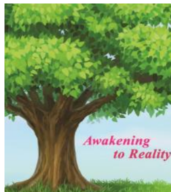

Available online at www.elixirpublishers.com (Elixir International Journal)

Aquaculture

Elixir Aqua. 118 (2018) 50634-50638

ISSN: 2229-712X

Strain selection, Identification and Growth Productivity of Six Microalgae with High Interest in Aquaculture from Dakhla Bay (Morocco)

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ARTICLE INFO

Article history:
Received: 28 March 2018;
Received in revised form:
26 April 2018;
Accepted: 5 May 2018;

Keywords
Microalgae,
Isolation,
Indigenous,
Dakhla bay,
Aquaculture.

ABSTRACT

The present work aimed mainly to isolate specific strains of microalgae potentially used in nutrition program in hatcheries. Six indigenous strains of microalgae with high interest were isolated herein using different techniques. Four species of diatoms were gender identified as *Navicula* sp1, *Navicula* sp2, *Nitzschia* sp and *Melosira* sp, while two species of flagellates were gender identified as *Tetraselmis* sp and *Isochrysis* sp. These native species have shown a high growth productivity comparing to other reference species mainly used as feeding source in aquaculture. Given that, hatcheries in Morocco are currently arising, a native algal collection is needed as well.

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1. Introduction

Microalgae are widely applied in various fields, because of their ability to produce an enormous high-value compound for human nutrition, medical applications, cosmetics and agrochemical industry **[1]**. Aquaculture is also considered as one of the most important field where microalgae - with high nutritional value - are mainly used as (mostly live) food for broodstock, juvenile and larvae of many species (mollusks. crustacean and live prey for fish) in hatcheries **[2]** **[3]**. Microalgae species should posse a number of key attributes to ensure high growth rate and survival of aquatic animals. Suitable species have been selected on the basis of many criteria such mass-culture potential. cell size **[2]** **[4]** and food value which depends on digestibility, biochemical composition and presence or absence of toxins **[1]** **[4]**. Moreover, they must also have rapid growth rates and be stable in culture to any fluctuations in temperature, light and nutrient in aquaculture hatcheries **[4]**.

The production of high quality of microalgae biomass in aquaculture hatcheries requires high human and financial investments and represents one of the highest cost units in many hatcheries **[1]**. Many types of microalgae cultivation system have been reviewed in aquaculture hatcheries over the past years **[5]** **[6]** **[7]**. For instance, open ponds or tanks (with or without aeration). Bubble columns (usually oriented vertically or less frequently horizontal) and closed photo-bioreactors **[8]**.

Since thirty years ago. many strains of microalgae identified by Person and Claus (1980) have been successfully used in bivalve hatcheries including *Isochrysis galbana*. Isochrysis sp. (T.ISO), *Pavlova lutheri* and *Teraselmis suecica*. Furthermore, the use of multi-specific diets including different microalgae strains gives the better nutritional results during broodstock conditioning process than mono-specific diets **[9]**. The isolation and purification of microalgae constitute a crucial step to obtain monoculture strains suitable for aquaculture uses. Many authors describe methods for their isolation and purification, for instance, Anderson in 2005 **[10]**, showed many conventional ways to get pure culture as single-Cell isolation by micropipette. on agar plate. dilution techniques and other automated techniques as flow cytometry with cell sorting and clonal isolation.

The major problems encountered with the use of natural (local) microalgae are the lack of any previous study/attempt for their isolation from the native water (Moroccan seawater) and therefore the ignorance of their nutritional value. The use of exogenous species in hatcheries still remains hazardous because of the ignorance of their impact on the natural ecosystems, especially their behavior versus the indigenous phytoplankton species. Consequentially, this study has been carried out in order to isolate local strains of microalgae from Dakhla bay - which is considered a virgin site for sustainable aquaculture development in Morocco- seawater for their further uses in feeding programs of local and national emergent hatcheries. The choice of isolation of native microalgae instead of using the exogenous strains from specialized culture collections is based essentially on the ability of these strains to grow up easily under local conditions **[11]**. For all these raisons, the isolation of pure local strains has become the main purpose of the present study in order to acquire the first national collection of

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microalgae with high nutritional value for a sustainable aquaculture.

2. Materials and Methods

2.1 Sampling and culture conditions

Water samples were collected from three distanced locations in Dakhla Bay: Boutalha (23°50'35"N. 15°51'82"W). Dunablanca (23°52'45" N. 15°44'49"W) and Puertito (23°34'68" N. 15°54'05"W).

Regardless of location, all water samples were collected and kept in clean and cool containers which guarantee the surviving of microalgae cells until their maintaining in the laboratory. On return to the laboratory, samples were divided on two groups and undertaken following two different treatments (W and WS). For the first group (W), water samples were diluted to 1/50 by filtered seawater, and then enriched by Walne medium (rate of 1 ml/L) **[12]**. On the other hand, the second group (WS) was also diluted to 1/50 but enriched by Walne medium (1 ml/L) and Sodium metasilicate solution (30g/l) (Na2SiO3.5H2O), at the rate of 1 ml/L in order to promote diatom's growth.

All samples under different treatments (W and WS) were maintained at 22 °C and under artificial lighting diffused by white fluorescent lamps (2400 lux). The photoperiod was fixed at 16/8 (Light/Dark cycle) and under sufficient aeration for atmospheric CO2 enrichment and for cells suspension.

2.2 Isolation of Microalgae

Dilutions techniques: In aseptic conditions, we have proceeded with dilution in ten labeled test tubes (from 10-1 to 10-10) containing 9 ml of enriched seawater with sterile automatic dispenser **[13]**. The same process has been followed for both sample material treatments (W and WS). After two weeks of incubation under the controlled conditions (see above), we have checked all tubes to see if mono-algal culture has been resulted.

Single cell isolation by Micropipette: For special details see **[13]**. The same process has been followed for both sample material treatments (W and WS). After two weeks of incubation under the controlled conditions (see above), we have checked all tubes to see if pure mono-algal culture has been resulted.

Isolation on Agar plaque: To isolate microalgae on plaque, we have used Petri dishes containing 1.5 % of Agar and enriched by microalgae culture medium 1ml/L of Walne medium (W) or 1ml/L of Walne medium and 1ml/L of silicate (WS). Three different manners were followed herein. Isolation on streak plaque. Decantation method and Decantation method with dimpled surface **[14]**.

2.3 Morphological identification

Cells of microalgae were initially separated microscopically based on, their morphology (for both dilution and single cell isolation methods), and the morphological observation of colonies on the agar plates. The identification of each microalga to the genus level was based on the morphology of cells using a similar method reported by Tomas **[15]**. Each microalgae strain was photographed at different magnification using Leica microscope DM1000 equipped with a digital camera (Leica D-LUX 3).

2.4 Growth monitoring of isolated microalgae

Each pure culture of isolated microalgae was grown following the initial treatment (W or WS). The flagellates were cultured in Walne medium only (W), while the diatoms were cultured in Walne medium and silicate (WS). An aliquot of each isolated microalgae with an initial density (x cell/mL) is added to the seawater, and the culture was continuously aerated using air pumps and the media was added only once at the beginning of the experiment.

Each microalgae culture has been monitored every two days for cellular growth parameters by direct cell counting using Malassez Cell counter. Some useful parameters were calculated during this work. such as growth rate (μ), generation time (k) and doubling time (d) following the equations (Tempest et al.1978).

$\mu = (\ln N_1 - \ln N_0) / (t - t_0)$

$k = \mu / \ln(2)$

$d = \ln(2)/\mu = 1/k$

N_0 and N_1 represent the number of cells at time t_0 and time t respectively.

3. Results

3.1 Isolation of microalgae

Six indigenous species, among more than 90 observed strains from three different field sampling locations in Dakhla bay were isolated regarding their importance in aquaculture purposes. Three microalgae have been isolated only following dilution method (One species using W medium and two species in WS) which showed mono-algal tubes from dilution 10-5 to 10-10 after 15 days of incubation, while single cell method (Capillary method) has endorsed the isolation of only one microalga using WS medium after a week of incubation. Whereas, two microalgae have been successfully isolated in both mediums (W and WS) following decantation (agar plate) and dilution methods (Table 1).

Table 1. Identified microalgae from different sampling areas at different Medias and following different methods (B: Boutalha, D: Dunablanca and P: Puertito)

Microalgae	Sampling area	Isolation method	Medium	Genus	Identification ref.
1	B	Dilution/solid media	W	***Tetraselmis* sp**	**[15] [16]**
2	B	Dilution	W	***Isochrysis* sp**	**[15]**
3	D, P	Dilution	WS	***Navicula* sp1**	**[17]**
4	D	Dilution	WS	***Navicula sp2***	**[17]**
5	B, D, P	Dilution/Solid media	WS	***Nitzschia* sp**	**[17]**
6	B	Capillary	WS	***Melosira sp***	**[15]**

3.2 Identification of Isolated strains

The isolates were categorized based on their morphological forms of colonies or cells and the colour of the culture. The microalgae isolated herein belong mainly to two high representative phylogenetic groups (Diatoms and Chlorophyta). The identification of these microalgae potentially used in feeding programs in aquaculture, was mainly based on morphological criteria specified in many references (Figure 1 and Table 1).

The dilution method was successfully used to obtain different strains of microalgae under both treatments. The two species *Tetraselmis* sp and *Isochrysis* sp were mainly obtained under W treatment of Boutalha Sampling water, while *Navicula* sp 1 and *Navicula* sp 2 were obtained under WS treatments of water samples of Dunablanca and Puertito. The species *Tetraselmis* sp and *Nitzschia* sp were the only microalgae isolated by two different methods (Dilution and essentially by agar plate technique). Whereas, *Melosira* sp was isolated using capillary method from water samples of Boutalha (Table 1 and Figure 1).

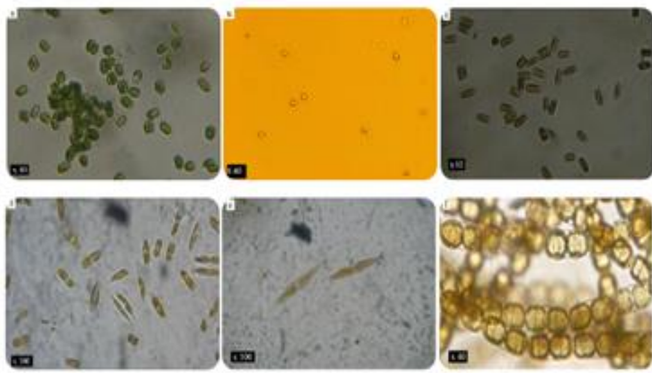


Figure 1. Photomicrographs of isolated microalgae from Dakhla bay seawater. (a) *Tetraselmis* sp, (b) *Isochrysis* sp, (c) *Navicula* sp 1, (d) *Navicula* sp 2, (e) *Nitzschia* sp, (f) *Melosira* sp.

3.3 Growth monitoring of microalgae

3.3.1 Growth pattern of the isolated microalgae

For the flagellate species (Figure 2a), we observed that *Isochrysis* sp has a short lag phase (2 days) and a large exponential phase (8days), contrary to Tetraselmis sp which has a long log phase (8 days) but a short exponential phase (2 days). After ten days of inoculation, both flagellate species have reached the end of exponential phase with 3.7 x 106 cell/ml and 1.4 x 106 cell/ml for Isochrysis sp and Tetraselmis sp respectively. So after the tenth day, the division rate decreases and the cells enter the stationary and death phases.

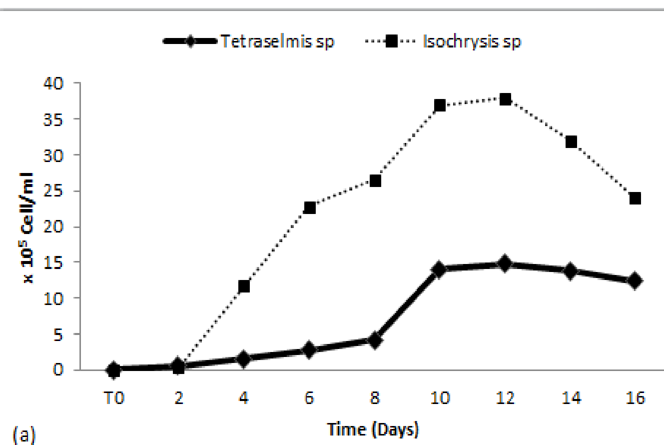
For diatoms species (Figure 2b), we observed four days of lag phase for all diatom species. During the exponential phase, *Melosira* sp cells divide faster than all the other species and reaches 3.1 x 106 cell/ml (4 days). The species *Nitzschia* sp has shown the same scenario of *Melosire* sp but with less division rate and reaches 2.32 x 106 cell/ml by the end of exponential period. Whereas, both species of *Navicula* sp have shown longer exponential phase (6 days), where *Navicula* sp 1 has shown a faster division of cells (3.4 x 106 cell/ml) than *Navicula* sp 2 which has reached only 2.1 x 106 cell/ml by the end of the exponential phase.

3.3.2 Growth parameters

Since all growth parameters are related to each other, we will discuss only the doubling time factor as it's the most reliable and important parameter in microalgae production in hatcheries. The two species *Isochrysis* sp and *Navicula* sp 2 have shown the highest doubling time among all species isolated herein (td = 3.64 d and td = 3.50 d respectively), followed by *Tetraselmis* sp, *Navicula* sp 1 and *Melosira* sp (td = 1.92 d, td = 1.36 d and td = 1.15 d respectively). While *Nitzschia* sp isolated herein, have shown the lowest doubling time in this study (≈1 d). Consequently, *Nitzschia* sp has the highest division rate (K = 1.01 d-1) followed by *Melosira* sp, *Navicula* sp 1, *Tetraselmis* sp and (K = 0.87 d-1, K = 0.73 d-1, K = 0.52 d-1 respectively) while *Navicula* sp 2 and *Isochrysis* sp have shown the smallest division rate in this study (K = 0.29 d-1 and K = 0.27 d-1 respectively).

Table 2. Growth parameters of each isolated microalgae after their mass culture under batch system (IGR: Instantaneous Growth Rate, DR: Division Rate, td: Doubling time, NW: Natural Water).

Isolated species	IGR (µ) (d^{-1})	DR (k) (d^{-1})	t_d (d)	Medium	Reference
Tetraselmis sp	0.36	0.52	1.92	Conway	Current study
Isochrysis sp	0.19	0.27	3.64	Conway	Current study
Navicula sp. 1	0.51	0.73	1.36	Conway	Current study
Navicula sp.2	0.20	0.29	3.50	Conway	Current study
Nitzshia sp	0.70	1.01	0.99	Conway	Current study
Melosira sp	0.60	0.87	1.15	Conway	Current study
Species reference					
Tetraselmis suecica	0.35	0.50	1.98	Conway	[16]
Tetraselmis sp M8	0.43	0.62	1.61	F/2	[18]
Isochrysis galbana	0.20	0.29	3.50	Conway	[19]
Navicula muralis	2.63	-	0.26	-	[20]
Nitzschia sp CP2a	0.22	0.32	3.15	F/2	[18]
Melosira granulate	-	-	10	NW	[21]
Melosira ambigua	-	-	14	NW	[21]
Melosira islandica	-	-	16-34	NW	[21]



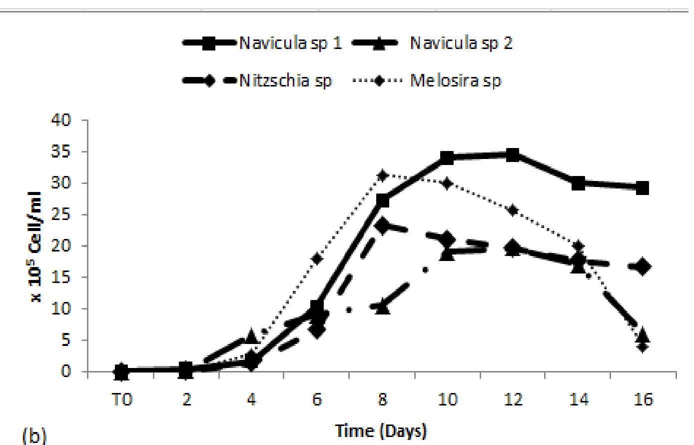


Figure 2. Growth pattern of the isolated microalgae (a): *Flagellates* and (b): *Diatoms*.

4. Discussion

In this work six microalgae potentially used in aquaculture's nutrition programs were isolated and purified to monoclonal strain (*Tetraselmis* sp, *Isochrysis* sp, *Nitzschia* sp, *Navicula* sp.1, *Navicula* sp.2 and *Melosira* sp). All microalgae isolated herein were obtained using different methods (dilution, single cell isolation and solid media methods). The observed specificity between the species and the method of isolation can be explained in different ways and point of views. The species isolated using dilution technique are generally unicellular not joined to neighboring cells and also their small size (from 2 µm 50 µm) makes them difficult to be isolated by capillary method. *Melosira* sp forms unbranched filamentous microscopic cells (visible when filament bends) which make it so easy to be isolated by capillary method. While, only two species (*Tetraselmis* sp and *Nitzschia* sp) have been revealed suitable to be isolated in solid media technique. The Walne medium used herein seemed to be appropriate for isolation and mass culture of microalgae while other studies have stated normally F/2 or SWM3 mediums are the most suitable media **[14] [18]**.

Since the growth rate parameters are the most important criteria to select the appropriate microalgae for nutrition programs in aquaculture **[4]**, we have observed a large diversity in terms of growth parameters (Dt, IGR and DR) among the six-isolated species. *Isochrysis* sp has shown the longest doubling time among all species (4.20 d) which is more or less congruent with the previous study under the same treatment using Conway medium for *Isochrysis galbana* (td = 3.50 d) **[19]**. The doubling time revealed herein for *Tetraselmis* sp (1.92 d) is very similar to the doubling time revealed by *Tetraselmis suecica* using Conway medium (1.98 d) and by *Tetraselmis* sp M8 using F/2 (1.62 d) **[16] [18]**. Generally, diatoms have shown a big difference between growth parameters of the isolated species and the reference ones, for *Navicula genus*, a big difference has been observed between the two-isolated species *Navicula* sp1 and *Navicula* sp2 regarding all growth parameters especially the doubling time (1.36 and 3.50 d respectively), which was as a matter of fact very long comparing it with *Navicula muralis* which is 0.26 d **[20]**. The *Nitzschia* sp have shown a very short doubling time (0.99 d) comparing with *Nitzschia* sp CP2a **[18]**. *Melosira* sp has shown a very short doubling time (1.15 d) comparing with the only reference find regarding this species which ranges from 14 d to 35 d in natural water **[21]**.

Such fact of similarity and discrepancy revealed between this study and previous ones could be justified by the difference of culture conditions used in the present work. Generally, it's well known that growth parameters of microalgae are highly affected by many factors, especially photoperiod **[22] [23] [24] [25]**, light intensity **[26]**, medium composition **[18] [27] [28]** and other environmental changes **[18] [29]**. Nevertheless, all strains isolated herein during this study are obtained from samples of Bay and Coastal waters, where the environmental conditions (Temperature, salinity, pH, nutrients and light) change frequently. These environmental changes require microalgae to produce and adapt more biochemical products to ensure their growth and survival **[30]**.

This work allowed the isolation of six indigenous microalgae with high aquaculture potential. Their biochemical composition (data not shown) has revealed their high nutritional value in aquaculture programs, while the systematic classification using cytofluometric and genetic analysis are still under process. The species belonging to *Tetraselmis* are widely used in aquaculture hatcheries for bivalves feeding **[8] [31] [32]** and larvae of penaeid shrimp, abalone and marine rotifers **[2]**. While species that belong to *Isochrysis* genus are generally used for over twenty years in aquaculture systems cultivation, mainly for molluscs broodstock and larvae feeding **[4]**, larval feeding of penaeid shrimp and also as a supply of freshwater prawns **[2]**. Furthermore, the combination of *Isochrysis* sp with other species such as *Pavlova* sp and *Chaetoceros* sp can provide a useful diet for bivalve nutrition in hatcheries **[33]**.

Benthic diatoms as *Navicula* sp and *Nitzschia* sp are recommended for Abalone and penaeid shrimp larvae nutrition **[1] [4] [8]** and also, they are useful for the nutrition of sea urchins **[8]**. During 1980s–1990s, microalgal feeds were used in relatively small amounts in aquaculture, mainly for the production of larvae, juvenile shell and finfish as well as for raising the zooplanktons required for feeding of juvenile fishes and other animals **[31] [32] [34]**. Possibility of partial replacement of fishmeal with algae has also been considered by several researchers **[35] [36] [37] [38]**, especially in tropical areas where they are found in abundant amounts. *Navicula* and *Haematococcus* were used in substantial amount (100 t year-1) as fish, shrimp and salmon feed during that period Lim and his collaborators (1991) **[39]** about co-culture of *Tetraselmis tetrahele* and *Skeletonema costatum* to feed rotifers and shrimp larvae in Singapore. *Tetraselmis tetrahele* and *Nitzschia gal* improved the food value of rotifers (*Brachionus plicatilis*) as live food for the milkfish fry **[40]**.

Acknowledgment

This work has been achieved partially thanks to the National Center of Scientific and Technique Research (CNRST) scholarship assigned to MAOULAININE Khadija.

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