail-like cell or in a folded flattened cell (Fig. 2-A).
4. Its color is a striking green with variable hues among its clearly defined chloroplast, nucleus and red spot. Other structures as granules and vacuoles can also be seen.
5. Its culture practically never fails giving encouraging growth from the second day and advancing fast.

Figure 4. Ciliated protozoa fed to satiation with *A. gracilis* cells. A- *Fabrea salina*. B- *Colpodella* sp.. C- *Condylostoma* sp..

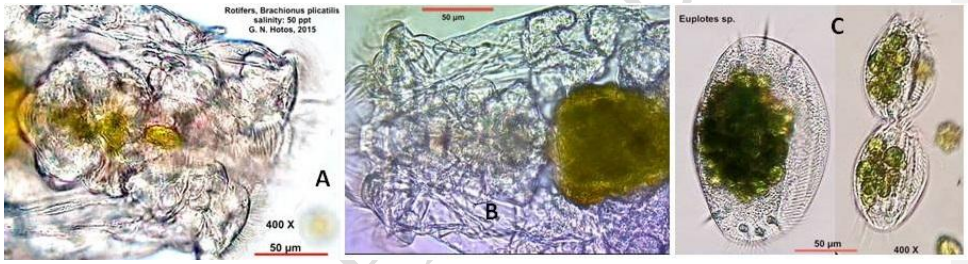

Figure 5. A- Rotifer *B. plicatilis* just "swallowing" a *A. gracilis* cell while many more are smashed in its mastax. B- Gut of *B. plicatilis* full with *A. gracilis* cells in digestion. C- Protozoa, *Euplotes* sp. fed to satiation with *A. gracilis*.

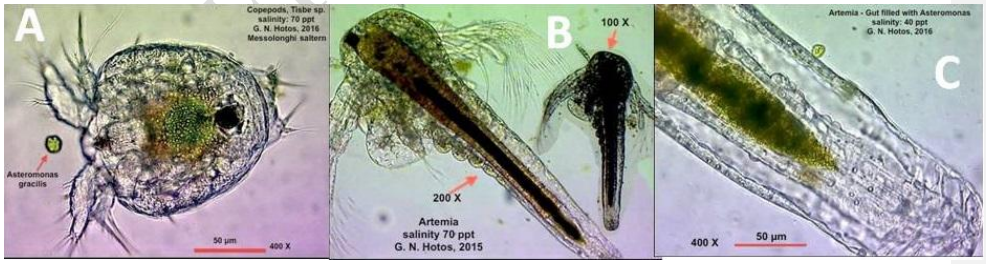

Figure 6. A- Copepod *Tisbe* sp. nauplius fed to satiation with *A. gracilis*. B- *Artemia* sp. with its alimentary canal full of *A. gracilis*. C- Close view of the *Artemia*'s alimentary canal full of *A. gracilis* cells.

As of its halotolerance, *A. gracilis* shares with *D. salina* and *T. marina* unique properties and advantages for a succesful monoculture. The exploitation of microalgae becomes more and more important with the aim of extracting valuable products from their cells for use in the biofuel industry, in health products, for food, or in pharmacy. They are rich in triglycerides, β-carotene, antioxidants, proteins. The main problem in the exploitation of microalgae is the standardization of their mass-culture as it is difficult to preserve them in monoculture or to avoid sudden crashes resulted from deterioration of environmental conditions (especially in open culture ponds). These constraints are

alleviated in the case of halotolerant microalgae. A lot of arid regions exist in many parts of the world. Their soil often rich in salt makes them inappropriate for agricultural use thus creating an opportunity there to reverse this adverse situation to an advantageous one. Advantageous in terms of using those areas as ponds filled with salt water in which cultures of species like *Asteromonas* could thrive. If the salinity is very high even rainfall could have little effect in the cultured alga provided the selected species apart from halotolerant is also sturdy in sudden fluctuations of salinity. From preliminary experiments became obvious that *A. gracilis* after an initial shock from any abrupt salinity change (in the region of 35-150 ppt), will soon adapt to the new salinity and will continue to grow. Contrary to *Asteromonas*, *T. marina* and *D. salina* suffer from salinity changes and although alive, need much longer time to adapt and reach full growth potential. Sometimes also, their culture fails totally. A lot of study is required for the clarification of their culture characteristics.

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Internet related videos courtesy of the author:
https://www.youtube.com/watch?v=acGUObR2ibQ (*Asteromonas*, *Tetraselmis*, *Dunaliella* live)
https://www.youtube.com/watch?v=ajz5JgvlYCA (rotifer *B. plicatilis* filtering *Asteromonas*)