Guide to cultivate *Dunaliellas* in high-school research laboratories

short title: *Dunaliella acidophila* cultivation

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Summary

Their easy manipulation, absence of toxicity and massive cultivation possibilities, made some of the unexplored unicellular microscopic *Dunaliellas* ideal for environmental bioresearch by high-school students. This guide describes the minimal concepts to understand *Dunaliellas* culture at mini-micro laboratory scales. The main purpose is to learn rigorous methods to evaluate scale-up procedures targeting defined applications. Although ~ 30 species of *Dunaliellas* are available for research, most of these unicellular microalgae species remain largely unexplored. On the other hand, 2 *Dunaliella* species are commercially cultivated in high salinity waters for animal feed, human food, medical products, or cosmetics. *D.acidophila* is one of the few *Dunaliella* species that can be vigorously grown in fresh water and it is unique under extreme acid waters. The Mars-like geochemical conditions at Rio Tinto (Huelva, Spain), is an exclusive source of potentially world-wide novel *D.acidophila* strains, whose practical applications remain totally unexplored. Apart from the exciting curiosity on such peculiar primitive life at pH 1 (Mars life?), because of their properties and few contaminating microorganism competitors, *D.acidophilas* could be employed for effluent detoxification, efficient sink of global-warming CO_2, production of high-value carotenes (i.e., Vitamin A precursors), alternative sources of starchs, omega fatty acids, antibiotic or even biofuels, adsorption of heavy-metal water poisons as well as for yet unforeseen applications. Furthermore, there is not yet any genetic transformation methods which could allow exploration of many other possibilities. Among other topics, some tools to organize collaborative team-work, handling state-of-the-art bibliography, internet links, design of reproducible experiments, use of simple apparatusses, sterile work, and a few safety measures, among other topics, are briefly described here using *D.acidophila.* Some preliminary results using mini-micro cultures are also included to trigger further explorations using this biomodel by "learn by doing", hands-on training strategies. Most of the procedures described can be applied to many other microalgae.

Keywords: learn-by-doing; bioresearch: Dunaliella; sulfuric acid; carotene; starch; decontamination; heavy metals; microalgae.

Introduction

Microalgae are light-dependent photosynthetic microorganisms that grow in fresh and marine waters. More than 40.000 microalgae species have been identified (http://www.jcoll.org/vida_microsubmarina/microalgas/microalgas.html). Around 20 microalgae species are now mass-cultivated to annual tons productions using them as bioreactors for animal feed including aquaculture, human food, medicines and cosmetics. Hundreds of microalgae species can be cultured at laboratory scales when provided with light, carbon dioxide (CO_2), Nitrogen (N), Phosphorous (P) and several micronutrients for photosynthesis.

Among microalgae, unicellular *Dunaliellas* have ~30 easy-to-grow species/strains deposited in international culture collections (Table 2). Molecularly classified as Chlorophytas (green algae) (Assuncao et al., 2012; Emami et al., 2015), their surprising ecological adaptability to variations in light intensity, salinity, pHs, extreme temperatures and heavy metals, together with their high efficient use of atmospheric CO_2, make *Dunaliellas* ideal models for exploring novel applications (Avron and Ben-Amotz, 1992; Ben-Amotz et al., 2009).

To grow photosynthetic *Dunaliellas* light must be supplied. At laboratory scales, the quality of artificial light (spectra), their quantity (intensity) and dark/light cycles (exposure), are the most important physical variables to control. *Dunaliellas* detect light through an eye-spot that signals swimming towards illumination when light is limited or move away when light is excessive.

Light quality is characterized by an spectra of photon fluxes of different wavelengths (expressed in nanometers, nm). Wavelenghts between ~400-700 nm constitute the visible spectra and are also among the required wavelengths for most photosynthesis. Each microalgae specie including *Dunaliella,* absorb light through their particular pigments such as chlorophylls and carotenes (Figure S3A). Green *Dunaliellas* use all wavelengths of the visible spectra (Figure S3D), except the greens. Therefore, to grow *Dunaliellas* with artificial lights, we must provide at least blue, red or both lights. Electricity-dependent lamps such as the warmer Fluorescents (Figure S3B) or the colder Light Emitting Diodes or LEDs (Figure S3C), provide light wavelengths. In addition to their spectra, light quality is also estimated by the so called "color temperature", a quality resume of their spectra. Color temperature refers to the color the metals acquire when heated to high temperatures. It is expressed in Kelvin degrees (ºK or K) from 1000 K (more reds) to 10000 K (more blues, best mimicking brilliant daylight). In fluorescent lights of ~4000 K, *Dunaliellas* divide once every 1-2 days with day/night cycles of 14/10 h. Complementing the day-light spectra with ultraviolet light (wavelength of 320-400 nm), reduces growth and may induce carotene accumulation (Garcia-Gonzalez et al., 2000).

Light intensity is the quantity of photons or photon flux density, that a surface receives during an amount of time. Light intensity is scientifically expressed in µmols of photons per square meter (m^2) per second (s). The average sun-light is ~ 2000 µmol photons/m^2/s. The light intensity is also expressed in luxes by luxometers or luminometers (~1 lux = 54 µmol photons/m^2/s). Optimal light intensities for most *Dunaliellas* are ~ 200 µmol photon/m^2/s (Figure S2B), while 20-fold stronger light may irreversibly inhibit their growth.

Like animals and humans sleep at night, microalgae also require some dark resting to recover from their photosynthetic activity. After millions of years of selection, microalgae are adapted to natural day/night light cycles and slow seasonal variations in light quality and quantity. At the laboratory, most microalgae growth is not increased by continuous light, nor much influenced by variations on day/night cycles. At each laboratory, microalgae has to be adapted. Nevertheless, short light-pulses could increase photosynthesis in some cases (Avron and Ben-Amotz, 1992; Ben-Amotz et al., 2009). Apparently such pulses allow microalgae to recover from their photosynthesis by taking some short "siestas". Experimentation would identify the best quality and quantity of light for our particular purposes.

Dunaliellas inhabit hypersaline coastal lagoons and salt lakes and may persist for years in saturated salt waters, such as those at the Dead Sea (Israel/Jordan) or Great Salt Lake (USA). In hypersaline summer environments (> 3.5 % NaCl, 0.6M), *Dunaliellas* produce space-visible large-scale orange-red blooms of carotenes (Figure 1) (https://en.wikipedia.org/wiki/Dunaliella) (Hosseini Tafreshi and Shariati, 2009; Oren, 2014; Raja et al., 2007). Because of their salt adaptability to >5 M sodium chloride (NaCl) and resistance to excessive sun-light intensities (>2000 µmol/m^2/s), annual tons of *D.salina* and *D.tertiolecta* are commercially cultivated with no toxicological environmental risks. These *Dunaliellas* may accumulate into their bodies ~ 40 % starch, 10 % glycerol, 5 % lipids and 10 % carotenes. Carotenes are high-added valued because they are oxygen scavenger antioxidants, quenchers of cancerigen free-radicals, and precursors of vitamin A. *Dunaliella* carotenes are included in animal feeds, food additives and human skin creams to protect against excessive sun-light exposures (Woyda-Plosszczyca and Rybak, 2021). *Dunaliellas* have the advantage of using for their growth waters that are hypersaline (for instance those produced by desalination plants) or acidic (many industrial waste effluents), which are of few other uses.

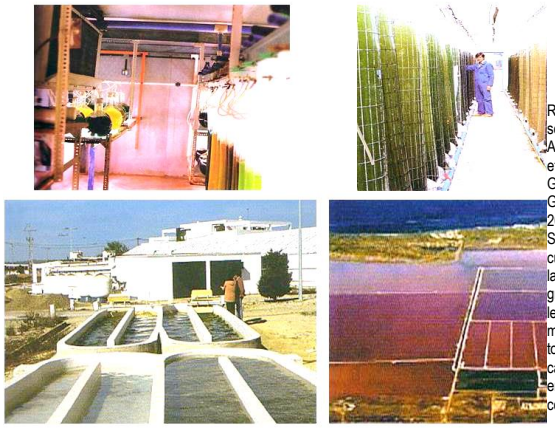


Figure 1
Mass cultivation of *Dunaliellas* to commercially produce red-orange carotenes

Reproduced from several sources (Avron and Ben-Amotz, 1992; Ben-Amotz et al., 2009; Garcia-Gonzalez et al., 2000; Garcia-Gonzalez et al., 2005; Prieto et al., 2011). Step-by-step scale-up cultures from initial laboratory scales in optimal growth conditions (upper left) are required for massive lagoon cultivations to produce orange-red carotens under environmental stress conditions (bottom right).

Green unicellular *Dunaliellas* show variable shapes, usually dominated by ~10-15 µm fusiform motile cells with 2 flagella in V. They are surrounded by flexible proteinaceous non-cellulosic walls in contrast to most other microalgae (Figure 2). Their *in vitro* grow and photosynthesis requires light, inorganic Carbon dioxide (CO_2), Nitrogen (N), Phosphorous (P) and traces of micronutrients. *Dunaliellas* photosynthesis fix the C to starchs (polysaccharides), N to proteins and P to nucleic acids. Photosynthesis also produces O_2 which is required for their own respiration. Some *Dunaliellas* can use CO_2 at highly extreme conditions of light (ten times more than the average sun-light), salinities (ten times more than the average sea water), toxic heavy-metals and/or hydrogen concentrations (one million times more than their average internal pH of 7-8).

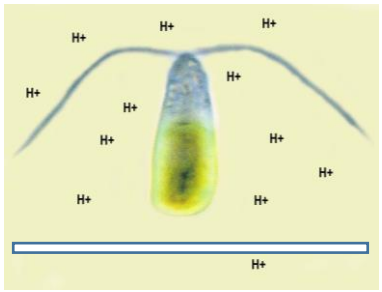


Figure 2
Typical *Dunaliella* at the optical microscope showing its 2 apical flagella

The flagella and the eye-spot are located to the apical part of their unicellular cells. Their yellow posterior part may be filled with starchs and carotenes. The white horizontal line at the bottom of the figure corresponds to ~15 µm (15 million shorter than 15 meters, 15 thousand shorter than 15 millimeters).Microphotography modified from the web. *D.acidophila* grow in high ionic Hydrogen H^+ concentrations.

In contrast to many other microalgae, the absence of an external rigid cellulose wall allows *Dunaliellas* to rapidly shrink to rugosity when at high salinities (> 5 M NaCl) or swell to sphericity at low salinities (< 0.15 M), to maintain their internal osmotic pressure. The spheric *Dunaliellas* slowly convert their starch reserves to glycerol (Fuggi et al., 1988b), before slowly recover their fusiform appearance (Ben-Amotz et al., 2009).

In 1963 in Czechoslovakia, a new specie of *Dunaliellas, D.acidophila* (Kalina, Massjuk, 1971) was discovered in extremely acid waters. It was then described in Italy and more recently in Rio Tinto in Huelva, Spain (Souza-Egipsy et al., 2008; Souza-Egipsy et al., 2021), but it is probably present in any non-thermal (< 30 ºC), moderate salinity (< 0.5 M NaCl) and acid waters all through the world. To note that, in contrast to other local microalgae living in acid waters, *D.acidophilas* at the iron-rich acid waters of Rio Tinto were associated with a minimum of bacteria in the biofilm sediments (Souza-Egipsy et al., 2008). The unique environment of iron-rich sediments in the absence of silicates, makes Rio Tinto a world-wide unique analog of the Mars-like geology, justifying recent NASA-financed projects to study the origins of life.

D. acidophilas grow optimally at acid waters (high hydrogen ion positive H^+ concentrations or low pH) corresponding to ~ 0.3 M of sulfuric acid (Fuggi et al., 1988a). This acid requirement has many advantages for experimentation since there are very few microbiological competitors that could interfere with their growth. These greatly reduces one of the main problems for massive productions, which for most other microalgae need physiological ~ pH 7-8 values (Figure S2A). In contrast, *D.acidophilas* cell division, occurring at pH 1 (Figure 3, left), has not been observed at pH 7-8, where they are progressively and irreversibly damaged during the first 8 h to burst-explode after 24 h (Figure S2A). On the other hand, *D.acidophilas* grow on heavily contaminated acid waters with positively charged (cationic) heavy-metals such as Iron (Fe), Copper (Cu), Arsenic (As), Cadmiun (Cd), Chromium (Cr), Niquel (Ni), Zinc (Zn), and Aluminum (Al) (Puente-Sanchez et al., 2016), suggesting that they could be used for bioremediation of such effluents (Balzano et al., 2020; Diaz et al., 2020). Furthermore, *D.acidophilas* take atmospheric CO_2 much more efficiently than any other *Dunaliellas* since at low pH, the maximal CO_2 solubility bypass the bicarbonate conversion which otherwise is required at higher pHs (Ben-Amotz et al., 2009; Raven, 2009).

Natural acidic waters, like those at RioTinto (Huelva, Spain) (http://jcoll.org/celukob/RioTinto.resume.pdf), are generated by bacterial oxidation of pyrites (FeS_2) to Fe^{+++}, and SO_4H_2 sulfuric acid ($SO4^-$ and H^+). At low pH >30 ºC, microalgae diversity is reduced to *Galderia sp* (red microalgae) and *Cyanidium sp* (*C.merolae* is the smallest eukaryotic genome known). At low pH < 30 ºC, only *Euglena mutabili, Chlamydomonas acidophila* and *D.acidophila* have been described. While most natural waters have H^+ concentrations between 10^{-6} to 10^{-8} M (corresponding to pHs 6 to 8, respectively), *D. acidophilas* grow at 10^{-1} M (pH 1), ~ 1 million more H^+ ions. Strong energetic efforts are required to continuously pump out such tremendous excess of H^+ for *D. acidophilas* to survive to maintain their intracellular H^+ at physiological levels (~10^{-7} M or pH 7). To do that, the external membranes of *D.acidophilas* contain abundant energy-dependent H^+ pumps (Weiss and Pick, 1996), capable of taking K^+ ions (required for survival) but resisting the abundant cationic heavy metal ions (Capasso and Pinto, 1982). Similar H^+ pumps have been described in other acid water yeast and bacteria.

Because studies are relatively scarce in *D.acidophilas* compared to other *Dunaliellas*, it is most probable that some of their applications remain to be discovered. Thus there is not yet any massive cultivation of *D.acidophilas,* despite their low pH advantage in eliminating competitors (Ben-Amotz et al., 2009). It is highly remarkable that the possible production of carotenes by *D.acidophilas* has not been yet investigated, not even at laboratory scales. Also, the abundant extracellular matrix produced by other microalgae that may be implicated in heavy metal adsorption in Rio Tinto (Aguilera et al., 2007; Aguilera et al., 2008a; Aguilera et al., 2008b), suggests *D.acidophilas* could participate on such productions (Puente-Sanchez et al., 2016). The existence of extracellular matrix-forming biofilms by acid microalgae, suggests their possible use for bioremediation of such polluted waters (Balzano et al., 2020). Although the high SO_4H_2 requirement could be an additional difficulty for mass cultivation in large lagoons, and *D.acidophilas* are rapidly killed by alternative hydrophobic organic acids, like acetic acid (Gimmler and Weis, 1992), using close-circuit controlled cultivation, screening for other acids, or reducing/adapting mutants to the SO_4H_2 /acetic acid changes, may be some of the alternatives that deserve more investigation. On the other hand, no genetic transformation has been yet reported for any *D.acidophilas*.

Although we are far from understanding all their acid, salinity, light, and/or heavy-metal resistance secrets, *D.acidophilas* offer an extreme and primitive life, highly-interesting for biostudies with many possible novel applications.

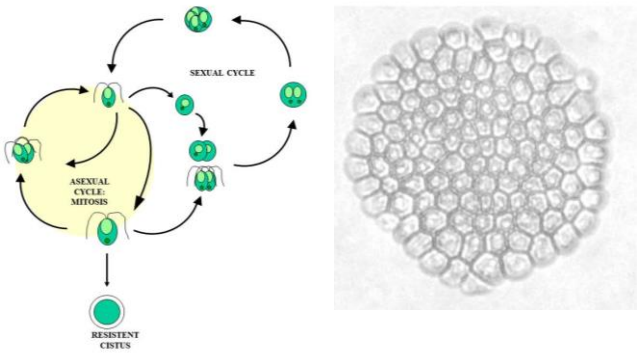


Figure 3
Reproductive sexual and asexual growth cycles of *Dunaliellas* (left, scheme from the web) and colony of 3-4 days after cloning in semisolid media containing 1.5 % agar (right, unpublished)

Materials and Methods

Previous chemical and biological concepts

These or any other similar sources are recommended for reading:

Table 1
Preliminary Concepts

Concepts	Suggested links
1. Atoms, molecules. Molecular weight (MW)	https://en.wikipedia.org/wiki/Molecular_mass
2. How to calculate MWs:	https://www.lenntech.com/calculators/molecular/molecular-weight-calculator.htm
3. Concentrations of chemicals (%, M, mM):	https://en.wikipedia.org/wiki/Molar_concentration
4. How to calculate chemical concentrations:	https://en.wikipedia.org/wiki/Molecular_mass
5. Molecular sizes in biochemistry:	https://en.wikipedia.org/wiki/Molecule
6. Microbe sizes:	https://bio.libretexts.org/Bookshelves/Microbiology/Book%3A_Microbiology_(Boundless)/3%3A_Microscopy/3.1%3A_Looking_at_Microbes/3.1A%3A_Microbe_Size
7. Prokaryotic and eukaryotic cells:	https://www.technologynetworks.com/cell-science/articles/prokaryotes-vs-eukaryotes-what-are-the-key-differences-336095

Recommendations for a minimal organization for collaborative work

A minimum set of initial recommendations to perform common rigorous scientific experimentation may be as follows:

1. Set up a short group of 4-6 high school students with enthusiasm and ready to devote some of their free hours per week to focus on an interesting research biotopic. Obtain some type of formalization, commitment, parental permission or registration. Compile their personal contacts such as phones and email to distribute among the group. Choose a responsible leader. Choose a nickname a logo for the biogroup and the project which will be descriptive of the target biotopic.

2. Acquire a hand writable "notebook" for common use with blank pages of DinA4 size. Write down everything done or to be done in one page, leaving blank its back side. Use colors and fluorescent labels (Stabilo) for easier

and more rapid localization. Paper remains the cheapest most important part of any bioresearch.

3. Open both a common directory on a single computer and a web-box for use at a virtual cloud (dropbox, google, etc) to include common protocols, research articles in pdf, more data, ideas, notifications to the group, etc. That information will complement the day-to-day notebook hand-writing notes.

4. Reserve a specific location at the biolaboratory for any special products, containers or manipulators required for the work (tweezers, crystals, chemical compounds, etc). These should be used only by the group.

5. Schedule a weekly meeting with a defined periodicity, time and place. Write down everywhere all conclusions, protocols, results, routemaps, etc.

6. Subscribe to personal email monthly alerts for bibliography using key-words describing your interest at https://scholar.google.es/schhp?hl=es or at https://pubmed.ncbi.nlm.nih.gov/. Use EndNote or similar programs to handle the bibliography. Obtain pdf copies of the most important papers. The most important pdf used in this work can be obtained at http://www/jcoll.org/celukob/pdf.

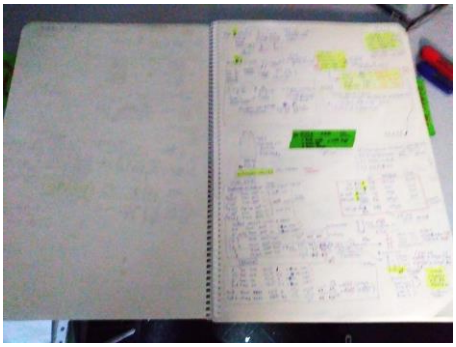
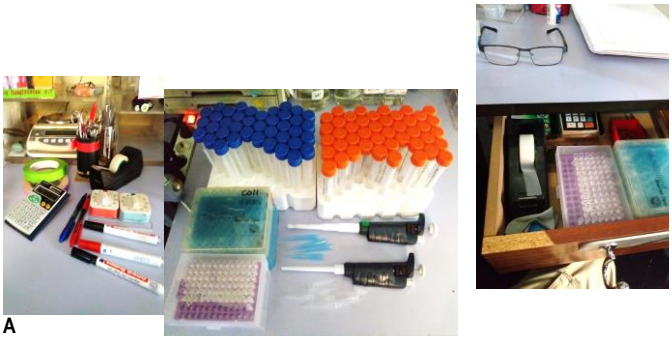


Figure 4
Example of hand-written laboratory notebook
Always include the data in a right page and write the name and the date of writing at the top right corner. The blank left page will be used for later additions, to record forgotten or new data, photos, elaboration of results, new ideas, etc. Do not trust human memory, it is insufficient to retain all the details involved which need to be written down before, during and after experimentation. Most human memories are highly volatile, like computer's RAM tend to disappear with disconnection.

Minimal required handling instruments

It is necessary to have appropriated means to label all flasks, tubes, and containers used for any experiment with water-proof labels (edding 8000 for fine labels and edding 3000) and tapes of different colors to decorate them with clear descriptions and dates. Best use duplicated labels to prevent deletion (for instance, some non-voluntary spoiled alcohol may eliminate the labels). The more the better. Add tubes of different sizes and colors to reduce the pipetting mistakes that otherwise occur. Use color codes (at least red and black) and group the tubes of the same experiment in a labeled boxes clearly labeled with names, dates, etc. Use always the same recognizable order of the tubes (for instance, 1 to 10, left to right). Label everything!. Take all the possible ideas which will increase their future identification from tomorrow on. Even so, the best experimenters make mistakes and usually forget to write down an apparently small detail of the procedure which after a few days will make the results irreproducible or doubtful. Be aware of this small but powerful enemy of any reproducible research.



A **B** **C**

Figure 5
Examples of instruments to best handle mini-micro-experimentation
Take order and carefully write down labels at several places.
Don't work for the "*Journal of Irreproducible Results*" !.
A) Colored and transparent adhesive tapes together with water-proof edding pencils (3000 and 8400) to label tubes and flasks with different colors. Use stop watches to control timings.
B) Use automatic pipetman pipettes with tips of 1mL and 200µL to handle small amounts of liquids with precision.
C) Save bench space and maintain order by placing tips and other commonly required instrumentation at the same drawer to help rapid experimentation.

Source banks for microalgae strains

Microalgae as well as many other microorganisms can be obtained from several international collections deposited in banks (Table 2).

Most of the experiments described here have been performed with *Dunaliella acidophila* strain CCAP19/35 (kindly supplied by Prof.Javier Fernandez-Portal, Retamar). Other *Dunaliellas* were aso obtained from the culture collection of algae and protozoa (CCAP) at the United Kingdom (Table 2).

Table 2
Sources to obtain Dunaliellas (actualized from Ben-Amotz (Ben-Amotz et al., 2009)

Name, country	Internet link or email researchers
Culture Centre of Algae and Protozoa(CCAP), United Kingdom	https://www.ccap.ac.uk/catalogue
Culture Collection of Algae at Goettingen University (SAG)	https://www.uni-goettingen.de/en/culture+collection+of+algae+%28sag%29/ 184982.html
Dipartamento Biologia Vegetal University of Naples "Federico II at Italy	http://www.mimi-it.it/index.php/associated/dth-unina2/
Woods Hole Culture Collection (WHOI), Woods Hole	dkulis@whoi.edu
Provasoli Guillard National Center for Culture of Marine Phytoplankton	https://ncc.carleton.edu/resources/20043.html
Univ. Toronto Culture Collection of Algae and Cyanobacteria (UTCC)	https://uwaterloo.ca/canadian-phycological-culture-centre/about/history
UTEX Culture Collection. Univ.Texas	https://utex.org/
North East Pacific Culture Collection (NEPCC) Univ British Columbia.	https://ircom.botany.ubc.ca/products/algae/marine/
Coimbra Collection of Algae (ACOI)	http://acoi.ci.uc.pt/
American Type Culture Collection	http://www.atcc.org/
Microalgal culture collections	http://seaweed.ucg.ie/cost/MicroAlgalCultures.html
Culture Collection, Univ. New South Wales, Australia	https://www.acronymattic.com/University-of-New-South-Wales-Culture-Collection-(UNSWCC).html
CSIRO Microalgae Research Centre	https://www.csiro.au/en/about/facilities-collections/collections/anaco/australian-national-algae-supply-service
Harmful Algae Culture Collection (UTAS)	Judi.Marshall@utas.edu.au
Cawthorne Culture Collection of Microalgae (CAW)	Krystyna@cawthorn.org.nz
University of Rhode island Culture Collection (URI)	pharg@gso.uri.edu
Plymouth Culture Centre (PLY)	mpg@mail.pmf.ac.uk

"Incubators" for mini-micro-miniculture of microalgae

Small and simple incubators can be used to supply artificial light (fluorescent or LED) and to maintain some control of optimal temperature to ~25 ºC. For *Dunaliellas*, there is no need for an exact control of temperature other than the room temperature around 25 ºC. Since most microalgae die after a few days at darkness and grow best with light cycles of day (to produce their organic material) /night (to divide), and maintaining temperatures relatively low at ~ 25 ºC, the simplest way to cultivate them is to place the microalgae cultures nearby a home window with indirect day light at room temperature.

To best control light intensity and day/night cycles, a source of 3-4 fluorescent or LED lights may be sufficient. Stronger intensities could be obtained by illuminating not only from the top but also from the lateral or the bottom sides with additional fluorescent lights. Holding fluorescent ~ 50 cm or LEDs ~20 cm lights above the cultures may be used to avoid excessive heat but ventilation may be necessary to maintain low the temperatures during the summers. A high-low temperature thermometer helps to control the temperature. Light fluorescent sources with similar to sun-light spectra (wave-length spectra between ~400-700 nm of visible lights) are preferred. Further investigations of different wave-length sources including ultraviolet lights and leds may be experimented and optimize for different microalgae and according to the purpose pursued (for instance to produce carotene, UV lights may be used to complement).

To maintain microalgae stocks to avoid losing or changing the inoculum with different experimentations, the cultures may be maintained at slow growth rates during months. For that, a weak light (i.e., < 20 µmol photon/m^2/s) could be introduced into a refrigerator maintained at a minimum cooling rate resulting in 7-10 ºC, and/or some components of the culture media or CO_2 may be eliminated. Changing or adding more media should be done periodically to maintain the stocks in good shape ready for experimentation.

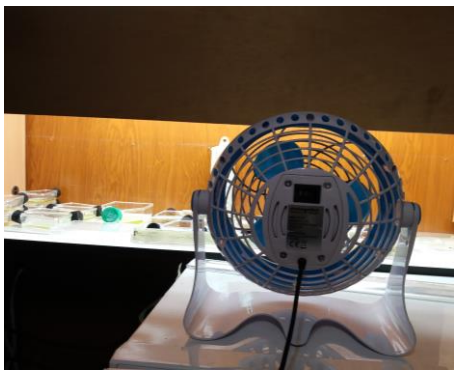


Figure 6
Example of simple "incubator" with ventilation to cultivate microalgae
In this example, the fluorescent lights are at the top of the cultures, protecting eyes from bright exposure with a front cover. To reduce temperature, their high heat-producer reactants may be installed far away and/or a ventilator installed to the side of the cultures. A white color bottom may be used to reflect light. Light intensity. Light intensity is measured in µmol photon/m^2/s. A recommended starting value of light intensity to cultivate microalgae may be ~50-100 µmol photon/m^2/s.

Mini-micro methods for culturing microalgae

To culture microalgae in a first static batch approximation, all their requirements should be provided (Table S1). Static micromethods of 2 to 50 mL volume were used to optimize some of the many requirement variables aiming to scale-up to agitated >liter cultures.

The initial culture media adapted in this work for *D.acidophila* (Table 3) was experimentally modified from the acidified Jaworski enriched with the highly irreproducible soil extract (AJS) to a final soil-extract-free AJAS media enriched with Algal (Fabregas et al., 1988) (Table S2) and synthetic marine salts from Instant Ocean (Table S3). To observe any *D.acidophila* growth, AJ was required at 10x higher concentrations than those recommended by CCAP recipies. A comparison of Instant Ocean salts (similar to natural sea water), with Jaworski and Algal media has been included (Figure S1).

To grow microalgae at high densities, all their chemical-physical requirements need to be provided (Table S1), such as: light, hydrogen concentration (pH), carbon dioxide (CO_2), temperature, and salinity/osmolality (electrical conductivity). For optimal growth, each microalgae specie has narrow

margins for each of these variables. Furthermore, optimal combinations are required because many of them are interdependent. Any failures in the value of only one of such variables either kill the microalgae or very often require months of microalgae adaptation. Therefore, designs and instruments (Figure 7 A,B,C,D), are needed to estimate the values of these chemical-physical variables. Microcultures would result in rapid finding the right conditions to grow specific microalgae lowering their adaptation time to any laboratory conditions. The best growth conditions at large scale are easier to explore using micro/mini cultures.

Table 3
AJA (Acidulated Jaworski, Algal) media used for initial *D.acidophila* cultures

Nº	CHEMICAL PRODUCTS	~ MW	g/50mL x100	mM x100	mL per 1000mL H_2O
1	Cl_2Ca	147	0.6	84.0	10
2	KH_2PO_4	136	0.6	92.0	10
3	$MgSO_4.7H_2O$	246	2.5	203.0	10
4	$NaHCO_3$	84	0.8	190.0	10
5	EDTA Na_2	372	0.22	12.0	10
	Cl_3Fe	162	0.04	5.0	10
6	H_3BO_3	62	0.12	40.0	10
	$MnCl_2.4H_2O$	198	0.06	7.6	
	$MoNa_2O_4.2H_2O$	242	0.05	4.1	
7	Tiamine	337	0.002	0.1	10
	Biotin	244	0.002	0.1	
	Cobalamine	1355	0.002	<0.1	
8	$NaNO_3$	85	4	941.0	10
9	NaH_2PO_4	120	0.6	100	10
10	+ALGAL Nitrogen	----	Table S2	500.0	20
11	H_2O				to 945
	0.2 µm filter				
12	$+SO_4H_2$	98		1800.0	55

In our hands, this was the best media to initiate culture of *D.acidophila* strain CCAP19/35. The chemical products (**red**, macronutrients Nitrogen N and Phosphorous P) and Vitamins (**green**) were weighted and dissolved 100x concentrated in H_2O. To prepare the modified AJ, the 100x concentrates, instead the 1000x as recomended by CCAP, were added to 945 mL of distilled water and the mix filtered through 0.2 µm (Millipore filters). Sulfuric acid was then added to a final concentration of 100 mM. The 36 M concentrated sulfuric acid SO_4H_2 was pre-diluted 20x to 1.8 M for safer handling.
MW, rounded molecular weights.
mM, miliMolar concentrations.

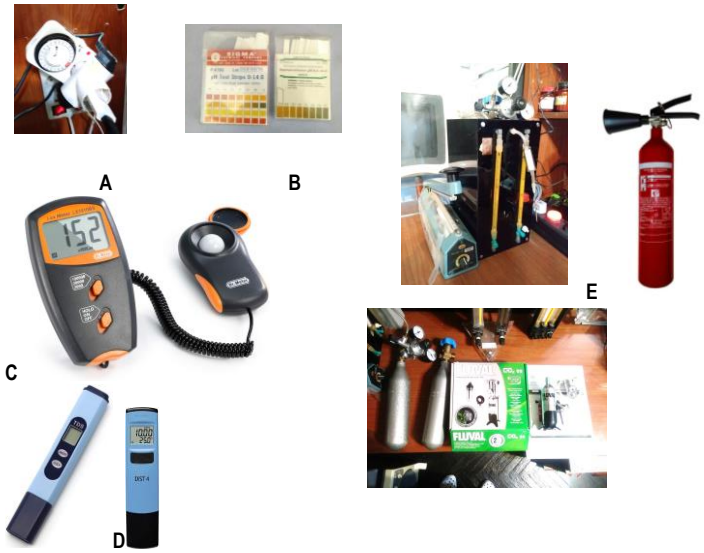


Figure 7
Minimal equipment to measure and control the main chemical-physical variables involved in microalgae miniculture
A) Electric switch to control day/nigh 14/10 h cycles by turning on/off fluorescent lights.
B) pH sticks to estimate hydrogen H^+ concentrations of the culture media.
C) Example of luxometer to measure light intensity in luxes (~25 €).
D) Examples of conductivemeters to measure the electrical conductivity of the medium in mSiemens/cm (~50 €).
E) Example of a home-made generators of ~5-20 % CO_2. It mixes atmosphere air from one aquarium pump with adjustable 0-100 % controlled flows from a CO_2 tank used for aquaria. Well-plates or flasks are gassed for 2 minutes and sealed in plastic bags A plastic bag sealer is used to envelop 96 or 24-well plates to study large number of samples in small 200 µL or 2 mL volumes. Small tanks of 100 % CO_2 compressed to ~50 atmospheres are widely used by aquarists to grow water plants (i.e., low cost Fluval CO2 controllers of different sizes). A cheapest source could be CO_2 fire extinguishers of 2-6 Kg provided with a pressure controller. Guidelines to use them are available at several webs. Because 100% may be toxic to most microalgae, care should be taken to reduce such excess (a CO_2 meter would provide the highest accuracy to measure the concentration of CO_2).

To estimate light intensity by luxes and salinity by ionic electric conductivities, simple instruments are commercially available at low prizes (20-50 €) (Figure 7C, D). The pH is even easier to measure using paper sticks covering several pH ranges rather than by instruments (Figure 7B).

Microalgae grow best in static cultures when feed with 2-20 % of CO_2 in hermetically closed containers. This is because the CO_2 concentration in the atmosphere is only ~ 0.03 % and microalgae need CO_2 and water to fix the Carbon to organic molecules and produce Oxygen. The CO_2 solubility and their reaction with water to form bicarbonate and carbonate depends on the CO_2 abundance (concentration) in the air phase above the cultures, and the temperature, pH and the salinity in the culture media (Figure S5A). At 25 ºC, maximal amounts of CO_2 that dissolved in water are ~ 10-20 mM (Figure S5B). Maintaining a high 20-40 % percentage of CO_2 in the gas phase in hermetically closed flasks assures the amount CO_2 is not limiting the microalgae growth. However, since excess of CO_2 may inhibit photosynthesis and growth the percentage of CO_2 should be carefully assayed before growing the microalgae. At low densities (<0.5 x 10^6 cells/mL), such as when starting the culture, an excess of CO_2 may delay the growth while more CO_2 would be required when the culture is actively growing (2-10 x 10^6 cells/mL). Home-made devices can be constructed to mix 100 % of CO_2 provided by high pressured small tanks (for instance those usual of fire extinguish) and air provided by pumps used by aquarists (Figure 7E). Control and mixing of both flows allows for an approximated supply of the desired percentage of CO_2 in the culture media. Alternative methods to provide CO_2 are commercially available.

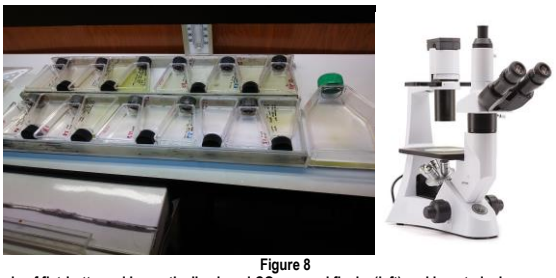


Figure 8
Example of flat-bottomed hermetically closed CO_2-gassed flasks (left) and inverted microscope (right)
Flasks of 50 (black hat) and 200 mL (green hat) capacity were used for 15 or 50 mL culture volumes, respectively. Flasks with different degrees of green colors can be visualized (left) while microscopic details can be observed without disturbing the cultures with inverted microscopes at 100x to 400x (right).

To best visualize the microalgae appearance, viability and growth can be monitored with microscopes (http://www.jcoll.org/microscopio/index.html). To best control the atmosphere by gassing closed containers with CO_2, the flat-bottom culture flasks of 50 mL provided with hermetic caps (such as those used for mammalian cell culture), were adapted to grow microalgae.

Estimation of microalgae growth

Microalgae growth can be monitored by visual, microscopic count, and absorbance-based estimation methods, among many other possibilities.

In the case of *Dunaliellas*, the simplest and more rapid method consist in a visual evaluation of the green color intensity of the cultures. As a first approximation, with some experience, a relationship between intensity of green colors and the approximated number of microalgae per mL can be estimated. The most exact method is to count the numbers of microalgae at the microscope (using 200-400x) with an hemocytometer. Diluting in 1 % formaldehyde, 0.1% acetic acid and 0.1 M sodium phosphate pH 5.6 1:1 v:v, may be necessary to kill the microalgae if they move excessively (Fabregas et al., 1999). However, that is work-intensive and time-consuming for large number of samples. To count a larger number of samples (for instance, ~100 samples in 96-well plates), the light absorbance intensities at 400-450 nm wavelengths may be estimated, according to the chlorophyll spectrum which does not absorb in the greens (https://www.mpsd.mpg.de/17628/2015-04-chlorophyll-rubio). Photoshop or similar drawing programs could also be used to estimate aproximated RGB color intensities. An example of how to use smarthpone "spectrophotomer" or "colorMeter Free" applications (personal communication Guillermo Canosa) is described in youtube https://www.youtube.com/watch?v=LzYhyXYcs0M&t=15s. Although any of these measurement alternatives could be used as first aproximations, more exact measurements would require readings by expensive plate spectrophotometers. Old, yet usable but officially discarded plate spectrophotometers may be obtained from some research laboratories.

Safety precautions

Because this type of work does not involved any pathogens, only some precautions are required. Nevertheless, it is an opportunity to familiarize with good and safe experimental practices. Just to mention, dress appropriated white lab coats, use hand gloves, never mouth pipette, dispose all used material in plastic closed containers, often clean surfaces and hands with ethanol 70 % and do not eat at the laboratory. Also, handle under a hook with gloves any toxic chemicals like sulfuric acid (safer if used diluted 20-fold) and potentially cancerigen (any chemical with strong colors). Maintain all flasks labeled with the chemical contained, mixture compositions, date and name of author. For additional safety precautions, please consult several of the pdfs included at my web page, including international and national guides and spanish legal norms (http://www.jcoll.org/celukob/SAFETY).

Figure 9
Example of a highly toxic chemical and easy dispensable liquids
A crystal bottle to hold 20-fold diluted corrosive sulfuric acid SO_4H_2 and 500 mL flasks for 70 % ethanol (to disinfect hands), distilled water, and 10 % bleach (to disinfect surfaces).

Results

Adaptation of *D.acidophila* strain CCAP19/35 to our laboratory conditions

Plates of 24-wells with 2 mL of media per well (Figure 10B), sealed in plastic bags and gassed with different CO_2 concentrations, were preferred to investigate variables to favor scale-up. Numerous variable-values and combinations could be tested during a few days or weeks in such mini-cultures to rapidly adapt *D.acidophilas* to vigorous growth under the chemical-physical conditions available at any laboratory. In our case, after initial unsuccessful attempts, growth of the *D.acidophila* inocula obtained from CCAP (kindly supplied by prof. Javier Fernandez-Portal, Retamar, Madrid, Spain) was first detected by the appearance of slightly green colored wells (Figure 10A,B). Any change on physical values or composition of the culture media required for *D.acidophila* 1-2 weeks to adapt. Small colonies with a number of *Dunaliellas* alive and others dead, visualized the processes of their adaptation.

Experiments were initially performed with illumination obtained from 4 fluorescent 30W lamps situated at 30-40 cm of distance to the cultures, providing a light intensity of ~ 50 µmol photons/m^2/s (Figure 8A). When 2 fluorescent lamps were complemented with one ultraviolet UV lamp (for reptiles, highest UV10), the *D.acidophila* migrated to the fluorescent side of the wells. Therefore, the UV light was omitted for initial experimentations.

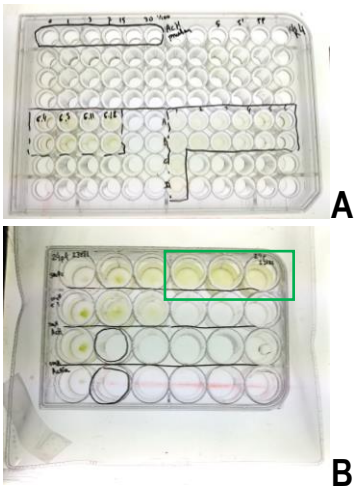


Figure 10
Examples of adaptation of *Dunaliella* initial inocula to chemical-physical variables in plates of 96 (A) or 24 (B)-wells
Wells were filled with 200 µL of culture media in 96-plates (**A**) or 2 mL in 24-plates (**B**). The higher the volume, the better to reduce water evaporation during ~15 days incubations. To maintain CO_2 concentrations, the plates were thermally sealed into CO_2-impermeable high-density polyethylene 17.5 x 22 cm plastic bags (Esselte, Madrid Spain), injected with CO_2 and resealed. The examples show results after 7 days of incubation at 27 ºC and 50 µmol photons/m^2/s of fluorescent light in different culture media. The transparent wells, have few *Dunaliellas*. In contrast, slighly-green wells have ~ 1 million of *Dunaliellas*. Of the upper row wells in B, containing increasing concentrations of sulfuric acid SO_4H_2 from left to right, those inside the green rectangle were ready to be transferred to 5-10 mL media to scale-up in hermetically closed 50 mL flasks (Figure 8, left).

After some light, temperature, culture media components (Table 3) and CO_2, were preliminarily optimized, their best values were combined to initiate 10 mL volumes in hermetically closed 50 mL static triangular flasks (Figure 8, left). Scale-up first atempts were made into 50-100 mL in 250mL flasks (Figure 10A). Indication of growth were the appearance of faint-green colors at the bottom of undisturbed cultures after 2-3-days (Figure 11A), confirmed by microscopic evidence of small colonies of 20-40 homogeneous cells per colony (Figure 11B). Some of cells showed continuous vibrations but remained together, while others moved freely throughout the media including its upper surface. A few days later, greener cultures appeared (Figure 11C) and their growth confirmed by the presence of slowly swimming *D.acidophilas* all around the microscope field (Figure 11D, video at https://youtu.be/1gR9_PMzC6M). The first experiments reached maximal cellular densities of 1-2 millions of *Dunaliellas* per mL (Figure 11D).

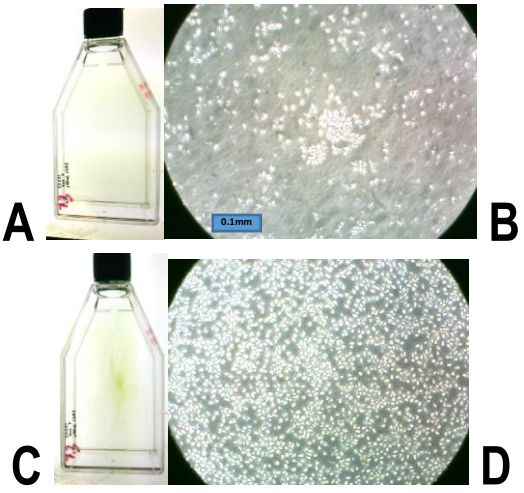


Figure 11
Visual estimation of growth (A,C) and with the inverted microscope (B,D) in 5% CO_2-gassed, hermetically-closed 250 mL flasks

Comparative cultures of *D.primolecta* and *D.acidophila and optimization of media components*

Our previous studies identified *D.primolecta* among 10 other *Dunaliella* species as the best growing in Sea Salt Enriched Algal (Table S2) SSEA media, reaching concentrations of 60 millions (60 x 10^6) per mL (Santin-Montanya et al., 2007). Such highest densities were targeted for *D.acidophila* because they would facilitate any scale-up application.

However, the SSEA media had to be diluted 4-fold, to obtain a minimal growth of *D.acidophila* which remained <1 x 10^6 per mL for weeks. Additionally, *D.acidophilas* grew poorly in the CCAP supplied Acidulated Jaworski Soil (AJS) media in our hands, specially when enriched with their provided Soil extract. To obtain similar cellular densities than SSEA media, 10-fold higher AJ concentrations (without the soil extract) than recommended by CCAP were required. Therefore, to increase cellular densities and reproducibility, formulations were designed by combining several concentrations of AJ media, Algal mix and SO_4H_2 and screened for vigorous growth (AJA media: Acidulated, Jaworski, Algal)(Table 3). Results showed that to obtain densities >1 x 10^6 growth, both ~ 100 mM SO_4H_2 (Figure 1, **blue**) and 10-fold concentrated AJ components (Figure 1, **black**), were required. An improvement of cellular densities was obtained after adding 10-20 mM Algal mix (Figure 1, **red**). Later on, the addition of artificial sea salts (Instant Ocean, IO), duplicated the *D.acidophila* densities to ~6 x 10^6 per mL (Figure 1, **green**) defining the AJAS media (Table S3).

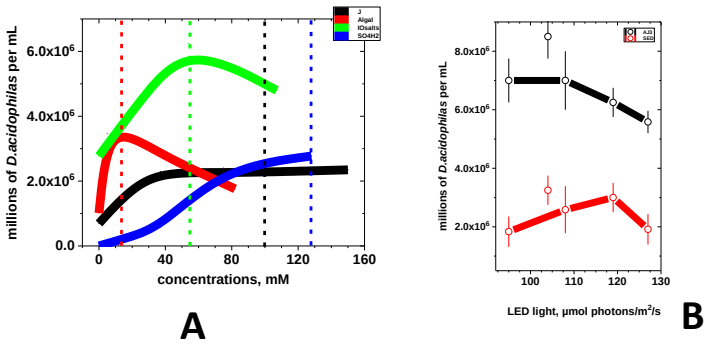


Figure 1
Optimization of main components of media for *D.acidophila* and comparison of growth at two different media using LED lights
Cultures were inoculated with 0.5x10^6 *Dunaliellas* per mL in 10 mL of media. Cultures were gassed with CO_2 at 5 % in 50 mL hermetically sealed flasks. The results were counted with an hemocytometer after 7 days of incubation at 28 ºC with 50 µmol photon/m^2/s of fluorescent light (**A**) or with different LED light intensities (**B**). Average and standard deviations were represented (n=3)
A: Black line, Jaworski media in mM of N
Red line, ALGAL mix in mM of N
Green line, Instant Ocean sea salts in mM of NaCl
Blue line, Sulfuric acid, SO_4H_2 in mM
Vertical dashed lines, concentrations of highest cellular densities. All the components not varied were added to the media at their optimal concentrations, except IO salts.
B: Black line, AJAS media
Red line, SED media
Circles & vertical lines, mean ± std. dev. (n=3)
Unbound data, independent experiment at 104 µmol photon/m^2/s

From all the results commented above, SO_4H_2 and CO_2 were found absolutely required to observe any *D.acidophila* growth. Other components could be provided by either acidulated Jaworski (AJ) or by Algal Vitamin-rich media. In an attempt to further simplify the culture media, the Instant Ocean (IO) salts were chosen as an starting basal component. IO was chosen because IO salts increased the densities obtained with AJ+Algal (Figure 1A, **green**), contained many of the required oligoelements in a reproducible manner and are world-wide available to mimic natural sea water. However, detailed analysis of the IO salt composition, revealed that N, P and Fe were not provided (Table S1, Table S2 and Figure S1).

Therefore, for a first evaluation of the relative importance of each of the nutrient requirements for the maximal cellular densities obtained for *D.primolecta*, the Algal mix components were tested one-by-one by re-mixing different concentrations individually with fixed concentrations of the rest of the IO components and monitoring their impact on growth (Figure S4). The results showed that for maximal *D.primolecta* growth, the Algal mix mainly provided KNO_3 as a source of Nitrogen (~10 mM), KH_2PO_4 for Phosphorous (~1 mM) and EDTA-Fe for Iron (~ 0.1 mM, EDTA was required to solubilize Fe at pH 7 optimal for *D.primolecta*). The minimal growth requirement for Vitamins (from 10^{-5} to 10^{-3} mM) and other published data (Ben-Amotz et al., 2009), suggested that their omission from the media could be attempted. Since other micronutrient requirements have also small influences on growth and since most of their concentrations (from 10^{-3} to 10^{-1} mM) could be provided by IO salts (Figure S1), perhaps the AJ and ALGAL oligoelements could be also eliminated in a minimal media.

Therefore, a new minimal media for *D.acidophila* (Salt Enriched *Dunaliellas*, SED) was designed by substituting both AJ and ALGAL oligoelements by IO salts. The minimal media was enriched also with different concentrations of KNO_3, KH_2PO_4, and $FeCl_3$, (EDTA is not needed to solubilize Fe^{+++} at pH 1) and 100 mM SO_4H_2. Experiments were designed to comparatively evaluate *D.acidophila* densities when grown in AJAS (Table S3) and SED media at different

LED-light intensities. Results showed that optimal LED-light intensities for 10 mL cultures were at ~100 µmol photon/m²/s, confirming previous observations by others (Gimmler and Weis, 1992) (Figure 1B). However, the highest densities were still obtained with the complex AJAS rather than with the minimal SED media formulation. To identify the component(s) that are still missing in the IO salts to obtain the higher AJAS densities, further experimentation will be needed.

Then, we assayed for the influence of the starting CO_2 concentrations on the culture atmosphere above the cultures using the SSEA media for *D.primolecta* (pH 7) and the AJAS media for *D.acidophila* (pH ~ 1) in static and sealed flasks. Both *D.primolecta* and *D.acidophila* showed an strict growth requirement for any CO_2 concentrations above the 0.03 % on natural atmosphere (Figure 2). After 6-8 days in culture, *D.primolecta* reached 5-6-fold higher cellular densities than *D. acidophila*. Concentrations > 5 % CO_2 apparently inhibit the initial growth of *D.acidophila* confirming earlier reports by others (Gimmler and Weis, 1992). After 12 days, however, unexpected much higher *D.acidophila* densities reaching ~ 15 x 10^6 cells per mL were obtained with 20 % CO_2 (Figure 2). Maintainig the same cultures for longer times confirmed that ~ 20 x 10^6 *D.acidophila* could be obtained with 40 % CO_2 (not shown) at 10 mL scales. These results, suggested that *D.acidophila* may require an adaptation period to high CO_2 concentrations rather than been inhibited by those high concentrations, as reported before (Gimmler and Weis, 1992).

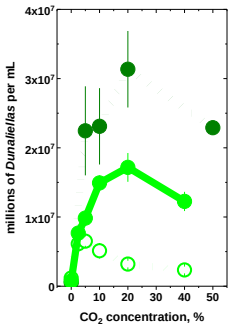


Figure 2
Comparison of growth of *D.primolecta* and *D.acidophila* at several CO_2 concentrations in culture
Cultures were inoculated with 0.5x10^6 *Dunaliellas* per mL in 10 mL of AJAS media for *D.acidophila* or SA for *D.primolecta* (B)(Santin-Montanya et al., 2007). Cultures were gassed with CO_2 at different percentages. The number of cells were counted with an hemocytometer after 6 (*D.primolecta*), 8 or 12 (*D. acidophila*) days of incubation at 28 ºC with 50 µmol photon/m²/s of fluorescent light.
Dark green, *D.primolecta* after 6-days
Light green, dotted line, *D.acidophila* after 8-days
Light green, solid line, *D.acidophila* after 12-days

Discussion

Because *D.acidophilas* grow in acid fresh or saline waters, they offer a unique useful alternative for such natural or industrial effluents. Because their extreme requirements, *D.acidophilas* have few competitor microorganisms (bacteria, fungus, viruses), favouring mass cultivation and research. Despite those advantages, *D.acidophilas* mass cultivations remain unexplored (Table 2).

D.acidophilas require CO_2 (0.03 % in the atmosphere) as their main nutrient solubilized in water to photosynthetically incorporate inorganic C to organic C. In water at pH 7-8, the CO_2 becomes bicarbonate (HCO_3^-), that needs to be reconverted to water CO_2 to be used for microalgae. In contrast, in water at pH 1-3, all CO_2 is soluble in water (Figure S5A), facilitating its use by *D.acidophila*. Microalgae capture of the CO_2 produced by wine or beer industrial fermentations, has been proposed to reduce global warming CO_2 contamination (CO_2 sink) (Woyda-Plosszczyca and Rybak, 2021). *D.acidophila* could be empoyed for that more efficienty (personal communication, prof. Javier Fernandez-Portal).

Photosyntheis also requires light. Auto-shading growth limitations, electric light costs or natural light variations, are well known challenges for high-density production of *Dunaliellas*. Perhaps, bioluminiscence could introduce an exciting light-source environmentally-friendly novel alternative for microalgae. Numerous light-generating bacteria (Vannier et al., 2020) or dinoflagellates (Valiadi and Iglesias-Rodriguez, 2013) and several ideas using bioluminiscence lamps in the absence of electricity, have been put forward mostly for human use (https://www.researchgate.net/post/Bioluminescent-bacteria-lamp-self-sustaining). Would bioluminiscence and microalgae work together?. Why not?. Could similar "biobulbs" be used for *Dunaliella's* photosynthesis?. Could a bacteria or dinoflagellate specie be selected to be co-cultured with *Dunaliella*s?.

To obtain large amounts of ~ 50 millions per mL of *D.acidophila* strain CCAP19/35 remains our target challenge. Simplifying their culture media and CO_2 atmosphere may help. However, we still need to identify the minimal chemical components of the Jaworski and Algal mix that supported maximal growth up to 15 x 10^6 cells per mL. With respect to CO_2, perhaps increasing amounts of CO_2 concentrations with the age of the cultures, could be used to increase the final cellular densities at larger-scales (Figure S6). On the other hand, since the maximal amount of dissolved CO_2 in water is limited (at 25 ºC, concentrations of CO_2 above the cultures of 20-40% or ~ 4-9M, only obtained 7-14 mM to be dissolved in water) (Figure S5B). Perhaps 40 % CO_2 will last for a long-term and static larger culture, if dissolved little-by-little in the aqueous media. Gentle movement of large-scale cultures will be still needed to homogenize the exchange of O_2 / CO_2 with the surface/atmosphere and also to reduce auto-shading light at high-densities. Because of the absence of rigid membranes in *Dunaliellas,* any movement of the culture media should be gentle (bubling CO_2 may be excesive). Periodic re-opening to renovate O_2 and re-gass CO_2, may be another alternative. Most probably, the size of the inocula (small split sizes when amplifying to larger scales), will have also great impacts on the final cellular densities. All these practical details require further experimentation to obtain >10 L cultures at high densities.

D.acidophilas are resistant to many toxic cationic heavy-metals (Gimmler and Weis, 1992). Heavy-metals are dissolved by the H^+ ions generated in natural and contaminated acid water effluents (such as those from Rio Tinto) when iron and sulfur-oxidizing bacteria degrade FeS_2 pyrites. *D.acidophilas* must balance their access to heavy-metal concentrations between micronutrient deficiency and toxic excess. Metal excess provokes increase in number / size of vacuoles, accumulation of starch / lipid reserves, synthesis of other heavy-metal ligands (such as polyphosphates and sulfide-containing phytochelatins), and finally extracellular export of such vacuoles to release the heavy-metal-complexes (Balzano et al., 2020; Diaz et al., 2020). The biofilms in acid waters may be caused by such extracellular exports (Aguilera et al., 2007; Aguilera et al., 2008a; Aguilera et al., 2008b), however, the participation of *D.acidophilas* in these proccesses has not been demonstrated. On the other hand, the high iron contamination of Rio Tinto together with its world-wide unique absence of silicon, may be a source of unexplored *D.acidophila* strains resulting from many centuries of adaptation. Such unique *D.acidophila* strains or mutants may offer novel applications to treat heavy-metal contaminated waters or to develop more sensible heavy-metal biosensors.

In *D.acidophila* cultures, N has been supplied by Nitrates NO_3^- or Ammonia NH_3^+. Nevertheless, available massive effluents having large amounts of Urea (CH_4N_2O), such as those from pig farms, could be a N-supply alternative. However, there are no studies on these possible decontaminations.

It was described that *D.acidophilas* accumulate intracellular starch-like molecules when grown at optimal conditions (Gimmler and Weis, 1992). In preliminary experiments, large amounts of ethanol-precipitable material that stained like polysacharides was detected in the extracellular supernatants of mini-cultures of the *D.acidophila* strain CCAP19/35 (unpublished). Additional first observations showed that those supernatants could substitute sugar in kombucha cultures (unpublished). Testing any other *D.acidophilas* like those strains from RioTinto and subsequent optimization of their culture conditions, may help to increase the production of similar extracellular materials for particular applications, including novel anti-microbials. For instance, dose-dependent anti-viral activities could be demonstrated in the extracellular polysacharides obtained from several microalgae cultures, including *D.tertiolecta* (Fabregas et al., 1999). No data do exist on possible antibiotic or anti-viral activities on the extracellular materials from *D.acidophilas*.

Table 2
Possible applications of microalgae, *Dunaliellas* and *D.acidophilas*

Application	*Other microalgae*	*Dunaliellas*	*D.acidophilas*
pH of culture	pH7-8, abundant microbial contaminations	pH7-8 abundant microbial contaminations	pH1-2 absence of other microorganisms
Atmospheric CO_2 sink	Require HCO_3 conversion for solubility	Require HCO_3 conversion for solubility	Atmospheric CO_2 is soluble
Heavy metal contamination	Most of them toxic	Resistance by binding to extracellular starchs?	Resistant by extracellular starchs?
Starchs (polysacharides)	Extracellular accumulation	Extracellular accumulation	Unexplored
Carotene (vitamin A precursors)	A few species	Some species commercially exploited	Unexplored
Omega fatty acids	Some species	Some species	Unexplored
Antibiotics/antivirals	Some species	Some species	Unexplored
Waste-water treatments	Some attempts	Unexplored	Unexplored
Ecotoxicity testing	Some species	*D.primolecta*	Unexplored
Genetic transformation	Some species	Some species	Unexplored

It was observed that the green color of some *D.acidophila* cultures slowly changed to light-brown (unpublished). Although the degree of browning observed was minimal, the culture conditions could be further screened to select those which increase that brown intensity, since the brown color could characterize high-value carotenes and/or other compounds. Any high value by-products from *D.acidophila* cultures would raise their impact and deserve further studies.

D.primolecta has been used for sensitive ecotoxicity herbicide testing (Santin-Montanya et al., 2007). In a similar way, *D.acidophila* could be used to test for toxicities of diverse acid effluents which otherwise may be very difficult to evaluate because their low pH will kill possible marker microorganisms. On the other hand, specific heavy-metal sensible *D.acidophila* mutants could be used for detection of each heavy-metal, rather than for unspecific resistance. Specific heavy-metal mutants of other microalgae are presently being developed for commercial kit assays (personal communication from Dr. Rafael Blasco).

Additional applications could be developed if *D.acidophilas* could be genetically manipulated. For instance, recombinant pathogen-free vaccines with a high degree of purification could be mass produced in absence of any usual contaminant microorganisms or viruses. However, no protocols for *D.acidophila* transformation and very few for any other *Dunaliellas* have been reported (reviewed by Coll (Coll, 2006)). Although the genome of *D.salina* is actually been mapped (Avron and Ben-Amotz, 1992), to our knowledge, that of *D.acidophila* is not. In the future, *D.acidophilas* genomic investigation may help to discover more applications of these unique microalgae.

Supporting information

Table S1
Requirements for most microalgae culture

Requirement	~Range values	Main Functions
Water	80-90%	Maintain flow of nutrients, light exposition, etc
Light	10-200 µmol photons/m²/s	Photosynthesis, incorporation of CO_2 to organic molecules
H+ concentration	pH 1-10	General metabolism, osmotic pressure, enzymes, etc
CO_2	2-20 %	Photosynthesis, incorporation of CO_2 to organic molecules
Temperature	~25 ± 10 ºC	Optimal speed for metabolic functioning
Ion concentration	0.15 to 8 M	Intracellular osmotic pressure, enzymes

Atoms	Microalgae	Main molecules and functions
Carbon, C	~40%	Carbohydrates, many organic compounds
Nitrogen, N	~30%	Proteins
Oxygen, O	~20%	Most organic compounds, energy and respiration
Phosphorous, P	20mM	Nucleic acids
Sulfur, S	10mM	Some organic compounds
Sodium, Na^+	15mM	Cation. Osmotic pressure, enzymes
Potassium, K^+	150mM	Cation. Osmotic pressure
Calcium, Ca^{++}	50mM	Divalent cation. General metabolism, enzymes
Magnesium, Mg^{++}	50mM	Divalent cation. Photosynthesis, osmotic pressure
Vitamins,	Traces	General metabolism, enzymes
Oligoelements	Traces	Micronutrients for general metabolism, enzymes

Most variations are due to the specie, seasonal temperature, light exposure, and/or physiological state.Data were rounded from published values (Avron and Ben-Amotz, 1992; Ben-Amotz et al., 2009; Gimmler and Weis, 1992)

Table S2
Composition of ALGAL mix (Fabregas et al., 1984)

PRODUCTS	MW	g/L x100	mM x100
KNO_3	101	50.40	500.0
NaH_2PO_4	120	3.36	27.5
EDTA Na_2 /KOH	372	0.48	1.29
FeCitrato	379	0.53	1.40
Cl_2Zn	136	0.03	0.22
$MnCl_2.4H_2O$	197	0.26	1.32
$MoNa_2O_4.2H_2O$	242	0.06	0.24
$CuSO_4.5H_2O$	249	<0.01	0.02
$CoCl_2.6H_2O$	238	<0.01	0.13
Tiamine	337	0.10	0.32
Biotin	244	0.65	0.001
Cobalamine	1355	0.09	0.01

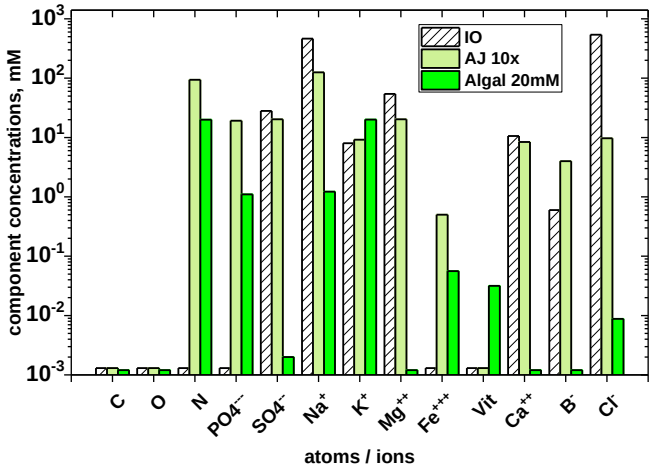


Figure S1
Comparison among the composition of nutrients and micronutrients of synthetic Instant Ocean sea salt IO, AJ and ALGAL added to experimental media to cultivate *D.acidophila*

IO, Instant Ocean main components at 1x are at similar concentrations of natural sea water at 3.5 % total salts, corresponding to ~ 0.6 M NaCl. Additionally IO contains traces of many other inorganic ions, but lacks Nitrogen N, Phosphorous P, Iron Fe and Vitamins (Tiamine, Biotin, Cobalamine) (https://aquacraft.net/marine-aquarist/issue3-2000/ma3-instant-ocean/) (https://www.amazon.com/-/es/Sal-de-mar-Instant-Ocean/dp/B000256EUS)

AJ, contains N and P, EDTA to quelate Fe and traces of the inorganic ions described in Table 1.

Algal, contains N and P, EDTA to quelate Fe, and traces of Zn, Mn, Mo, Cu, Co and Vitamins (green).

For optimal growth of *D.acidophila*, 100mM sulfuric acid SO_4H_2 should be added to a final conductivity of ~40 mS/cm². Intracellular physiological isotonicity was ~ 300 mOsm/L corresponding to 0.9 % (0.15 M) NaCl.
Carbon C (CO_2), and Oxygen O limit microalgae growth in any media.

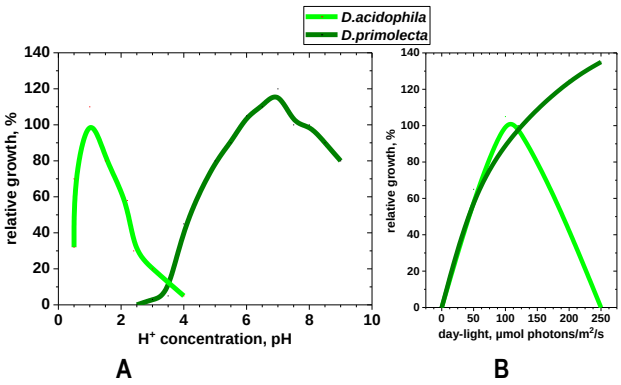


Figure S2
Comparison of pH and light dependence among *D.acidophila* and *D.primolecta*
Relative growth was calculated by the formula, 100 x millions of Dunaliellas per mL /maximum millions of *D.acidophila* per mL, data modified from Gimmler and Weiss (Gimmler and Weis, 1992). H^+ concentration is 10^{-pH} M. Light intensity irradiation is given in µmols of photons of day-light spectra per m² per second (multiply for 54 to obtain their corresponding value in luxes).

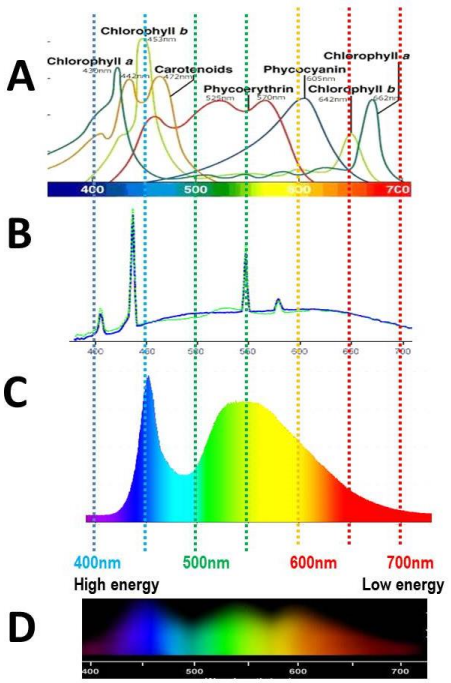


Figure S3
Microalgae pigments (A), fluorescent light spectra (B) LED spectra (C) and sun-light spectra (D)

A) Microalgae absorb light according to their major pigments. Green *Dunaliellas* don't use greens but blue and red wavelengths.

B+C) Fluorescents and light emitting diode LEDs are electricity-dependent alternatives to mimic sunlight spectra.

D) Sun-light spectra. Any light is made of photons of different wavelengths. Photons, between 400-700 nm constitute the visible electromagnetic spectra. The photon intensity has been drawn at the Y axes in the figure.

The light intensity is the amount of photons that a surface receives during a time. It is scientifically measured in µmols of photons per square meter (m²) per second (s). The average sun-light is ~ 2000 µmol photons/m²/s.

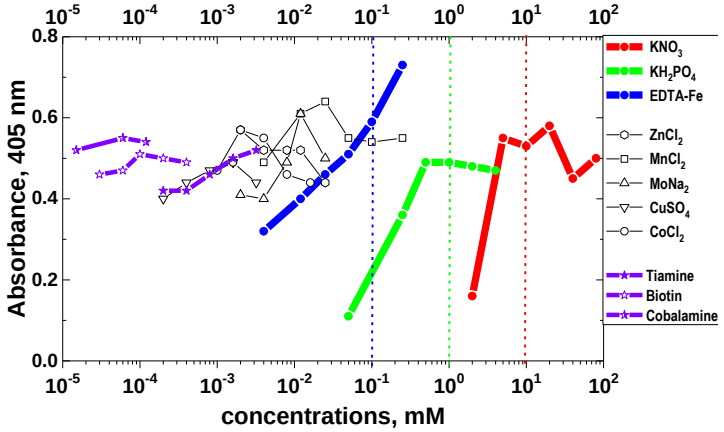
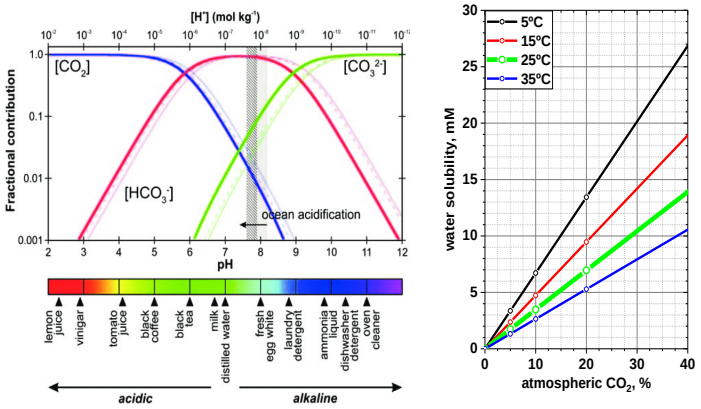


Figure S4
***Dunaliellas* growth at different concentrations of ALGAL components**
D.Primolecta at 0.5 x 10^6 cells per mL were grown in 300 µL wells of 96-well plates in 100 µLSA media at 28 ºC and fluorescent lights of 40 µmol photons/m²/s (Santin-Montanya et al., 2007) with different concentrations of one of the ALGAL components while maintaining the rest of components at their corresponding mM concentrations when in 20 mM of Nitrogen N of ALGAL. The plates were gassed with 20 % of CO_2 , sealed and incubated during 4 days. Growth was estimated by their Absorbance at 405 nm with a spectrophotometer plate reader.



A **B**

Figure S5
Solubility of CO_2 in water at different pHs (A) and atmospheric percentages (B)
Decreasing water pHs first convert precipitates of Calcium Ca carbonates CO_3^{2-} to bicarbonate HCO_3^- and finally to carbon dioxide CO_2. Since 40 % of atmospheric CO_2 (44g/mol of molecular weight) would correspond to ~9M, only ~ 1000-fold is solubilized in water (modified from (Wolfbeis et al., 1998). Other components in the microalgae media contribute to slightly decrease the concentrations of solubilized CO_2. Growth of *D.acidophila* was maximal at 25ºC and 20-40% CO_2 (Figure 1B).

Table S3
Composition of optimized AJAS media for *D.acidophila*

Component	Initial, mM	folds	Final, mM	µL for 1mL	mL for 50 mL
H_20	--------	---------	--------	780	39
SO_4H_2	1800	x16	112.5	60	3
Jaworski (Table 3)	941	x10	94.0	100	5
Algal (Table S2)	500	x25	20.0	40	2
Sea Salt (Instant Ocean)	2500	x50	50.0	20	1

Jaworski's chemical products were weighted and concentrated 100x in H_2O (Table 3 and S2). The concentrates of AJ and Algal are expressed as N Nitrogen content of 941 and 500 mM, respectively. Instant Ocean salts (15 %) are expressed as 2500 mM of NaCl. IO salts are commercially available from aquarium pet-shops (https://www.instantocean.com/products/sea-salt-mixes/sea-salt-mixture.aspx).Sulfuric acid is finally added to a final concentration of ~100 mM. The 36 M concentrated sulfuric acid SO_4H_2 is pre-diluted 20x to 1.8 M for safer handling. **mM**, miliMolar. Because the high SO_4H_2 concentration, there is not need to further sterilize the media.

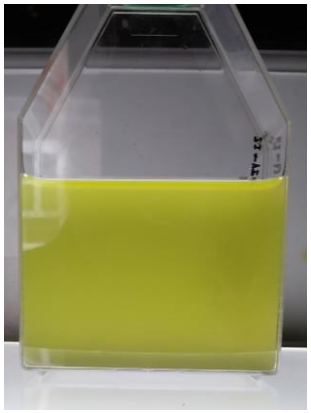


Figure S6
External aspect of the highest *D.acidophila* concentrations in higher volumes
The photograph shows ~8 x 10^6 *D.acidophilas* per mL. The reslts were obtained with 150 mL of AJAS in a 250 mL flask, gassed with 20 % CO_2. The mix was cultured statically with illumination ~50 µmol photons/m^2/s of LEDS during 12 days. The final culture was inoculated with 15 inoculations of 10mL of smaller cultures obtained in 50 mL flasks. The original mix yielded an initial concentration of ~5x10^6 *D.acidophilas*. Further scale-up will need switching from static to gently agitated cultures.

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Competing interests
The author declares no competing interests

Authors' contributions
JC designed, performed and analyzed the experiments and the data and drafted the manuscript.

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